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## Research Article

**Keywords:** Ansiedade, Serotoninérgico, Memória, Docking, Zebrafish

**Posted Date:** July 2nd, 2026

**DOI:** <https://doi.org/10.21203/rs.3.rs-9849150/v1>

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**Additional Declarations:** No competing interests reported.

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# **Natural Phenolic Acids Modulate Memory and Anxiolytic Behavior in Adult Zebrafish (Danio Rerio): Serotonergic Involvement, Oxidation-Dependent Activity, Quantum Calculations, and Molecular Docking Highlight Sinaptic acid**

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## **ABSTRACT**

This study investigated the anxiolytic and memory-preserving effects of cinnamic acid and its derivatives in adult zebrafish (*Danio rerio*). The compounds were administered at doses of 4, 20, and 40 mg/kg and showed no toxicity after 96 hours of monitoring. In behavioral experiments, it was observed that the highest dose of CiAc, as well as the lowest and highest doses of CaAc, compromised motor functions, reducing the animals' swimming, however, they demonstrated better responses than the reference drug. All compounds exhibited anxiolytic-like behavior in the light-dark test, evidenced by increased time spent in the light zone of the aquarium. All substances, each substance, were blocked by at least one serotonergic antagonist (pizotifen: 5-HTR1/5-HTR2A/2C, granisetron: 5-HTR3A/3B, or cyproheptadine: 5-HTR2A), which strongly suggests that the anxiolytic effect broadly involves the serotonergic system, acting on multiple 5-HT receptor subtypes. For the learning and memory analyses, the inhibitory avoidance test was conducted, showing a pattern in memory retention where, as specific functional groups were introduced into the reference molecule (in this case, CiAc), an improvement in memory retention of the animals was observed. The results indicated that the oxidation of these compounds was an important factor in their anxiolytic effects and memory preservation; these data are consistent with the results obtained from in-silico analyses. Given this, SiAc (sinapic acid), the most oxidized compound analyzed in this study, exhibited the best anxiolytic effect at all tested doses and was the compound that best retained memory.

## **KEYWORDS**

Ansiedade; Serotoninérgico; Memória; Docking; Zebrafish

## INTRODUCTION

Anxiety and depression are at the top of the list of diseases that most affect the population, both nationally and worldwide<sup>[1]</sup>. Conventional anxiolytic drugs, such as benzodiazepines, buspirone, and chronically administered selective serotonin reuptake inhibitor antidepressants (SSRIs), are widely used to reduce stress and clinical anxiety<sup>[2]</sup>. However, continued use of these medications may be associated with challenges such as dependence, tolerance<sup>[3]</sup>, and potential side effects related to memory loss<sup>[4]</sup>.

Natural products constitute an important source of new drugs for the treatment of different pathologies, including depression, in which some compounds have already demonstrated antidepressant activity, establishing themselves as promising therapeutic alternatives<sup>[5]</sup>. Among these compounds, cinnamic acid stands out, a polyphenol derived from phenolic acid and one of the most abundant plant secondary metabolites, present in several plants such as cinnamon, grape, tea, cocoa, spinach and celery<sup>[6,7]</sup>. This acid and its derivatives have the ability to interact with multiple therapeutic targets, which gives them broad-spectrum biological properties, including antibacterial<sup>[8,9]</sup>, antiviral<sup>[10,11]</sup>, antifungal<sup>[12]</sup> and anticancer activities<sup>[13]</sup>.

Hybrid molecules with two or more different pharmacophores are the most popular strategies for the discovery and development of new drugs, as hybrids have the ability to modulate multiple targets and counterbalance side effects<sup>[14,15]</sup>.

The biological activities of different cinnamic acid derivatives have been attributed to the nature and position of the substituent groups. Drug resistance and the absence of curative therapies with low adverse side effect profiles have led to research into the development of therapeutic compounds based on cinnamic acid, as well as its derivatives<sup>[16]</sup>.

