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Chalcones reverse the anxiety and convulsive behavior of adult zebrafish

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ABSTRACT

In the treatment of anxiety and seizures, drugs of the benzodiazepine (BZD) class are used, which act on the Central Nervous System (CNS) through the neurotransmitter gamma-aminobutyric acid (GABA). Flavonoids modulate GABAA receptors. The aim of this study was to evaluate the anxiolytic and anticonvulsant effects of synthetic chalcones and their mechanisms of action via the GABAergic system, using adult zebrafish (ZFa). The animals were treated with chalcones (4.0 or 20 or 40 mg/kg; 20 μL; i.p) and submitted to the open field and 96 h toxicity test. Chalcones that cause locomotor alteration were evaluated in the light and dark anxiolytic test. The same doses of chalcones were evaluated in the anticonvulsant test. The lowest effective dose was chosen to assess the possible involvement in the GABAA receptor by blocking the flumazenil (fmz) antagonist. No chalcone was toxic and altered ZFa's locomotion. All chalcones had anxiolytic and anticonvulsant effects, mainly chalcones 1, where all doses showed effects in both tests. These effects were blocked by Fmz (antagonist GABAA), where it shows evidence of the performance of these activities of the GABA system. Therefore, this study demonstrated in relation to structure–activity, that the position of the substituents is important in the intensity of activities and that the absence of toxicity and the action of these compounds in the CNS, shows the pharmacological potential of these molecules, and, therefore, the insights are designed for the development of new drugs.

1. Introduction

Anxiety is one of the common psychiatric comorbidities in epilepsy, with an incidence rate reported in up to 25% of patients. The severity of the seizures is strongly correlated with anxiety disorders in patients with epilepsy [1,2]. The tracking of antiepileptic drugs (AEDs) has been developed in rodents, and lately zebrafish larvae have been used to prospect for anticonvulsant drugs. Studies have shown that the pentylenetetrazole (PTZ) chemoconvulsant model induces seizure in a concentration-dependent manner [3,4]. This model was extended to adult zebrafish, using three main analyses: electrophysiological evaluation in immobilized animals [5], c-fos expression in the CNS [6], and behavioral outcome parameters [7]. In addition, Mussulini et al. [8] performed a detailed behavioral characterization of PTZ-induced seizures in adult zebrafish and analyzed parameters such as mortality rate and seizure severity.

In this regard, the PTZ-induced seizure model has been used to screen for anticonvulsant compounds of marine origin [9] plant derivatives [10–12] and synthetic [13]. Mussulini et al. [14] highlights the importance of the zebrafish model for studying epileptic seizures and epileptic syndromes and concluded that, although zebrafish larvae appear to be more attractive as a tool, adult zebrafish

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are also valuable as they can be used to study the impact of genetic and drug manipulation on behavioral changes after seizures.

For the treatment of anxiety and seizures, drugs of the benzodiazepine class (BZD) are used, which include lorazepam and diazepam. In the past 2 decades, concerns about the short- and long-term risks associated with the use of BZDs have increased. They act on the Central Nervous System (CNS) through the neurotransmitter gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, and bind to $GABA_A$ receptors where they potentiate the inhibitory action of GABA [15].

Zebrafish (*Danio rerio*) has gained wide popularity in behavioral neuroscience and research in psychopharmacology [16]. The genetic compositions of zebrafish are comparable to humans with 70% genetic similarity, while 84% of the genes known for human diseases are widely expressed in zebrafish (Norton and Bally-Cuif [17]). It has emerged as a robust animal model for several neurological diseases, including epilepsy [8,18]. In addition, it has contributed to a better understanding of the role of several genes that have been implicated in the disease [19].

Of interest, flavonoids and their glycosides have been shown to have a mild to potent activity in several animal models of seizure [20,21]. Upon investigating the mechanism of action, these structures were found to modulate allosteric $GABA_A$ receptors by binding to the BZD receptor site [22,23]. For this reason, studies have been carried out to investigate the anxiolytic and anticonvulsant potential of these compounds. [24,25]. Its neuroprotective activities are due to antioxidant properties, which stimulate neuronal regeneration inducing neurogenesis and preventing apoptosis of these cells due to oxidative stress [26]. In nature, chalcones (subclass of flavonoids) are normally found as chalcone aglycone and chalcone *O*-glycosides, but can also be modified by hydroxylation, condensation, or methylation. They are present in numerous families of dicotyledonous plants and in some monocotyledons, pteridophytes, and gymnosperms [27], but are synthesized as main components in the families Asteraceae, Moraceae, Fabaceae, and Aristolochiaceae [28]. The molecular structure of chalcones is a starting point for the synthesis of newly derived compounds. In recent years, interest in these molecules has increased, mainly due to their potential use as drugs against several human diseases already reported [29,30]. Previous study demonstrated that chalcones used in this study showed promising antimicrobial activity [31]. The chemical structure of chalcones contains several replaceable hydrogens that allow a large number of derivatives to be obtained and a variety of promising biological activities to be generated, for example, anti-inflammatory and neuroprotective [32,33] antidepression and analgesic effect [34] antioxidant [35] anxiolytic [36] antiviral and anticancer [37].

In view of the relationship between flavonoids or subclasses and GABAergic neurotransmission in the modulation of anxiolytic and anticonvulsant activities, this study investigated the effect of synthetic chalcones. In order to elucidate the possible direct interactions of these molecules with $GABA_A$ receptors, a molecular docking study was carried out.

2. Material and methods

2.1. Drugs and reagents

The following substances were used: diazepam (Dzp, Neo Química®), flumazenil (Fmz; Sandoz®), dimethyl sulfoxide (3% DMSO; Dynamic®), and pentylenetetrazole (PTZ, Sigma-Aldrich).

2.2. Synthesis and chemical characterization of chalcones

The description of the procedure of the synthesis of chalcones is shown in Scheme 1. Chalcones (1–4) were synthesized by a Claisen–Schmidt condensation reaction in a basic medium. At ethanol (5 mL) solution of 2-hydroxy-3,4,6 trimethoxyacetophenone (2 mmol) was added to a solution of benzaldehyde and the derivatives (2 mmol), followed by the addition of ten drops of 50% w/v aq. NaOH with stirring for 48 h. The solid that formed was filtered under reduced pressure, washed with cold water, and analyzed by TLC.

2.2.1. (E)-3-(2,4-Dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (**1**)

Yellow solid (Yield: 73, 93%), m.p. 164.3–164.8 °C. ^{1}H NMR ($CDCl_3$, ppm): 3.98 (s, MeO-3′); 3.94 (s, MeO-4′); 3.85 (s, MeO-6′); 6.01 (s, H-5′); 7.62 (d, *J* = 8.5, H-3); 7.46 (d, *J* = 1.5, H-5); 7.30 (d, *J* = 1.5, H-6); 7.80 (d, Hα, *J* = 15,5 Hz); 8.06 (d, Hβ, *J* = 15.5 Hz). ^{13}C NMR ($CDCl_3$, ppm): 192.6 (C═O); 60.7 (MeO-3′); 56.1 (MeO-4′); 56.1 (MeO-6′); 106.8 (C-1′); 158.7 (C-2′); 130.4 (C-3′); 159.4 (C-4′); 87.1 (C-5′); 158.6 (C-6′); 134.2 (C-1); 136.8 (C-2); 135.5 (C-3); 136.0 (C-4); 131.7 (C-5); 128.5 (C-6); 127.5 (Cα); 130.1 (Cβ). MS (EI) *m*/*z* ($M^{+\cdot}$ 383), calcd for $C_{18}H_{16}Cl_2O_5$/383.

2.2.2. (E)-3-(4-Chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl) prop-2-en-1-one (**2**)

Yellow solid (Yield: 74, 68%), m.p. 94.3–96.8 °C. ^{1}H NMR ($CDCl_3$, ppm): 3.96 (s, MeO-3′); 3.96 (s, MeO-4′); 3.94 (s, MeO-6′); 6.02 (s, H-5′); 7.38 (d, *J* = 8.35, H-2/6); 7.53 (d, *J* = 8.35, H-3/5); 7.79 (d, Hα, *J* = 15.6 Hz); 7.83 (d, Hβ, *J* = 15.6 Hz). ^{13}C NMR ($CDCl_3$, ppm): 193.5 (C═O); 60.9 (MeO-3′); 60.9 (MeO-4′); 60.9 (MeO-6′); 106.5 (C-1′); 159.0 (C-2′); 129.7 (C-3′); 159.6 (C-4′); 87.4 (C-5′); 159.2 (C-6′); 134.2 (C-1); 131.2 (C-2/6); 131.7 (C-3/5); 136.2 (C-4); 128.2 (Cα); 141.3 (Cβ). MS (EI) *m*/*z* ($M^{+\cdot}$ 348), calcd for $C_{18}H_{17}ClO_5$/348.

