











Anxiolytic-like and Anticonvulsant Effect in Adult Zebrafish (*Danio rerio*) through GABAergic System and Molecular Docking Study of Chalcone Derived from Natural Products

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Received: 27.01.2021; Revised: 22.02.2021; Accepted: 25.02.2021; Published: 2.03.2021

Abstract: Anxiety and seizure are mental disorders commonly treated in the clinic with benzodiazepines (BZDs). However, long-term treatment with these drugs causes side effects such as decreased motor coordination, drowsiness, sedation, and, when used for a long time, leads to dependence and withdrawal syndromes. This study's objective was to evaluate the anxiolytic-like and anticonvulsant effect in adult zebrafish (*Danio rerio*) through GABAergic system of synthetic chalcone derived from cinnamaldehyde and natural acetophenone isolated from *Croton anisodontus* and their mechanisms of action via the GABAergic system, using adult zebrafish. The animals were treated with chalcone (0.1 or 0.5 or 1.0 mg / kg; 20 μ L; *i.p.*) and subjected to the open field test. The chalcone caused locomotor alteration when evaluated in the light & dark anxiolytic test. The lowest effective dose was chosen to assess possible involvement in the GABA_A receptor. The chalcone showed anxiolytic and anticonvulsant effects in all doses in both tests. The chalcone presented anticonvulsant activity, a possible inhibitor of Carbonic Anhydrase and an anxiolytic effect dependent on the GABAergic system, coupling it in a different region from the diazepam inhibitor.

Keywords: cinnamaldehyde; chalcone; Zebrafish; anxiety; seizures.

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1. Introduction

Zebrafish (*Danio rerio*) is a fish found in fresh water and has been used for pharmacological investigation of new bioactive compounds. This animal's use as an

experimental model is because it is a small animal, with easy maintenance, good reproductive rate, besides presenting a sequenced genome with 70% homology to that of the human being and anatomy of the central nervous system known. These advantages make using this model satisfactory in behavioral, toxicological, and pharmacological studies to elucidating mechanisms of several human diseases [1]. The literature points out that Zebrafish comes being used for studies of anxiety [2], seizure [3], Alzheimer's [4], inflammation [5], diabetes [6], cholesterol [7], fatty liver [8], cardiovascular disease [9, 10], oxidative stress [11] and antimicrobial and antifungal activities [12-14].

Anxiety and seizure are mental disorders commonly treated in the clinic with benzodiazepines (BZDs); these drugs were introduced into therapeutic practice more than 50 years ago due to their central nervous system depressing effects [15]. However, long-term treatment with these drugs causes side effects such as decreased motor coordination, drowsiness, sedation, and, when used for a long time, leads to dependence and withdrawal syndromes, and in high doses, they can be fatal. The anxiolytic-like effect shown by chalcones derivatives makes this class of compounds an important tool to discover new agents with increased strength, low cost, superior pharmacokinetic properties, and minimum side effects [16]. Its structure comprises at least two aromatic rings connected by an unsaturated α - β bridge, probably the most responsible for its biological activities [17-20]. They can be obtained from natural sources or by synthesis and are widely distributed in fruits, vegetables, and tea [21, 22]. This compound class has aroused much interest due to the wide spectrum of pharmacological activities present, including antinociceptive, anti-inflammatory, antitumor, antibacterial, Anxiolytic, antifungal, antileishmanial, and antioxidant activity [23, 24].

This work aimed to evaluate the anxiolytic-like and anticonvulsant effect in adult zebrafish (*Danio rerio*) through GABAergic system of chalcone (2*E*, 4*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-5-phenylpenta-2,4-dien-1-one derived from cinnamaldehyde and natural acetophenone isolated from *Croton anisodontus*.

2. Materials and Methods

2.1. Drugs and reagents.

Diazepam (Dzp, Neo Química®), Flumazenil (Fmz; Sandoz®), Dimethyl sulfoxide (3% DMSO; Dynamic®), Pentylentetrazole (PTZ, Sigma-Aldrich).