Given the recognized biological efficacy of cinnamic acid derivatives, the use of animal models becomes essential to validate their pharmacological potential and understand their mechanisms of action. In this context, zebrafish stands out for preserving the main glutamatergic and inhibitory excitatory neurotransmission pathways, in addition to expressing muscarinic cholinergic receptors<sup>[17]</sup>. Reports of neurobehaviors associated with learning and memory in this model date back more than two decades<sup>[18]</sup>. Since then, a broad repertoire of behaviors comparable to humans, including locomotor activity, anxiety-like responses, learning processes, memory retention, spatial and object recognition, fear reactions, as well as social preference and interaction, has been characterized in this animal<sup>[17]</sup>. This evidence has consolidated zebrafish as a relevant vertebrate model in preliminary research on cognitive decline, especially in the search for new drugs.

Thus, the present study aimed to evaluate the anxiolytic effect, as well as the relationship with learning and memory, of four cinnamic acid derivatives: p-coumaric acid, caffeic acid, ferulic acid and sinapic acid, in adult zebrafish (*Danio rerio*), aiming to understand their therapeutic potential in relevant pathophysiological conditions.

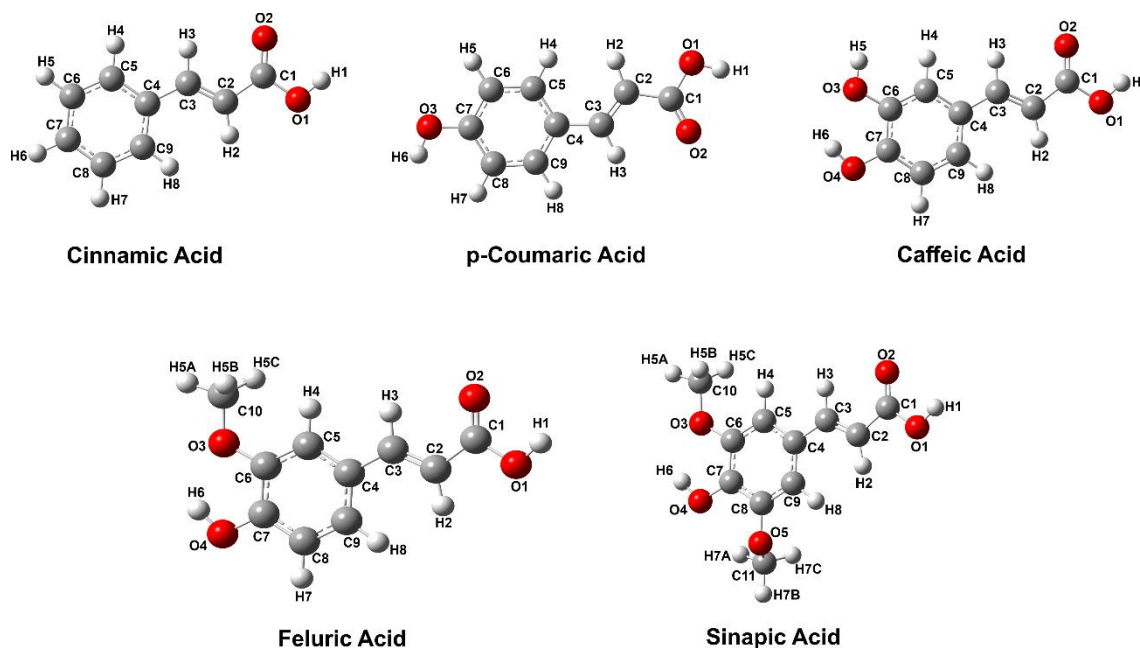


**Figure 1.** Chemical structure of derivados do ácido cinâmico.

## EXPERIMENTAL SECTION

### Drugs and Reagents

The following substances were used: Diazepam (DZP, Neo Química), Flumazenil (Fmz; Sandoz), Dimethyl sulfoxide (3% DMSO; Dynamic), Fluoxetine (Sandoz), Ethanol (Sigma-Aldrich), and Donepezil (DNZP) (Sigma-Aldrich). Cinnamic acid (CiAc), p-Coumaric acid (CuAc), Caffeic acid (CaAc), Ferulic acid (FeAc), and Sinapic acid (SiAc) were also obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