2.2.3. (E)-3-(2-Fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl) prop-2-en-1-one (**3**)

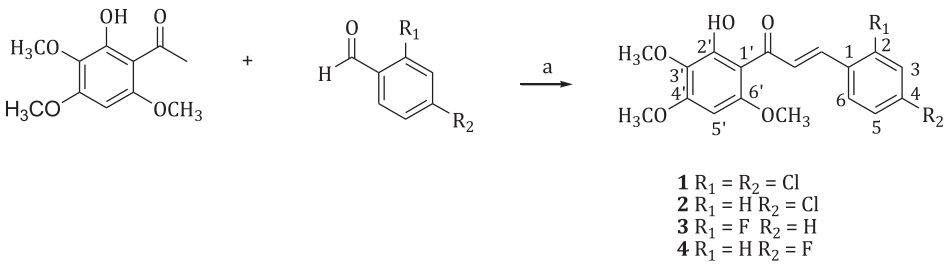
Yellow solid (Yield: 84, 07%), m.p. 144.3–144.8 °C. ^{1}H NMR ($CDCl_3$, ppm): 3.96 (s, MeO-3′); 3.96 (s, MeO-4′); 3.84 (s, MeO-6′); 6.07 (s, H-5′); 7.67 (m, H-3); 7.44 (m, H-4); 7.25 (m, H-5); 7.18 (m, H-6); 7.82 (d, Hα, *J* = 15,5 Hz); 8.06 (d, Hβ, *J* = 15.5 Hz). ^{13}C NMR ($CDCl_3$, ppm): 193.5 (C═O); 60.0 (MeO-3′); 56.2 (MeO-4′); 56.1 (MeO-6′); 107.1 (C-1′); 158.9 (C-2′); 130.4 (C-3′); 159.6 (C-4′); 87.3 (C-5′); 158.8 (C-6′); 131.7 (C-1); 135.4 (C-2); 131.6 (C-3); 131.1 (C-4); 116.3 (C-5); 116.6 (C-6); 124.7 (Cα); 135.5 (Cβ). MS (EI) *m*/*z* ($M^{+\cdot}$ 322), calcd for $C_{18}H_{17}FO_5$/332.

2.2.4. (E)-3-(4-Fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl) prop-2-en-1-one (**4**)

Yellow solid (Yield: 69, 53%), m.p. 144.3–144.8 °C. ^{1}H NMR ($CDCl_3$, ppm): 3.96 (s, MeO-3′); 3.96 (s, MeO-4′); 3.84 (s, MeO-6′); 6.07 (s, H-5′); 7.63 (d, *J* = 8.58, H-2/6); 7.65 (d, *J* = 8.58, H-3/5); 7.82 (d, Hα, *J* = 15,6 Hz); 7.83 (d, Hβ, *J* = 15.6 Hz). ^{13}C NMR ($CDCl_3$, ppm): 193.2 (C═O); 60.9 (MeO-3′); 56.3 (MeO-4′); 56.2 (MeO-6′); 107.1 (C-1′); 158.7 (C-2′); 130.4 (C-3′); 159.6 (C-4′); 87.4 (C-5′); 158.7 (C-6′); 131.9 (C-1); 131.2 (C-2/6); 131.9 (C-3/5); 141.5 (C-4); 127.4 (Cα); 141.5 (Cβ). MS (EI) *m*/*z* ($M^{+\cdot}$ 322), calcd for $C_{18}H_{17}FO_5$/332.

2.3. Animals

Zebrafish (*Danio rerio*), wild, adult, of both sexes (60 and 120 days; 3.5 ± 0.5 cm; 0.4 ± 0.1 g) were obtained from a local store (Fortaleza, Ceará, Brazil). The animals were acclimated for 24 h in glass aquariums (30 × 15 × 20 cm), containing dechlorinated water (ProtecPlus) and air pumps with submerged filters, at 25 °C and pH 7.0, with a circadian cycle of 14:10 h (light/dark). Animals were anesthetized before drug applications and after the experiments, the animals were sacrificed by immersion in ice water (2 and 4 °C) for 1 min until the loss of opercular movements.



Scheme 1. Preparation of chalcones (**A–D**). a) NaOH 50% w v^{-1}, ethanol (5 mL), room temperature, 48 h.

The work was approved by the Ethics Committee for the Use of Animals of the State University of Ceará (CEUA-UECE; # 3344801/2017), being in accordance with the Ethical Principles of Animal Experimentation.

2.4. Toxicity in adult zebrafish (ZFa)

The test was based on the [38] methodology with adaptations. The Zfa ($n = 6$ group) were treated with chalcones (4,0 or 20 or 40 mg/kg; 20 μL; i.p). As a negative control, dimethyl sulfoxide (DMSO 3%; 20 μL, i.p) was used. After 24, 48, 72, and 96 h, the number of animals killed was counted and the data were subjected to statistical analysis, using the Trimmed Spearman–Karber method with 95% confidence intervals, where the lethal dose to kill 50% was estimated (LD_{50}) of animals.

2.5. Open-field test

Animals ($n = 6$/group) were pre-treated (20 μL; i.p) with 4 chalcones, in the same doses analyzed in Section 2.4. Diazepam (Dzp; 40 mg/kg) or vehicle (DMSO 3%) were used as positive and negative controls, respectively. After 30 min of treatment, animals were

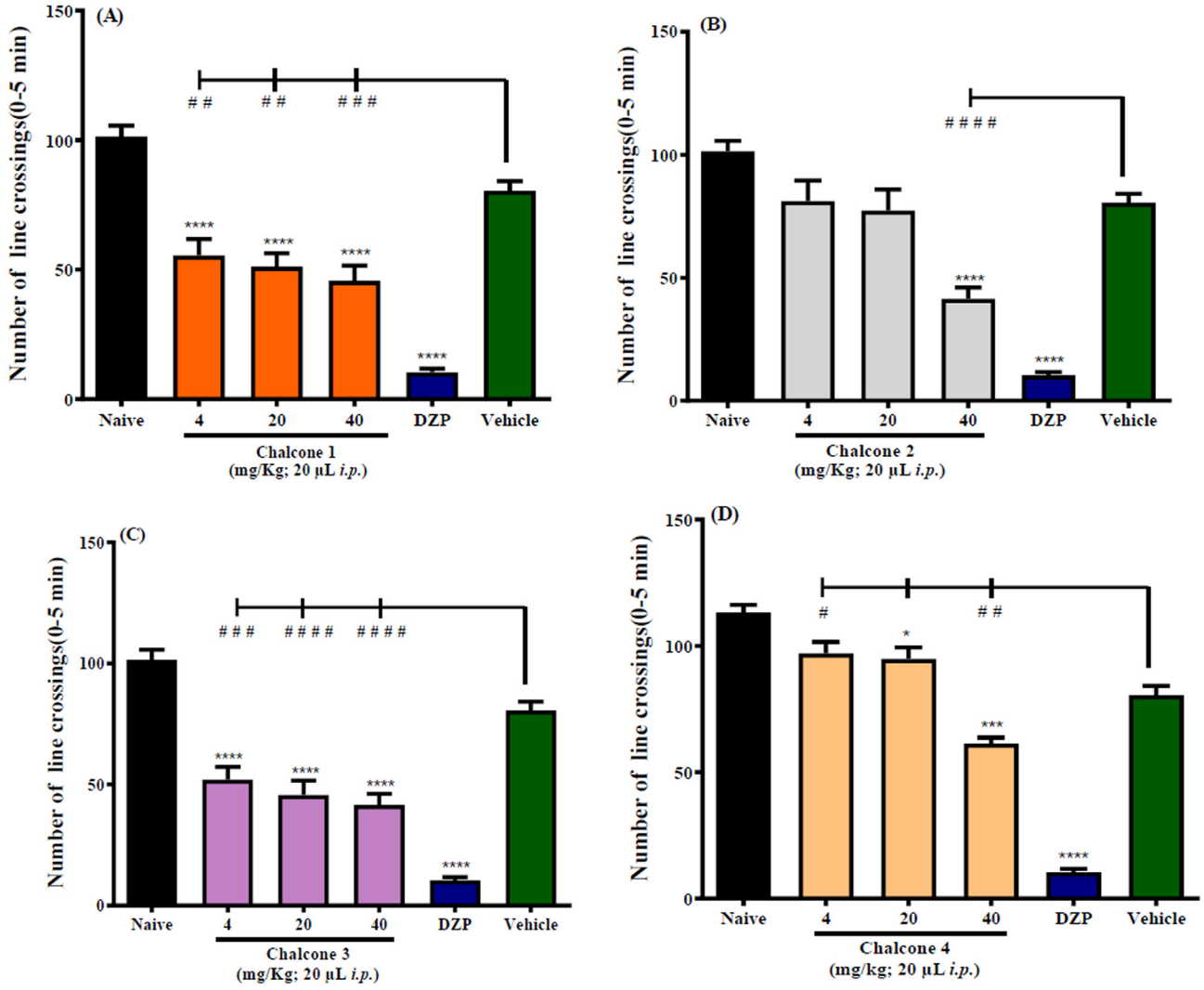


Fig. 1. Effect of chalcone 1 (A), chalcone 2 (B), chalcone 3 (C) and chalcone 4 (D) on the locomotor behavior of zebrafish (*Danio rerio*) in the Open-Field Test (0–5 min). Naive – Animals without treatment. Dzp – diazepam (40 mg/kg; 20 μL; i.p). Vehicle (DMSO 3%) (20 μL; i.p) The values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Turkey ($^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. Naive; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ vs Vehicle or DZP).