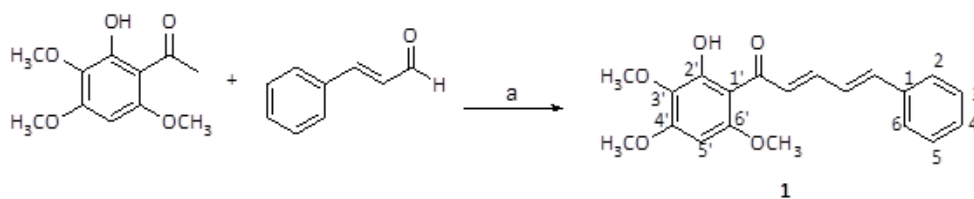
2.2. Synthesis and structural data.

The compounds 2-hydroxy-3,4,6 trimethoxyacetophenone (2 mmol) and cinnamaldehyde (2 mmol) were placed in a volumetric flask (25 mL). Then 5 mL of ethanolic NaOH (50%) solution was added and mixed with stirring for 48 h at room temperature. The progress of the reaction was checked by TLC (n-hexane: ethylacetate, 2:1). After 48 h the reaction mixture was neutralized with dilute HCl (10%) and ice water added. The product was obtained as a yellow solid (yield: 35.4%, m.p. 144.8–145.2 °C) filtered under reduced pressure, washed with cold water, and recrystallized from ethanol (Scheme 1) [25].

(2*E*, 4*E*)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-5-phenylpenta-2,4-dien-1-one (1)

Yellow solid (Yield: 35.4%), m.p. 144.8-145.2°C. IV (KBr, $\nu_{cm^{-1}}$): 1634, 1600, 1588, 1575, 1480, 1167. ¹H RMN (CDCl₃, ppm): 3.84 (s, MeO); 3.93 (s, MeO); 3.95(s, MeO); 6.99 (s, H-5'); 7.44 (m, H-4); 7.38 (d, H-3/5, J = 7,1 Hz.); 7.51 (d, H-2/6, J=7,2 Hz); 7.62 (d, H α , J=14,6 Hz); 7.64 (d, H β , J=14,6 Hz); 7.40 (d, H α , J=14,6 Hz); 7.43 (d, H β , J=14,6 Hz). ¹³C

RMN (CDCl_3 , ppm): 193.2 (C=O); 60.9 (MeO-3'); 56.1 (MeO-4'); 56.2 (MeO-6'); 107.0 (C-1'); 158.7 (C-2'); 131.0 (C-3'); 159.6 (C-4'); 87.2 (C-5'); 158.5 (C-6'); 136.5 (C-1); 127.7 (C-2/6); 129.1 (C-3/5); 131.0 (C-4); 129.2 (C α); 143.5 (C β); 127.0 (C α); 141.8 (C β). MS-EI m/z = 340).



Scheme 1. Preparation of chalcone. a) NaOH 50 % w v⁻¹, ethanol, t.a., 48 h.

2.3. Involvement of the GABAergic system in anxiety.

To assess the involvement of the GABAergic system in anxiolytic activity, a group of animals ($n = 6$ / each) received the GABAA antagonist flumazenil (4.0 mg/kg; *i.p.*) 15 minutes before the chalcone cinnamaldehyde or diazepam. After 30 min of the treatments, the animals were submitted to the light & dark test [16].

2.4. Pentylentetrazole-induced seizure (PTZ).

PTZ-induced seizure reversal was investigated ($n = 6$ / group) and the animals were treated with chalcone (0.1; 0.5 and 1 mg / mL; 20 μL ; *ip*), Diazepam (1.0 mg / mL; 20 μL ; *ip*), vehicle (3% DMSO; 20 μL ; *ip*). An untreated group ($n = 6$ / group) was included (Naive). After 30 min, the animals were exposed to PTZ at 7.5 mM, and the behavior similar to seizure in three stages was evaluated: 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 [26, 27]. At the end of the analysis of the 3 stages of the test, the animals were euthanized on the ice.