### Computational details

The geometry optimization of the acids molecules was performed by using Gaussian 16 code (M. J. Frisch et al., 2019). The exchange-correlation functional M062-X (Zhao & Truhlar, 2008) by Truhlar group with Pople set basis 6-311++g(2d,2p) (Krishnan et al., 1980) was chosen as density functional theory level for calculations. To ensure that the minimum energy was reached in geometry optimization, the normal modes were calculated. No presence of the

imaginary mode shows that the molecule reached a structural configuration that represents minimum energy. The structural optimization was carried out considering the following convergence threshold:  $2 \times 10^{-6}$  Ha/Bohr for maximum force with RMS  $1 \times 10^{-6}$  Ha/Bohr and maximum displacement of  $6 \times 10^{-6}$  Å with RMS of  $4 \times 10^{-6}$  Å. The geometry was used to analyze the HOMO and LUMO energies and shapes with GaussView 6 visualizer (Roy Dennington et al., 2016). The quantum descriptors (Wang et al., 2021) were calculated using the values of HOMO/LUMO energies. For Fukui indexes, the Multiwfn 3.8 (Lu, 2024; Lu & Chen, 2012) was used to calculate the values of  $f^0$ ,  $f^+$ ,  $f^-$  and dual descriptors  $f^{DD}$ .

### **Ensaio *in vivo* and acute toxicity (96 h)**

The experimental procedures were approved by the Animal Ethics Committee of the State University of Ceará (CEUA-UECE) under protocol number 04383945/2021. Adult zebrafish (*Danio rerio*), wild-type strain, were obtained from a local supplier in Fortaleza, Ceará. The animals were acclimated for 24 hours in a 10 L tank at 25 °C and a pH of approximately 7.0. After the experimental analyses, the animals were euthanized by immersion in ice-cold water (2–4 °C) for 10 minutes. Adult zebrafish (ZFa;  $n = 6/\text{group}$ ) received intraperitoneal (i.p.) treatment with 20 µL of the samples at doses of 4, 20, or 40 mg/kg. As a negative control, 3% DMSO (20 µL; i.p.) was used. After 24, 48, 72, and 96 hours, the number of dead animals was recorded, and the data were statistically analyzed to estimate the lethal dose capable of causing the death of 50% of the population ( $LD_{50}$ ) using the Trimmed Spearman–Kärber mathematical method with 95% confidence intervals [49].

### **Assessment of locomotor activity (open field test - OFT) and anxiolytic assessment (Light/Dark Test - LDT)**

To evaluate changes in motor coordination, the open field test was performed [4,50]. In this test, animals ( $n = 6/\text{group}$ ) received intraperitoneal (i.p.) treatment with 20 µL of the samples at doses of 4, 20, or 40 mg/kg. The vehicle group (control, 3% DMSO; 20 µL) and DZP (4 mg/kg; 20 µL) were also evaluated. Thirty minutes after treatment, the animals were transferred to Petri dishes divided into quadrants containing the same water as the aquarium. Locomotor activity was assessed by counting the number of line crossings (CL) over a 5-minute period. The anxiolytic effect was analyzed using the light/dark test. Animals ( $n = 6/\text{group}$ ) received i.p. treatment with the samples (4, 20, or 40 mg/kg), vehicle (3% DMSO; 20 µL), or

DZP (4 mg/kg). Thirty minutes after administration, they were transferred to the light zone of an aquarium (30 × 15 × 20 cm), equally divided into light and dark areas and filled with dechlorinated tap water to a height of 3 cm. The anxiolytic effect was determined by the time spent in the light zone during a 5-minute observation period. For serotonergic neuromodulation assessment, animals (n = 6/group) were pretreated with the antagonists cyproheptadine (0.8 mg/kg; 10 µL; i.p.), pizotifen (0.8 mg/kg; 10 µL; i.p.), and granisetron (0.5 mg/kg; 10 µL; i.p.). After 15 minutes, they received the lowest effective dose of each derivative. Control groups were treated with DZP (4.0 mg/kg; 20 µL; i.p.) or vehicle (3% DMSO; 20 µL; i.p.). Thirty minutes after treatment, the animals were again subjected to the light/dark test [50,51].