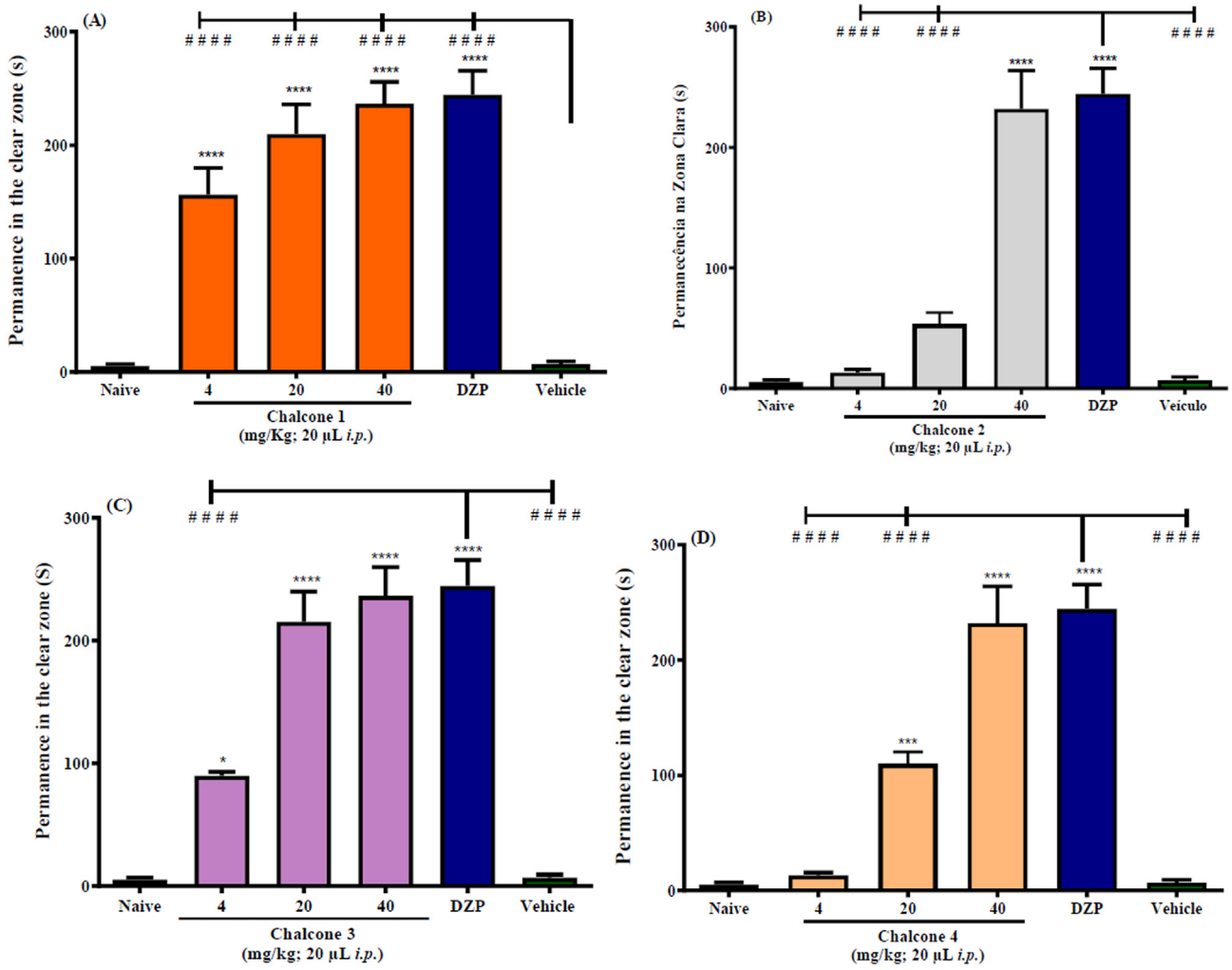


Fig. 2. Anxiolytic effect of chalcone 1 (A), chalcone 2 (B), chalcone 3 (C) and chalcone 4 (D) in adult zebrafish in the light and dark test (0–5 min). Naive – Animals without treatment. Dzp – diazepam (40 mg/kg; 20 µL; i.p). Vehicle (DMSO 3%) (20 µL; i.p) The values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Turkey ($^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. Naive; $^{\#\#\#\#}p < 0.0001$ vs Vehicle or DZP).

added individually in glass Petri dishes (10 × 15 cm; with quadrants at the bottom of the plate), containing the same water from the aquarium (Magalhães et al. [39]). A group without treatment (Naive) was included. The number of line crossings was recorded during 0–5 min.

2.6. Anxiolytic evaluation via GABAergic system

A glass aquarium (30 × 15 × 20 cm) was used with a light and dark area filled with tap water pretreated with anti-chlorine, until it reached a height of 3 cm. The animals (n = 6/group) were pretreated with chalcones, DZP (40 mg/kg; i.p) and 3% DMSO (see 2.5) and submitted to the light and dark test [40]. After 30 min of treatment, animals were placed individually in the light zone of the aquarium and the anxiolytic effect was quantified as time (s) of permanence in the light zone during 5 min of analysis. A naive group was included. The Gabaergic mechanism of action was performed with the lowest effective dose. The animals (n = 6/group) received Fmz (4.0 mg/kg; 20 µL; i.p) and after 15 min they were treated with the lowest effective doses of chalcones: A (4 mg/kg; i.p); B (40 mg/kg; i.p); C (4 mg/kg; i.p); and D (20 mg/kg; i.p). Dzp (40 mg/kg; 20 µL; i.p), and vehicle (3% DMSO; 20 µL; i.p) were included as controls. After 30 min of treatment, animals were submitted to the light and dark test, described in the section.

2.7. Pentylenetetrazole-induced seizure (PTZ) via GABAergic system

The behavior related to seizure crises was analyzed in three stages [41]. Stage I – dramatically increased swimming activity; stage II – swirling swimming behavior; stage III – clonus-like seizures, followed by loss of posture when the animal falls to one side and remains immobile for 1–3 s. The doses of chalcones analyzed in the anxiety test (Section 2.6) were also subjected to the anticonvulsant test. Diazepam (40 mg/kg; 20 µL; i.p) and vehicle (3% DMSO; 20 µL; i.p) were included. After 30 min, the animals were exposed to PTZ at 7.5 mM, and the behavior similar to seizure in three stages was evaluated. The anticonvulsant action mechanism was performed with the effective dose in the three stages. The animals (n = 6/group) received Fmz (4.0 mg/kg; 20 µL; i.p) and after 15 min, they were treated with the lowest effective doses at each stage of seizure. Groups treated with Dzp (40 mg/kg; 20 µL; i.p), and vehicle (3% DMSO; 20 µL; i.p) were included in the statistical analysis of the data.

2.8. Molecular docking

2.8.1. Ligand preparation

The two-dimensional chemical structures of chalcones were plotted from the plugins installed in the MarvinSketch © code

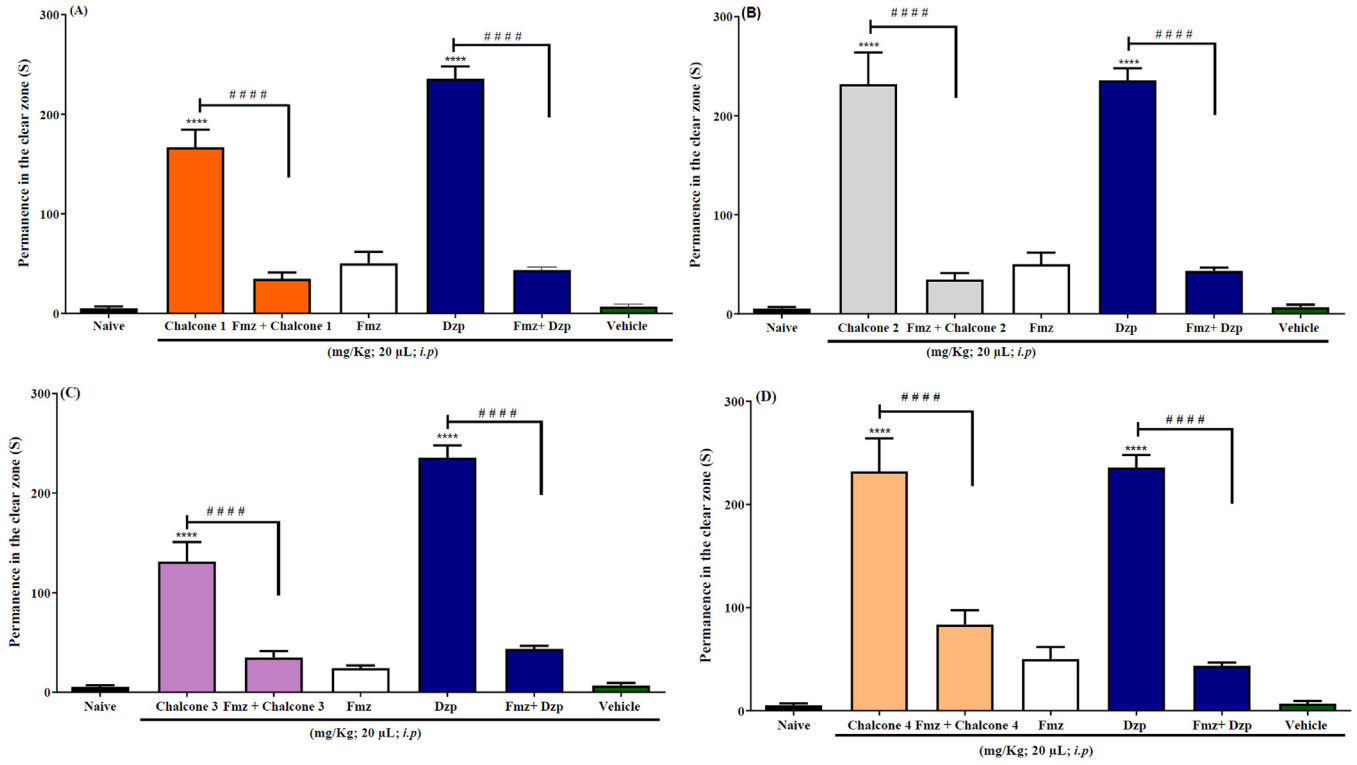


Fig. 3. Effect of flumazenil (Fmz) under the anxiolytic effect of chalcone 1 (A), chalcone 2 (B), chalcone 3 (C) and chalcone 4 (D) in the light and dark test. Dzp – diazepam (40 mg/kg; 20 µL; i.p). Fmz – flumazenil (4 mg/kg; 20 µL; i.p). The values represent the mean ± mean error (E.P.M.) for 6 animals/group. ANOVA followed by Tukey ($^{****}p < 0.0001$ vs. naive or vehicle; $^{\#\#\#\#}p < 0.0001$ vs. Fmz + Dzp or Fmz + chalcone 1 or Fmz + chalcone 2 or Fmz + chalcone 3 or Fmz + chalcone 4.