2.5. Docking of the anxiolytic effect on the GABAergic system.

For the study of molecular docking involving anxiolytic activity, the GABA_A receptor was selected. Its structure was obtained from the Protein Data Bank database (<https://www.rcsb.org/>), identified as “CryoEM structure of human full-length $\alpha 1\beta 3\gamma 2\text{L}$ GABA (A) R in complex with diazepam (Valium), GABA and megabody Mb38” (PDB 6HUP), deposited with a resolution of 3.58 Å, determined by electron microscopy, classified as a membrane protein, *Homo sapiens* organism, and *Homo sapiens* and *Escherichia coli* expression system. Computer simulations were performed using the AutoDock Vina code (version 1.1.2), using 3-way *multithreading*, *Lamarckian Genetic Algorithm* [28]. The grid box was defined with parameters of 126Åx100Åx126Å, centered on the entire protein with the dimensions (x, y, z) = (125,281, 139,534, 136,018). 50 independent simulations were carried out with 20 poses each as a standard procedure. For the selection of simulations with better poses, the simulations that presented RMSD (Root Mean Square Deviation) value less than 2 Å [29] and free binding energy (ΔG) below -6.0 kcal/mol were used as criteria [30]. The Discovery Studio Visualizer [31] and UCSF Chimera codes were used to analyzing the results and generate the two-dimensional chemical interaction maps [32].

2.6. Docking of the anticonvulsant effect.

For the *in silico* study of the potential anticonvulsant effect of chalcone, the structure of the enzyme Carbonic Anhydrase II (AC II) (PDB 3F8E) was obtained from the Protein Data Bank database (<https://www.rcsb.org/>), identified as “Coumarins are a novel class of suicide carbonic anhydrase inhibitors”. The protein structure is deposited in the Protein Data Bank with the co-crystallized TE1 inhibitor (coumarin), with a resolution of 2.00 Å, determined by X-Ray Diffraction, classified as lyase and Homo sapiens organism. For docking simulations, the AutoDock Vina code (version 1.1.2) was used [28]. The grid box was defined with parameters of 94Åx96Åx106Å, centered on the whole protein with the dimensions (x, y, z) = (-8,086, -0,658, 17,136), being carried out 50 independent simulations with obtaining 20 poses each. For the selection of simulations with better poses, the simulations that presented RMSD (Root Mean Square Deviation) value less than 2 Å [29] and free binding energy (ΔG) below -6.0 kcal/mol were used as criteria [30]. The results were analyzed and visualized using the codes Discovery Studio Visualizer [31] and UCSF Chimera [32].

2.7. Statistical analysis.

The zebrafish results were expressed as mean \pm 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 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).

3. Results and Discussion

3.1. Mechanism of anxiolytic action.

Flumazenil reduced (### p <0.001) the anxiolytic effect of chalcone (0.5 mg / mL; 20 μ L; *ip*) and Diazepam (1.0 mg / mL; 20 μ L; *ip*) (Figure 1). The anxiolytic activity of chalcone performed by Xavier et al. (2020) aroused the interest in investigating the mechanism of anxiolytic action of chalcone. Thus, we performed a light & dark test using flumazenil, a GABAA receptor antagonist. Based on Fig. 1, pretreatment with flumazenil altered the effects of chalcone (0.5 mg / mL; 20 μ L; *ip*) and Diazepam (1.0 mg / mL; 20 μ L; *ip*) in adult zebrafish, that is, it significantly reduced (p <0.0001 vs. Vehicle and Naive) the anxiolytic/sedative effect of the animals, indicating that the chalcone’s anxiolytic activity is dependent on the GABAergic system.

3.2. Docking of the anxiolytic effect on the GABAergic system.

The best conformation simulation presented an RMSD of 1,746 Å and a high-affinity energy, in the order of -8.1 Kcal/mol, highlighting that the complex formed presented interactions varying between 1.98Å and 3.64Å (Table 1).