### **Learning and Memory - Inhibitory Avoidance Test**

The evaluation of CiAc and its derivatives in inhibitory avoidance was performed as described by [52]. The apparatus consisted of a glass tank (28 cm × 14.7 cm × 19 cm) containing 1.3 L of dechlorinated water. The tank was divided into two identical compartments (black and white) by a manually operated guillotine door (10 × 10 cm). In the black compartment, three sets of metal bars (1 cm diameter), spaced 3 cm apart, were connected to an electroshock device to deliver an aversive stimulus. Fish were exposed to pulsed shocks at 100 Hz for 5 seconds. Each group of animals (n = 6/group), previously isolated individually in 500 mL containers and identified, underwent one training session. During training, each fish was placed in the white compartment. After one minute of adaptation, the guillotine door was raised, and the latency time to enter the black compartment was recorded. Once the fish crossed into the dark compartment, the door was closed, and a mild electric shock (125 mA, 3 ± 0.2 V) was administered. Afterward, the fish were treated intraperitoneally with each derivative at three different doses (4, 20, or 40 mg/kg, one dose per group). An additional group was treated with donepezil (DNZP) (0.5 mg/L, i.p.), and another received 3% DMSO (negative control; vehicle). Twenty-four hours later, the test session was conducted following the same procedure as training but without shock administration.

### **Molecular Docking Procedures**

#### **Ligand and Receptor Preparation for Docking Studies**

The chemical structures of CiAc, CaAc, CuAc, FeAc, and SiAc were drawn using MarvinSketch (<https://chemaxon.com/marvin>). The lowest-energy conformers were saved at physiological pH and subsequently optimized using Avogadro (<https://avogadro.cc/>), configured to use the steepest descent algorithm and the MMFF94 force field (Merck Molecular

Force Field) [23]. To investigate the mechanism of action of the molecules on 5-HTR1A, 5-HTR2A, 5-HTR2C, and 5-HTR3A receptors, structures were obtained from the Protein Data Bank (<https://www.rcsb.org/>) [54], PDB IDs: 7E2Y [55], 6A93 [56], 6BQH [57], and 6NP0 [30], respectively. During receptor preparation, residues were removed, polar hydrogens were added, and Kollman and Gasteiger charges were assigned [58] using AutoDockTools™ [59]. For AChE, the target structure was also obtained from the Protein Data Bank [54], PDB ID: 4EY6. Residues were removed, polar hydrogens were added, and Kollman and Gasteiger charges were assigned [58] using AutoDockTools™ [60].

### **Molecular Docking Simulation and Data Output**

Molecular docking simulations were performed using AutoDock Vina [61] with the Lamarckian Genetic Algorithm (LGA), exhaustiveness set to 64 [62], and grid boxes covering the entire receptor structure. Grid parameters were as follows: 5-HTR1A: center (96.411, -107.337, 89.080), size (76, 84, 122); 5-HTR2A: center (46.160, 1.112, 39.550), size (117, 58, 114); 5-HTR2C: center (44.329, 33.258, 33.996), size (90, 70, 126); 5-HTR3A: center (159.618, 159.700, 161.062), size (96, 84, 120). Comparative simulations were performed using fluoxetine (reference drug) and the antagonists employed in experimental assays: pizotifen (5-HTR1A and 5-HTR2C), cyproheptadine and pizotifen (5-HTR2A), and granisetron (redocking, 5-HTR3A). Fifty simulations were performed, generating 20 poses per simulation. Best poses were selected based on RMSD values  $\leq 2.0$  Å [63] and binding affinity energy  $\leq -6.0$  kcal/mol [29]. Binding site and cavity predictions were obtained using the ProteinsPlus virtual platform (Zentrum für Bioinformatik: Universität Hamburg - Proteins Plus Server) [64]. Docking simulations for AChE were also performed using AutoDock Vina [33], LGA, exhaustiveness 64 [38], and grid box with center (-0.430, -51.377, 4.120) and size (85, 93, 120). Fifty simulations were conducted, generating 20 poses per simulation, with selection based on RMSD  $\leq 2.0$  Å [39] and binding affinity  $\leq -6.0$  kcal/mol [36]. Redocking was performed with the co-crystallized inhibitor galantamine to validate the docking protocol. Binding site and cavity predictions were also carried out using the ProteinsPlus platform [64].