(https://chemaxon.com/products/marvin) [42] being saved in .mol format. The structures were optimized using the Avogadro code [43] configured to use the force field of Merck Molecular Force Field 94 – MMFF94 [44] with descending steepest algorithm, configured to perform cycles of 50 interactions.

2.8.2. Protein preparation

The enzyme 4-aminobutyrate-aminotransferase inactivated by gamma-vinyl GABA (PDB ID: 1OHW) was selected in the protein database Protein Data Bank (https://www.rcsb.org/), deposited with a resolution of 2.30 Å, determined from X-ray diffraction, with *R*-Value Free: 0.215, *R*-Value Work: 0.188 and *R*-Value Observed: 0.190 [45]. In the preparation of target enzymes, the residues present in the structures were removed and the polar hydrogens were added.

2.8.3. Molecular docking

For docking simulations, the AutoDock Vina code (version 1.1.2) [46] was used. The grid box was defined with parameters of 120 Å × 106 Å × 126 Å, centered on the whole protein with the

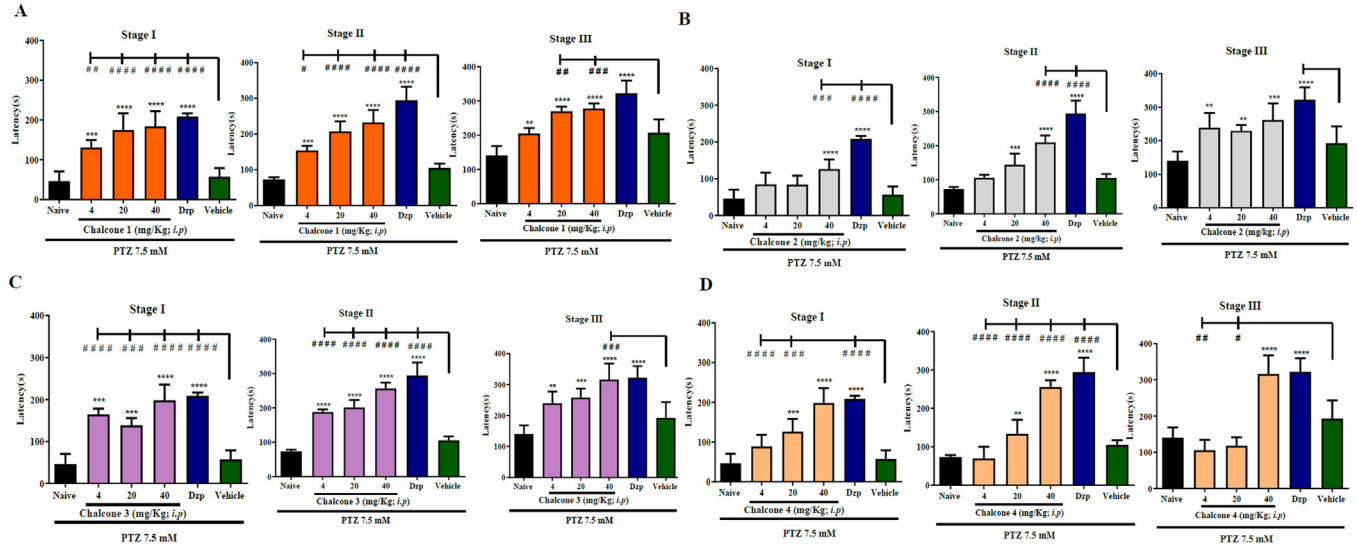


Fig. 4. Effect of chalcone 1 (A), chalcone 2 (B), chalcone 3 (C) and chalcone 4 (D) on the pentylenetetrazole-induced seizure (3 stages) in adult zebra fish. Dzp – diazepam (40 mg/mL; 20 µL, i.p); Vehicle – 3% DMSO (20 µL; i.p). The values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey ($^{**}p < 0.01$ $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. Naive; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$ vs. vehicle).

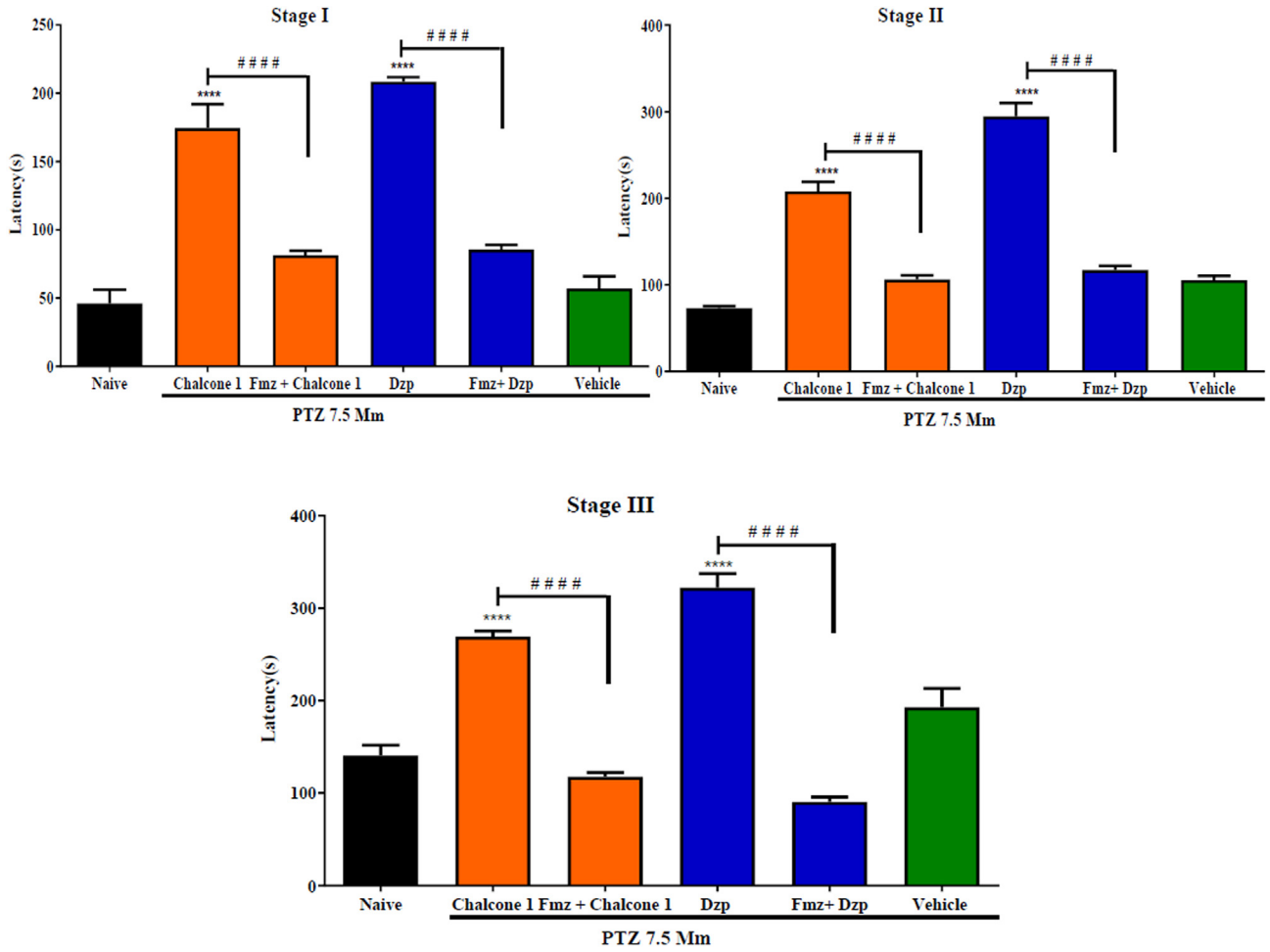


Fig. 5. Effect of flumazenil (Fmz) on the anticonvulsant action of chalcone 1 (4 mg/kg; i.p) in the pentylenetetrazole-induced seizure test in adult zebrafish. Fmz – flumazenil (4 mg/kg; 20 μL; i.p). The values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey ($^{****}p < 0.0001$ vs. Naïve and vehicle; $^{\#\#\#\#}p < 0.0001$ vs. Dzp + Fmz or Fmz + chalcone 1).

dimensions (x, y, z) = (30.812, 55.924, 70.500), 100 independent simulations being carried out obtaining 10 poses each. For the selection of simulations with better poses, the simulations that presented RMSD (Root Mean Square Deviation) value less than 2 Å and free bonding energy (ΔG) below −6.0 kcal/mol were used as criteria [47,48]. The results were analyzed and visualized using the codes Discovery Studio Visualizer and UCSF Chimera [49,50].