In order to investigate the potential anxiolytic activity of chalcone in the GABAergic system, docking simulations were carried out with the GABA_A receptor, one of the most important drug targets for presenting several allosteric sites, which allow the binding of compounds with anxiolytic properties, such as benzodiazepines, modulators typically positive [33]. Regarding the interactions (Figure 2), it was possible to identify the formation of four hydrogen bonds with the GABA_A receptor, three of which were classified as strong [34] with

the LYS1191D, TYR753C, SER890C residues, and a moderate interaction with the ASP887C residue. The chalcone also presented three hydrophobic interactions with residues TYR753 and PHE772, coupling in a different region from the inhibitor Diazepam (Figure 3), a classic agonist of the GABA_A receptor, thus being an indicator of a potential candidate for studies on synergism.

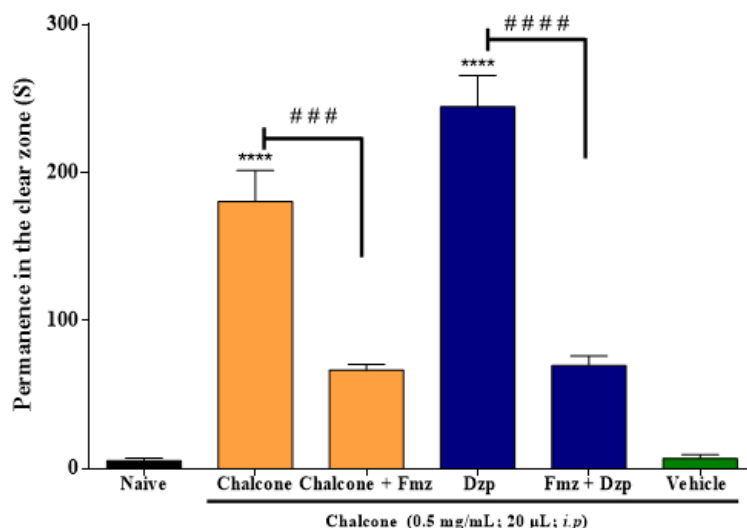


Figure 1. Effect of Flumazenil (Fmz) under the anxiolytic effect of the Chalcone - Cinnamaldehyde in the Light & Dark Test. Chalcone - Cinnamaldehyde (0.5 mg / mL; 20 μ L, *i.p.*). Dzp - Diazepam (1.0 mg / mL; 20 μ L, *i.p.*). Fmz - flumazenil (0.1 mg / mL; 20 μ L; *i.p.*). The values represent the mean \pm standard error of the mean (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).

Table 1. Interactions between the GABA_A receptor and the chalcone ligand.

Ligand	Receptor	Interaction	Distance (\AA)
Chalcone	TYR753C	H-Bond	2.36 \AA
	ASP887C	H-Bond	3.20 \AA
	SER890C	H-Bond	2.24 \AA
	LYS1191D	H-Bond	1.98 \AA
	TYR753C	Hydrophobic	3.61 \AA
	PHE772C	Hydrophobic	3.63 \AA
	PHE772C	Hydrophobic	3.64 \AA

3.3. Pentylene-tetrazole-induced seizure.

The anticonvulsant effect of chalcone was evaluated through seizures induced by PTZ in adult zebrafish. Chalcone (0.1; 0.5 and 1.0 mg / mL; 20 μ L; *ip*) as well as the positive control Dzp (1 mg / mL; 20 μ L; *ip*) increased latency time (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. vehicle) for the onset of PTZ-induced crises in the three stages, suggesting an anticonvulsant action (Figure 2). Pentylene-tetrazole is a chemoconvulsant that acts allosterically on the GABA_A receptor [35] and is used in Zebrafish to induce epileptic-like effects study seizures [36, 37]. We evaluated the anticonvulsant effect of chalcone and Dzp on PTZ-induced seizures in adult zebrafish. Both increased the latency time for the onset of PTZ-induced crises in the three stages, suggesting an anticonvulsant action (Figure 3). Antiepileptic drugs can have a sedative effect, and the treated animals show slow movements or alter locomotion [26, 27], as was demonstrated in another study carried out with chalcone cinnamaldehyde [25]; in our study, the highest doses of chalcone caused the motor impairment, which may justify its anticonvulsant action (Figure 3A).