### **Statistical Analysis**

Results are expressed as mean  $\pm$  standard error of the mean (SEM) for each group of six animals. After confirming normality and homogeneity of variance, differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. All analyses were performed using GraphPad Prism v.8.0. The level of statistical significance was set at  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Frontier Orbitals and Fukui index

The frontier orbitals for cinnamic, caffeic, ferulic, sinapic and p-coumaric acid are depicted in Figure X. For cinnamic acid, the highest occupied molecular orbital (HOMO) is spread in the entire molecule due to the presence of the electronic conjugation from aromatic ring to carbonyl group. The Figure 2 suggests that the atomic orbitals with main contribution to HOMO are  $\pi$ -type from C=C bonds and non-ligand electrons pair from Oxygen atoms. The presence of the -OH and -OCH<sub>3</sub> groups in the cinnamic acid derivatives increases the spreading of the electronic density, with contributions of the electronic pairs of the Oxygen atoms from both groups and  $\sigma^*_{C-H}$  of methyl group. The electronic density is more pronounced on substituent groups occupying *para* positions. The density of lowest unoccupied molecular orbital (LUMO) on cinnamic acid is spread on entire molecule. The shape and location of the electronic density suggest the contribution of the  $\pi^*_{C=C}$  and  $\pi^*_{C=O}$  for the LUMO composition. The presence of hydroxyl group at *para* position increases the spreading of the electronic density along the molecule.

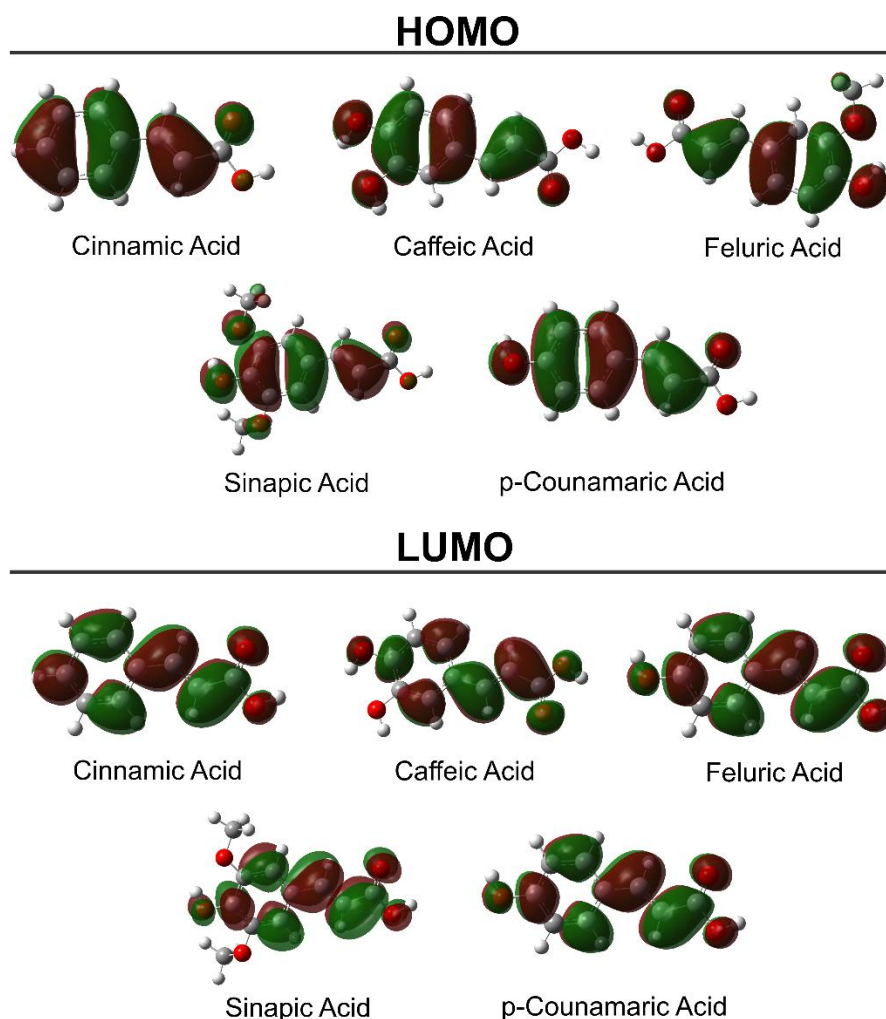


**Figure 2.** Frontier orbitals of cinnamic, caffeic, feluric, sinapic and p-counamaric acids calculated at DFT/M062-X/6-311++g(2d,2p) level theory