2.9. Statistical analysis

The results were expressed as mean ± standard deviation of the mean, for in vitro tests ($n = 3$), as well as mean ± standard error of the mean, for in vivo tests ($n = 6$/group). After confirming the normality and homogeneity distribution of the data, differences between the groups were submitted 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% ($p < 0.05$).

3. Results

3.1. Acute toxicity to adult zebrafish

None of the chalcones showed toxicity in adult zebrafish during the 96 h of analysis ($DL_{50} > 40$ mg/kg).

3.2. Evaluation of locomotor activity

The number of line crossings during the open-field test showed that the 3 doses of chalcone 1 reduced the animals' locomotion ($^{**}p < 0.01$; $^{***}p < 0.001$; Fig. 1A). In contrast, only the 40 mg/kg dose of chalcone 2 significantly reduced the number of crossbreeding animals ($^{****}p < 0.0001$, Fig. 1B). All doses of chalcone 3 also reduced the number of crosses ($^{***}p < 0.001$; $^{****}p < 0.0001$, Fig. 1C). Only doses of 20 and 40 mg /kg of chalcone 4 reduced animals' locomotion ($^{*}p < 0.5$; $^{***}p < 0.001$, Fig. 1D). These results regarding the synthesized chalcones were significantly similar ($p > 0.05$) to the positive Dzp control ($^{****}p < 0.0001$; 40 mg/kg) and significantly different compared to the controls (naive and vehicle).

3.3. Anxiolytic activity via GABAergic system

All doses of chalcone 1 induced a significant increase ($^{****}p < 0.0001$; Fig. 2A) in the time spent by animals in the clear area of the aquarium. However, only the dose of 40 mg/kg of chalcone 2 showed anxiolytic effect ($^{****}p < 0.0001$; Fig. 2B) in the Claro & Escuro test. The two highest doses of chalcones 3 and 4 showed statistically anxiolytic effect, causing the animals to stay longer in the clear zone of the aquarium ($^{***}p < 0.0001$; $^{****}p < 0.0001$; Fig. 2C and D). These results were significantly similar ($p > 0.05$) to the positive Dzp control ($^{****}p < 0.0001$; 40 mg/kg) and significantly different compared to controls (naive and vehicle).

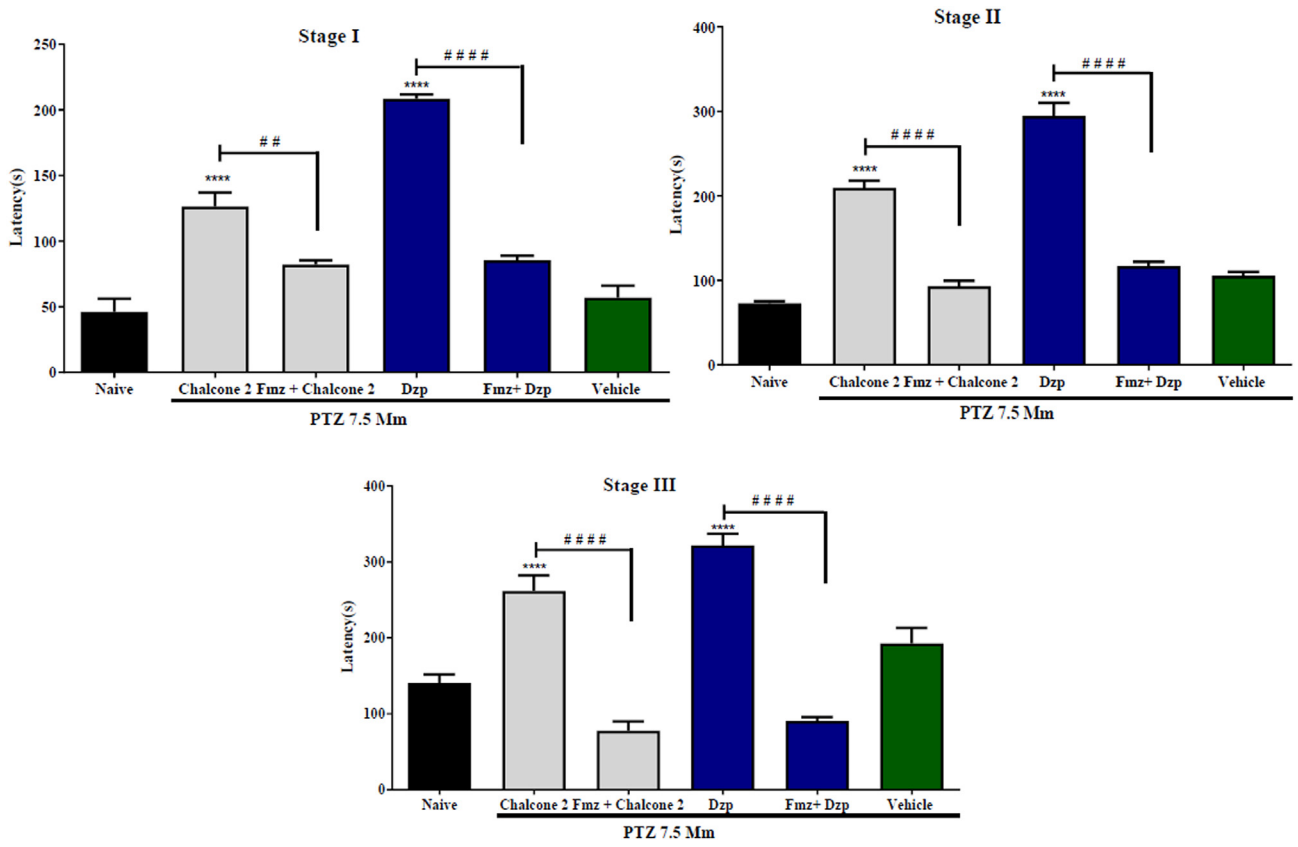


Fig. 6. Effect of flumazenil (Fmz) on the anticonvulsant action of chalcone 2 (40 mg/kg; i.p) in the pentylenetetrazole-induced seizure test in adult zebrafish. Dzp – diazepam (40 mg/mL; 20 μL, i.p). Fmz – flumazenil (4 mg/kg; 20 μL; i.p). The values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey ($^{****}p < 0.0001$ vs. Naïve and vehicle; $^{\#\#}p < 0.01$ $^{\#\#\#\#}p < 0.0001$ vs. Dzp + Fmz or Fmz + chalcone 2).