Flumazenil is well known as an antagonist of GABAergic receptors in $\alpha\beta\gamma$ subunits [1-3, 5]. It antagonizes the sedative effects caused by benzodiazepine overdoses and reverses these effects in addition to preventing respiratory depression by blocking GABA receptors [38, 39]. Thus, it is an excellent tool for the study of seizures in this receptor. We investigated the association of Diazepam and chalcone in the GABAA receptor in PTZ-induced seizures in Zebrafish (Figure 1). Our data show that pretreatment with flumazenil blocked the anticonvulsant effect of chalcone and Dzp in adult zebrafish in the three stages of the seizure (Figure 3).

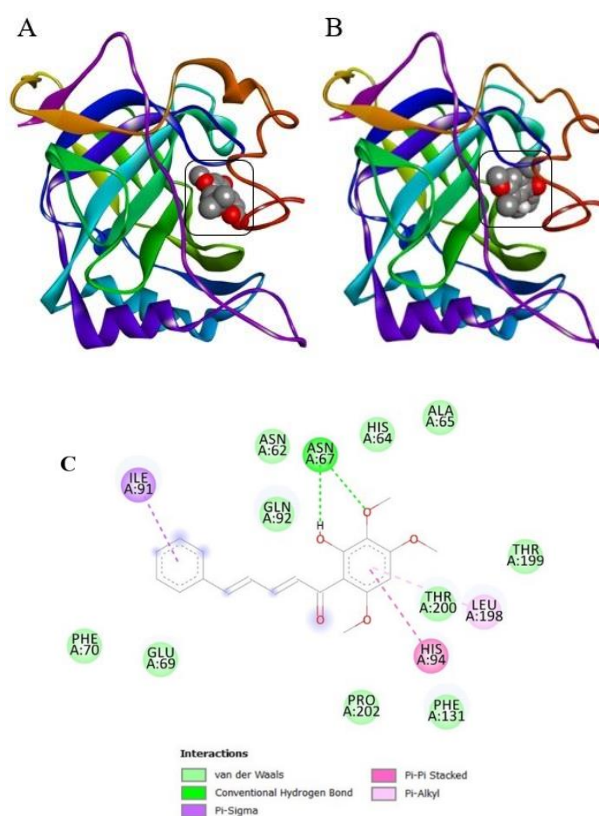


Figure 2. Interaction complex of inhibitor TE1 (A) and Chalcone (B) with the enzyme Carbonic Anhydrase II, and 2D map of interactions of the Carbonic Anhydrase II / chalcone complex (C).

3.4. Docking of the anticonvulsant effect.

The docking simulation between the chalcone ligand and the Carbonic Anhydrase II with better conformation presented RMSD of 1.72 Å and affinity energy in the order of -5.9 Kcal/mol. The formed complex presented interactions varying between 2.38Å and 3.72Å (Table 2).

Table 2. Interactions between the enzyme Carbonic Anhydrase II and the chalcone ligand.

Ligand	Receptor	Interaction	Distance (Å)
Chalcone	*ASN67A	H-Bond	2.46 Å
	*GLN92A	H-Bond	2.38 Å
	*ASN62A	H-Bond	3.47 Å
	*ILE91A	Hydrophobic	3.66 Å
	*ILE91A	Hydrophobic	3.69 Å
	*GLU69A	Hydrophobic	3.68 Å
	LEU198A	Hydrophobic	3.69 Å

* Residues belonging to the catalytic site of the co-crystallized inhibitor

For the study of the anticonvulsant effect, the enzyme Carbonic Anhydrase II was selected. Although the mechanisms that trigger seizures are not yet specifically known, pH changes in the brain are one of the related factors. The $\text{CO}_2 / \text{HCO}_3^-$ buffer is the main responsible for the buffering of the intra and extracellular spaces, with the Carbonic Anhydrase enzyme responsible for the balance of the two buffer species [40]. Carbonic anhydrase inhibitors have been studied as anticonvulsant drugs in epilepsy since the 1950s [41].