The energy values for HOMO and LUMO levels of cinnamic acid are -8.08 and -1.19 eV, respectively. The addition of the oxygenated groups in the benzenic ring have increased its energy, when we look for cinnamic acid derivates, where the greater variation is observed when there is presence of -OH or -OCH<sub>3</sub> groups, as was observed for feluric and sinapic acids, with feluric acid presenting the greater energy of LUMO level (-0.98 eV). The structural differences presented by cinnamic acid derivates cause the decreasing of the electronic gap, according to DFT/M062-X/6-311++g(2d,2p) results. Its indicate that the cinnamic acid derivates are less stable the precursor molecule and, consequently, more reactive than it. The molecules with greater sedative power have presented lower energy for LUMO level.

According to Koopman's theorem(Koopmans, 1934), the first ionization potential and electronic affinity are related to HOMO and LUMO levels, respectively, by  $IP = -E_{HOMO}$  and  $EA = -E_{LUMO}$ . So, analyzing the values of its levels, we can observe that the cinnamic acid derivates have lower values than it. The molecules that present better sedative activity have lower values of EA; FeAc (0.98), SiAc (1.00) and CuAc (1.00). Moreover, FeAc and SiAc molecules shows the lower values of the IP between studied molecules.

The quantum descriptors (Morell et al., 2005) derived from HOMO/LUMO levels are listed in Table 1. The addition of the -OH and -OCH<sub>3</sub> groups in the phenyl fragment decrease the chemical potential ( $\mu$ ) and, consequently, it makes the electronegativity less negative indicating lower tendency to loss electrons by cinnamic acid derivates. The molecule with better biological response presents low values of chemical potential and high values of the softness (S). Additionally, the -OH and -OCH<sub>3</sub> groups causes the lowering of the values of  $\omega$ ,  $\omega^-$  and  $\omega^+$ . These results suggests that the reduction of electrophilicity of the ligands can be associated with improved sedative activity and the memory retention.

**Table 1.** Quantum descriptors of cinnamic, caffeic, feluric, sinapic and p-counamaric acids calculated at DFT/M062-X/6-311++g(2d,2p) level theory

| Compound      | HOMO  | LUMO  | IP <sup>a</sup> | EA <sup>b</sup> | $\mu^c$ | $\chi^d$  | $\eta^e$ | S <sup>f</sup> | $\omega^g$ | $\omega^{+h}$ | $\omega^{-i}$ | Gap  |
|---------------|-------|-------|-----------------|-----------------|---------|-----------|----------|----------------|------------|---------------|---------------|------|
| Caffeic acid  | -7.41 | -1.03 | 7.41            | 1.03            | 4.22    | -<br>4.22 | 3.19     | 0.16           | 2.79       | 1.08          | 5.30          | 6.38 |
| Cinnamic acid | -8.08 | -1.19 | 8.08            | 1.19            | 4.64    | -<br>4.64 | 3.45     | 0.15           | 3.12       | 1.23          | 5.87          | 6.89 |
| Feluric acid  | -7.29 | -0.98 | 7.29            | 0.98            | 4.14    | -<br>4.14 | 3.16     | 0.16           | 2.71       | 1.04          | 5.17          | 6.31 |

|                 |       |       |      |      |      |      |      |      |      |      |      |      |
|-----------------|-------|-------|------|------|------|------|------|------|------|------|------|------|
| Sinapic acid    | -7.31 | -1.00 | 7.31 | 1.00 | 4.16 | -    | 3.16 | 0.16 | 2.74 | 1.05 | 5.21 | 6.31 |
|                 |       |       |      |      |      | 4.16 |      |      |      |      |      |      |
| p-Coumaric acid | -7.59 | -1.00 | 7.59 | 1.00 | 4.30 | -    | 3.30 | 0.15 | 2.80 | 1.06 | 5.36 | 6.59 |
|                 |       |       |      |      |      | 4.30 |      |      |      |      |      |      |