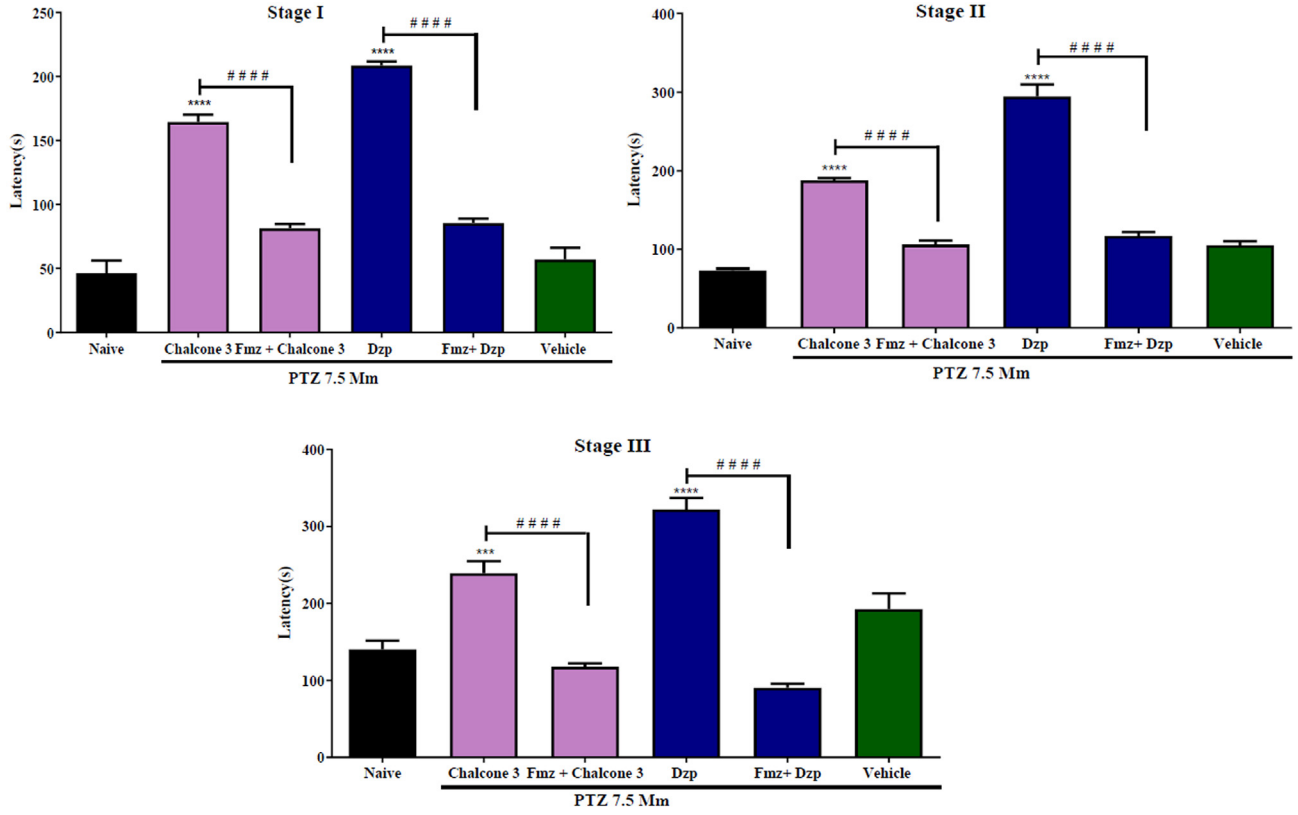


Fig. 7. Effect of flumazenil (Fmz) on the anticonvulsant action of chalcone 3 (4 mg/kg; i.p) in the pentylenetetrazole-induced seizure test in adult zebrafish. Dzp – diazepam (40 mg/mL; 20 μL, i.p). Fmz – flumazenil (4 mg/kg; 20 μL; i.p). The values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey ($^{***}p < 0.001$, $^{****}p < 0.0001$ vs. Naïve and vehicle; $^{\#\#\#\#}p < 0.0001$ vs. Dzp + Fmz or Fmz + chalcone 3).

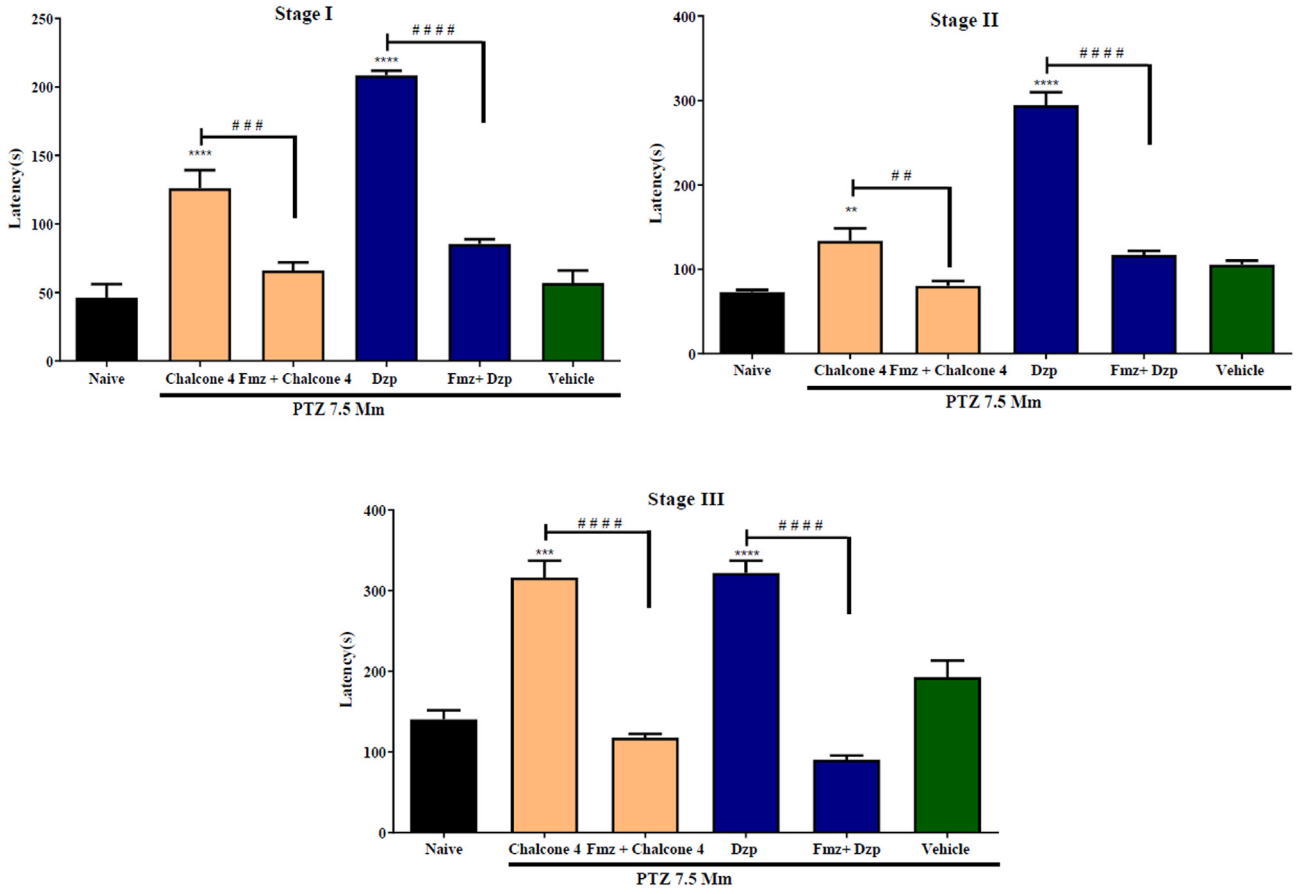


Fig. 8. Effect of flumazenil (Fmz) on the anticonvulsant action of chalcone 4 (40 mg/kg; i.p) in the pentylenetetrazole-induced seizure test in adult zebrafish. Dzp – diazepam (40 mg/mL; 20 μL, i.p). Fmz – flumazenil (4 mg/kg; 20 μL; i.p). The values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey ($^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. Naïve and vehicle; $^{\#\#}p < 0.0001$; $^{\#\#\#\#}p < 0.0001$ vs. Dzp + Fmz or Fmz + chalcone 4).

In the anxiety mechanism, all lower doses of chalcones that had an anxiolytic effect were significantly similar to the effect of diazepam (DZP; 40 mg/kg; ip), as there was a reduction in the anxiolytic effect of each dose by the antagonist Fmz ($^{\#\#\#\#}p < 0.0001$, Fig. 3A––D).

3.4. Pentylenetetrazole-induced seizures (involvement of the GABAergic system)

All doses of chalcone 1 delayed the onset of ZFa seizure stages ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs naive and vehicle). Interestingly, only the 40 mg/kg dose of chalcones 2 and 4 delayed the 3 stages of seizure ($^{***}p < 0.001$ $^{****}p < 0.0001$ vs naive and vehicle) (Fig. 4B and C). Results were similar to Dzp [(40 mg/kg; 20 μL; i.p; $^{****}p < 0.0001$ vs naive and vehicle)]. The anticonvulsant effect of the lowest effective doses in the 3 stages of chalcones A and C (4 mg/kg; 20 μL; ip), B and D (40 mg/kg 20 μL; ip), and Dzp (40 mg/kg; 20 μL; i.p) was reduced ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$; $^{\#\#\#\#}p < 0.0001$) by Fmz in the three phases (Figs. 5–8).

3.5. Molecular docking GABA-AT/chalcones

To investigate the potential effect of chalcones on the GABAergic system, docking simulations were performed on the gamma-aminobutyric acid aminotransferase (GABA-AT) protein. After performing molecular coupling simulations, all ligands showed affinity energy within the ideal parameter, less than −6.0 kcal/mol and RMSD (Root Mean Square Deviation) values less than 2 Å [47,48]. Chalcones 1 and 2, coupled in the region of the vigabatrin site (Fig. 9), showing interactions with residues PHE189, ARG192, GLU270, and PHE1141 (Fig. 11). However, chalcone 1 showed more favorable interactions (shorter distances) with residues ILE72 and TYR1138. Chalcones 3 and 4 coupled in a different region from the vigabatrin site (Fig. 10), showing interactions with residues GLU1060, PHE979, PHE351, and ILE862. Chalcone 4 showed a favorable interaction with TYR859 (Fig. 11).

4. Discussion

Notably, as far as is known, this is the first evidence showing anxiolytic and anticonvulsant effects of chalcones on the Central Nervous System (CNS) against PTZ seizure model. All doses of chalcones investigated in this study were subjected to an acute toxicity test (96 h) and showed no toxicity, which provides evidence for in vivo pharmacological tests, allowing the selection of safe doses. Thus, an open-field test was performed to analyze possible locomotor changes in the animals. Chalcones 1–4 altered zebrafish locomotion when administered intraperitoneally. The result is relevant because according to Gupta et al. [51] this behavior is characteristic of drugs that act in the CNS of the zebrafish. Changing locomotion could also be a sedation response caused by the influence of the drug's route of administration, usually influenced by the time of action, intensity, and duration [52]. In view of these questions, we investigated the anxiolytic effect of chalcones synthesized by the light and dark test.