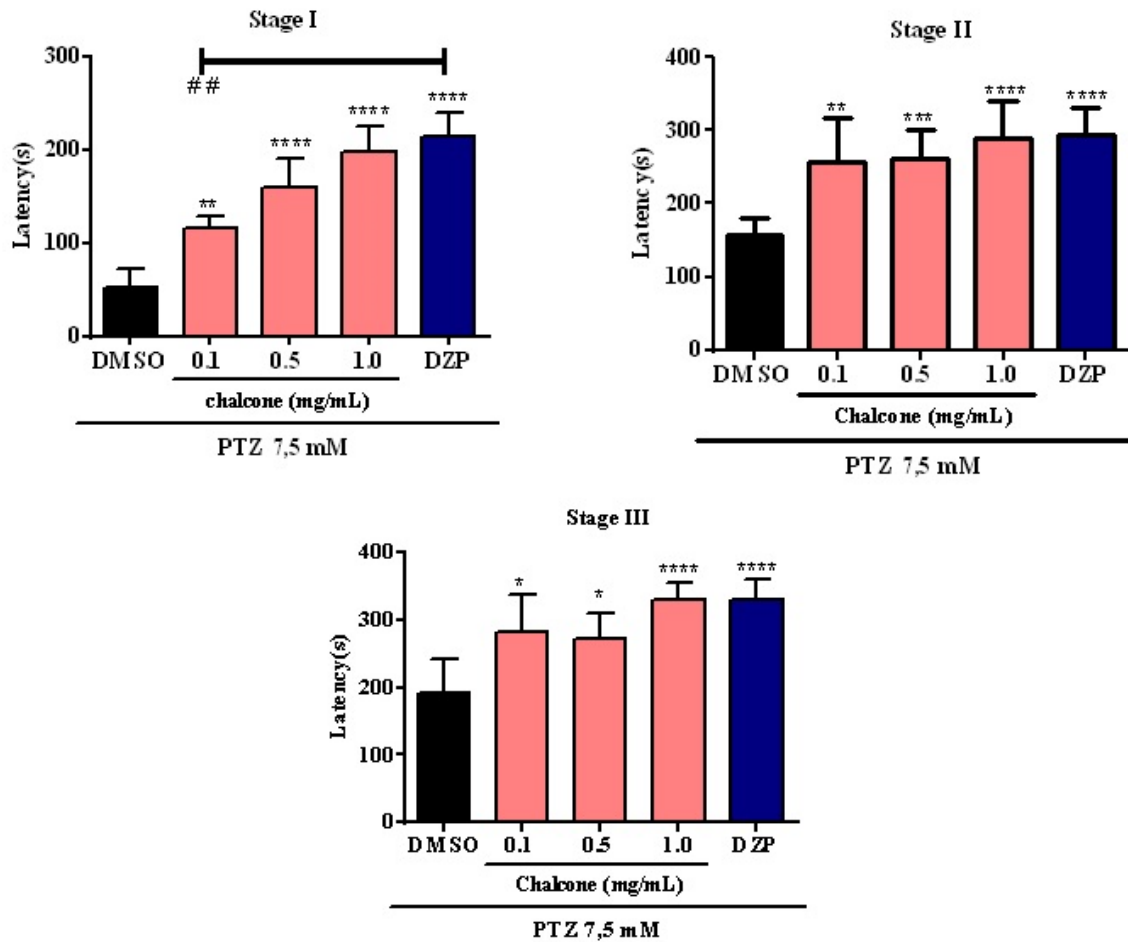


Figure 3. Effect of cinnamaldehyde chalcone on pentylenetetrazole induced seizure in adult zebrafish, stage I (A), stage II (B), stage III (C). Dzp-Diazepam (1.0 mg/mL; 20 μL ; *p.o.*); Vehicle – 3% DMSO (20 μL ; *p.o.*). The values represent the mean \pm standart error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by *Tukey* (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. Naive; ## $p < 0.01$ vs. Dzp).

The chalcone presents two hydrogen bonds, classified as strong [34] with residues ASN67A, GLN92A and medium intensity with residue ASN62A, being said residues belong to the catalytic site of the protein target. The AC II/chalcone complex also showed hydrophobic interactions with the ILE91A residue of the active site and GLU69A and LEU198A residues of the target protein (Figure 4). In this context, the chalcone ligand coupled in the same region as the co-crystallized inhibitor's active site (Figure 4), demonstrating a potential inhibitory effect on the carbonic anhydrase enzyme.

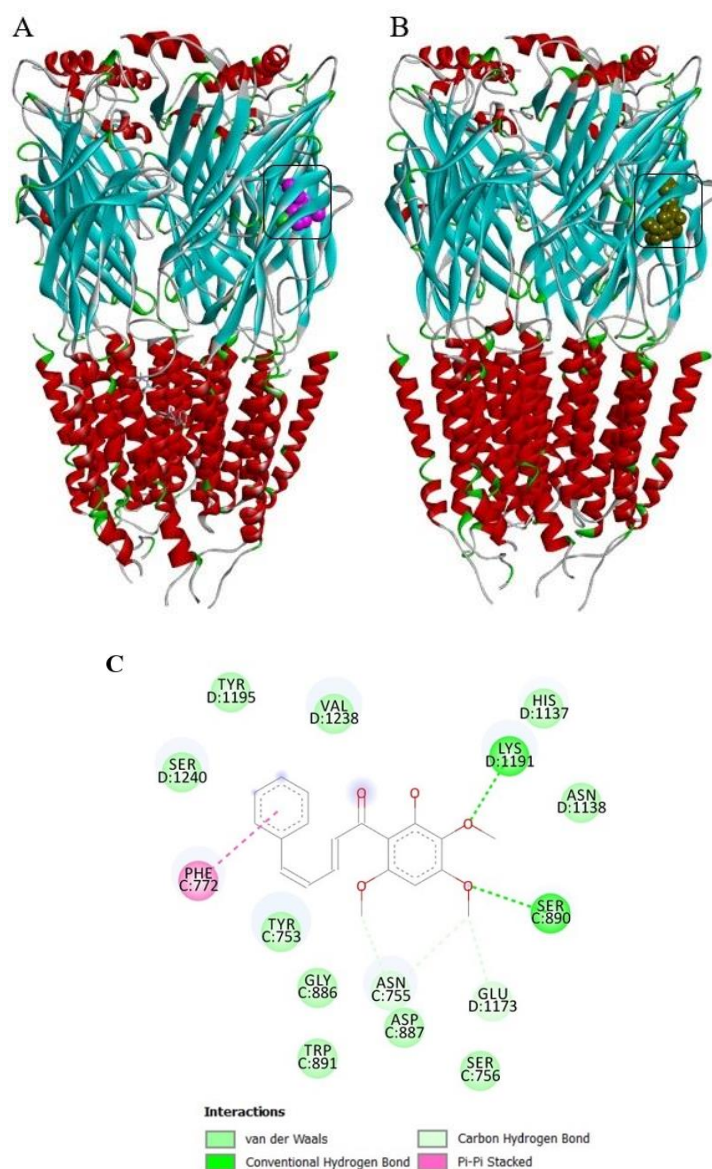


Figure 4. Diazepam (A) and Chalcone (B) interaction complex with the GABA_A receptor, and 2D map of interactions of the GABA_A / chalcone complex (C).

4. Conclusions

The chalcone presented anticonvulsant activity, being a possible inhibitor of Carbonic Anhydrase and an anxiolytic effect dependent on the GABAergic system, coupling it in a different region from the diazepam inhibitor, thus being an indication of a potential candidate for studies on synergism.

Funding

This research was funded by PQ/BPI-FUNCAP (Grant#: BP4-0172-00075.01.00/20) and CNPq (Grant: 305719/2018-1).

Acknowledgments

The authors thank FUNCAP, CAPES, CNPq, and CENAUREMN - Centro Nordestino de Aplicação e Uso da Ressonância Magnética Nuclear.

Conflicts of Interest

The authors declare no conflict of interest.

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