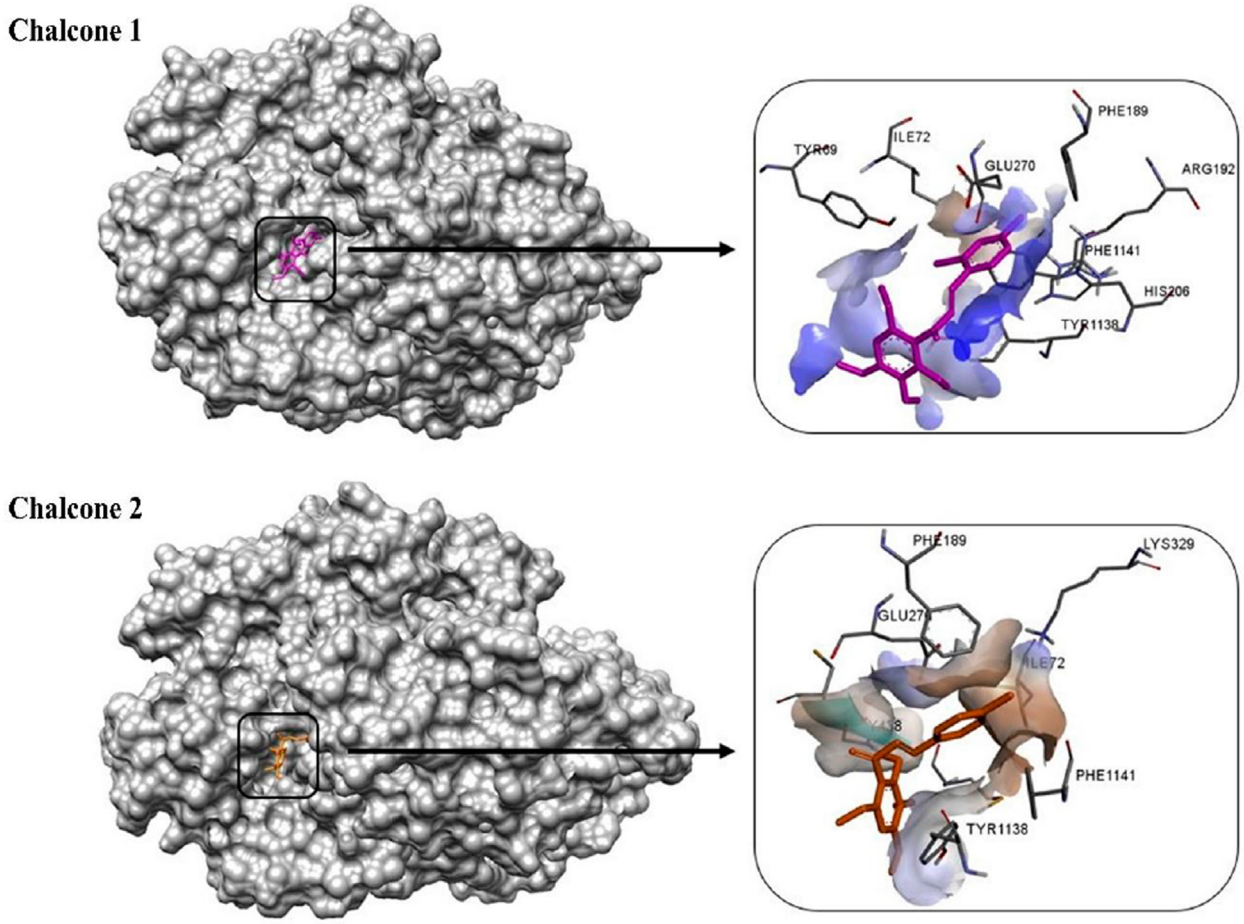


Fig. 9. Schematic 2-D representations the gamma-aminobutyric acid aminotransferase (GABA-AT) complexes with chalcones 1 and 2.

The scototaxis protocol (preferably light/dark) is used to assess the anxiolytic effects of pharmacological agents [53]. The chalcones had an anxiolytic effect, as there was an increase in the time the fish stayed in the clear area of the aquarium. When relating the structure–activity of the molecules and the nature of the Cl substituents in the R1 and R2 positions of the synthesized chalcones, it was evident that the substituent in the R1 position significantly potentiates anxiolytic activity (chalcone 1; Fig. 2A), in comparison to chalcone 2 (Fig. 2B), replaced only in R2, which differed from the control group Dzp only in the highest dose (40 mg/kg) evaluated. Substituent F in positions R1 and R2 influenced the anxiolytic effect of chalcones, showing effectiveness in all doses evaluated for substitution in position R1 (Fig. 2C), while chalcone with F in position R2 was effective only in the two largest doses (20 and 40 mg/kg) (Fig. 2D). In the ranking of the most common functional groups in bioactive molecules is fluorine atom (29.7%) and chlorine (19.5%) [54] the same substituents present in our molecules.

Anxiety is regulated by the inhibitory neurotransmitter GABA and this system is the target of BDZs and related drugs used to treat anxiety disorders [55]. The mechanism of the chalcone's anxiolytic effect was investigated by flumazenil, a GABAA antagonist. After pre-treatment, flumazenil reduced the anxiolytic/sedative effects of all analyzed chalcones, indicating that the anxiolytic activity of the molecules acts through the GABA system. Studies show that other series of synthesized chalcones also demonstrated anxiolytic effects in rodents and zebrafish larvae [34,56], respectively. Drugs that stimulate GABA receptors, such as BDZs and barbiturates, have anxiolytic and anticonvulsant effects by reducing GABAA-mediated neuronal excitability, causing changes in the α1 and γ2 subunits of this receptor [57,58]. The antiepileptic effects of drugs such as BDZs (anxiolytics) are accompanied by decreased locomotor activity and sedation [59]. For this reason, the anticonvulsant potential of these chalcones has been investigated, which alter the animals' locomotion and have an anxiolytic effect.

Seizures were induced by PTZ, a chemoconvulsant used to identify drugs effective in combating generalized seizures, in particular molecules that increase GABAergic neurotransmission [60]. In both zebrafish and rodent models, PTZ is the most characterized chemical model [61]. In the CNS, it antagonizes $GABA_A$ receptors, thus modifying the excitatory/inhibitory tone, which culminates in acute seizures [62]. It is important to note that several AEDs approved by the FDA (Food and Drug Administration) were initially discovered in PTZ models, suggesting some translational relevance for these acute models [63]. In zebrafish, it causes seizures in larvae [62] but several studies have extended this model to adults [6,8]. Even the zebrafish model was used recently to discover new insights into the first mechanisms of epileptogenesis [64].

During studies of anticonvulsant compounds, important advantages include the ability to conduct behavior analysis and electroencephalographic (EEG) records on zebrafish in the larval stage and adults [64–66]. Electroencephalographic records in the zebrafish confirm PTZ proconvulsive activity, showing epileptiform discharges similar to rodent and human EEG profiles [7,67]. These spontaneous epileptiform discharges have characteristics of amplitude, frequency, and duration that vary with time of exposure to PTZ [65,68]. However, these analyses are outside the scope of this article, but will be carried out later.

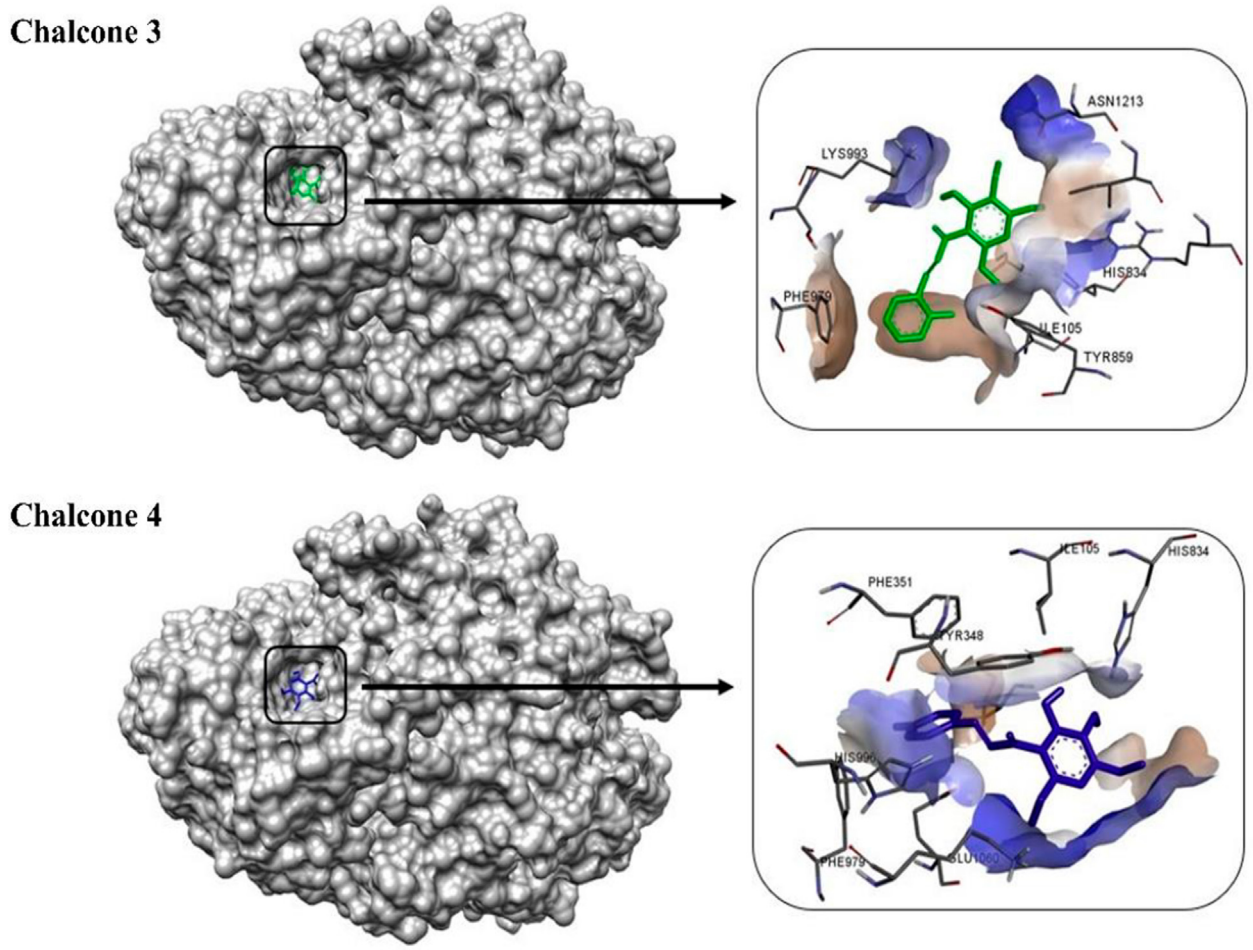


Fig. 10. Schematic 2-D representations the gamma-aminobutyric acid aminotransferase (GABA-AT) complexes with chalcones 3 and 4.

The results showed that 1 and 3 chalcone (Fig. 4Aand C) was effective in all doses as they increased the latency for the onset of convulsive stages, like Dzp. Chalcones 2 and 4 (Fig. 4B and D) also showed an anticonvulsant effect in one or two doses at each stage. Blocking the anticonvulsant effect of chalcones and Dzp by the antagonist flumazenil confirms previous findings and shows that these molecules act through the GABA system [69–71]. Regarding the structure–activity of the molecules, we can see that the presence of the two –Cl substituents provided chalcone with 1 effective anticonvulsant activity in all stages, when compared to chalcone 2 with only one –Cl substituent. This observation was also seen by Ibrahim [72] when analyzing the anticonvulsant activity of chalcone derivatives with a similar structure, and the derivative with –Cl substituent showed greater activity than the derivative with substituent (–F), as well as verified with chalcones 3 and 4 (Fig. 4C and D).

Chalcones are subclasses of flavonoid compounds, which are known to exert powerful anti-inflammatory effects on the brain through the activity of free radical scavenging [72] or by direct modulation of key components of the neuroinflammatory cascade. Consequently, this neuroprotective activity can be considered to explain the anxiolytic and anticonvulsant effect of these compounds [72–74].

Epilepsy has an inflammatory process in its etiology and thus studies have shown a protective effect of anti-inflammatories in animal models [25]. The anti-inflammatory activity of substituted chloro chalcone derivatives, with structures similar to chalcones 1 and 2 (Fig. 4A and B), showed anti-inflammatory activity and these effects were associated with the inhibition or suppression of inflammatory mediators such as TNF-a, NO, COX-2, and interleukins [75].

Structurally, all chalcones have 3 methoxy substituents (–OCH3). When investigating the anticonvulsant activity of methylated flavonoids by the PTZ-induced seizure model in zebrafish larvae, [24] observed that unmethylated flavonoids such as NRG (naringenin) and KFL (kaempferol) had only limited anticonvulsant activity and the methylation of NRGs (forming naringenin 7-O-methyl ether (NRG-M), and 4′,7-naringenin dimethyl ether (NRG-DM), had a clear impact on the result showing a better anticonvulsant effect. As with the previous methylated compounds, the results show that the synthesized chalcones had an anxiolytic effect and reversed all the stages of the seizure. It has been shown that the methylation of flavonoids favors their metabolic stability and membrane transport, where it facilitates absorption and positively affects their bioavailability [24,76].

The docking study can be used to illustrate the molecular interaction of new candidates at the protein–ligand interface. GABA-AT (γ-aminobutyric acid aminotransferase) is a validated target for AEDs and, being catabolic in nature, its selective inhibition increases GABA concentrations in the brain [77].

Docking studies were carried out to correlate structural changes with biological activities. In this context, the molecular interactions of chalcones with the protein GABA-AT (γ-aminobutyric acid aminotransferase), a validated target for AEDs, were analyzed and their selective inhibition increases GABA concentrations in the brain [49–51]. Regarding the chlorine substitutions in chalcones

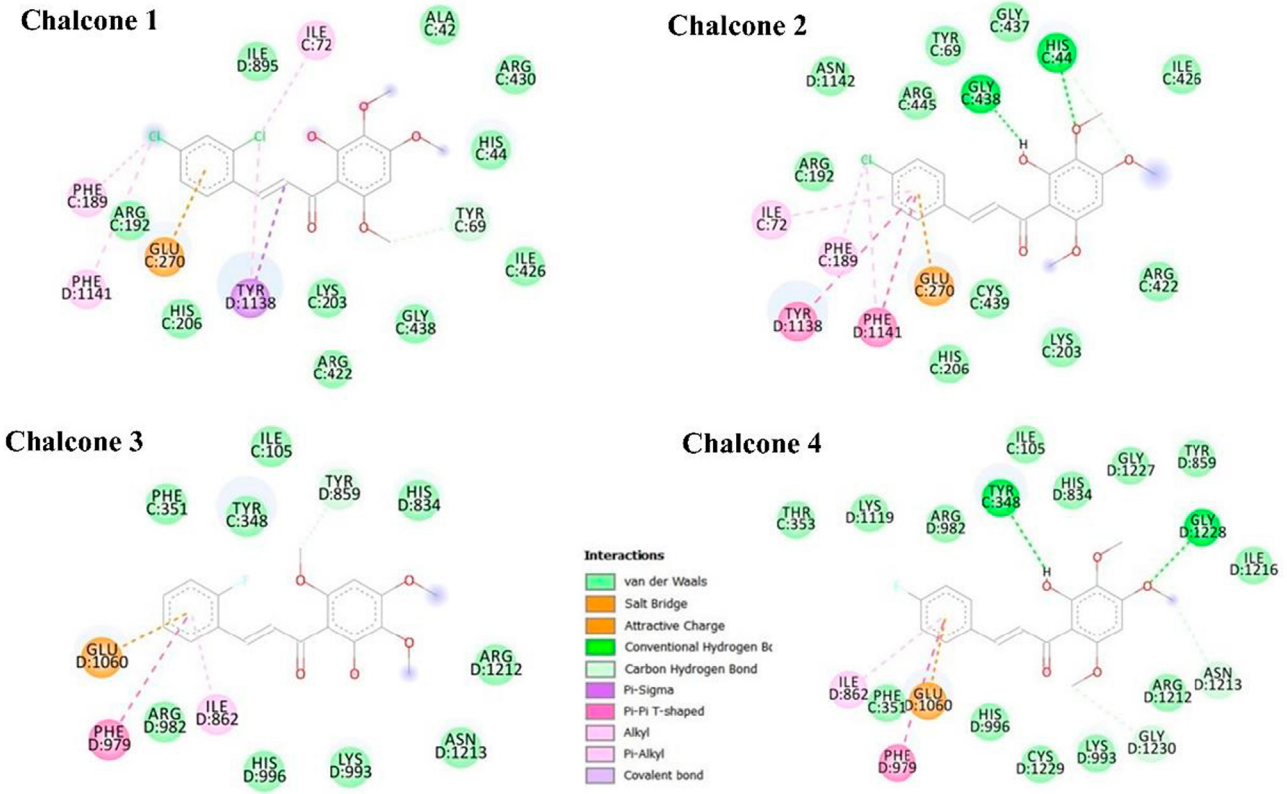


Fig. 11. 2D map of the molecular interactions of the Gamma-aminobutyric acid aminotransferase (GABA-AT) reeptor whit the chalcones 1–4.

1 and 2, in position R2, it was possible to observe that both interacted with residues ILE72, PHE189, ARG192, PHE1141, GLU270. However, there is a difference when there is a substitution in position R1 (chalcone A). The insertion of a chlorine atom at the R1 position increases the number of interactions with the TYR D: 1138 residue and changes the ligand accordingly, enabling interactions with the HIS44 and GLY438 residues. These interactions may be responsible for enhancing the activity of chalcone A. In the case of chalcone D, the replacement of fluorine (F) at position R2, causes a conformational change that makes the interaction of chalcone D difficult with the residue TYR D: 859 (>12 Å) and changes the arrangement of the interaction with the residue PHE C: 351. This new conformation hinders the interaction of chalcone D with the protein target. TYR859 has an important modulatory role in the activity, giving a greater effect to chalcone C, with 5.61 Å distance.

5. Conclusion

The synthesized chalcones presented anxiolytic and anticonvulsant effects, attenuating the convulsions induced by PTZ after pretreatment. The observed activities were completely antagonized by flumazenil, which implies indications of the involvement of GABA receptors. The interaction of the GABAA receptor will be confirmed by binding/activity assays. The study demonstrated in relation to the structure–activity, that the position of the substituents is important in the intensity of the activities since the substitutions in the positions R1 and R2 by chlorine potentiated the effects. It was also observed that the molecule with fluorine substituent in R1 showed efficacy in the lowest dose when compared to R2, that is, the position of the substituent significantly influenced the activity modulation. The absence of toxicity and the action of these compounds on the CNS evidence the pharmacological potential of these molecules and, thus, insights are projected for the development of new drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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