<sup>a</sup> $IP = -E_{HOMO}$ ; <sup>b</sup> $EA = -E_{LUMO}$ ; <sup>c,d</sup> $\chi = -\mu = \frac{IP+EA}{2}$ ; <sup>e</sup> $\eta = \frac{IP-EA}{2}$ ; <sup>f</sup> $S = 1/2\eta$ ; <sup>g</sup> $\omega = \frac{1}{N} = \mu^2 S$ ; <sup>h</sup> $\omega^+ = \frac{(IP+3EA)^2}{16(IP-EA)}$ ;  
<sup>i</sup> $\omega^- = \frac{(3IP+EA)^2}{16(IP-EA)}$

To improve the earlier discussion about reactivity of the cinnamic acid and its derivatives, the Fukui indexes ( $f^0$ ,  $f^+$  and  $f^-$ ) are calculated and compared, Table S1a-e. The first index analyzed is  $f^+$  which describes the electrophilicity behavior. The structure of the CiAc shows the higher values of its index in the doubly bounded (C2 and C3) carbon atoms with 0.1292 and 0.1527, respectively, indicating that these sites can act in nucleophilic attacks. Since the nucleophile is bound to C3, the negative charge can be stabilized by mesomeric effect with the carbonyl group and for C3 by mesomeric effect with phenyl group. For all molecules the C3 atom presents the higher value of  $f^+$ , while the presence of the substituent groups tends to decrease the values of this parameter for C2 in the cinnamic acid derivatives. The presence of the -OH groups reduce the values of the  $f^+$  for the C3, decreasing the capacity of this atom to act as electrophile. This result suggests that the presence of the -OH in the *para* position increases the negative charge density on these atoms. However, the presence of the -OCH<sub>3</sub> groups in the *meta* position presents contrary effect, occurring an increase of the values of  $f^+$  in the C3 atom from SiAc (0.1563) and FeAc (0.1541) indicating that these molecules have higher electrophilic character.

The structure of CiAc shows the highest values of  $f^-$  in C7 (0.1112) and C2 (0.1280) indicating that its sites are more vulnerable to electrophilic attacks. For molecules with -OH groups, CaAc (*para* and *ortho* positions) and CuAc (*ortho* position), the atoms with higher values of  $f^-$  are C7 (0.11376) and C4 (0.11212) for CaAc and C4 (0.12536) and C2 (0.12834). The molecules which have presence of the -OCH<sub>3</sub> groups present the C7 and C4 as atoms with higher values of  $f^-$ . The presence of this substituent in the *meta* positions relative to acid group becomes the C7 atom as sites with higher tendency to suffer electrophilic attacks, increasing  $f^-$  values in C7 and decreasing in C2 when compared with CiAc. This result suggests that the CuAc act better as nucleophile.

Finally, for all molecules, the greater reactivity index was observed for C2 and C3 atoms. This parameter measures reactivity of the sites for radical attacks. The obtained values of  $f^0$  for CiAc were 0.12857 (C2) and 0.10977 (C3). The presence of the -OH and -OCH<sub>3</sub> in the

cinnamic acid derivatives decreases the values of  $f^0$  for both atoms, making these molecules less susceptible to radical reactions.

### Acute toxicity 96 h

Acute toxicity is essential to ensure the initial safety of new therapeutic agents, guiding regulatory decisions and safe clinical development. It is traditionally assessed by testing on animals, in which doses of the compound are administered and adverse effects are monitored for days to weeks [19]. Table 1 presents the results of acute toxicity analyses of CiAc and its derivatives, obtained over a period of 96 hours.

The CiAc derivatives evaluated in this study did not demonstrate toxicity in adult zebrafish during the 96-hour observation period ( $LD_{50} > 40$  mg/kg), as no significant deaths or visible anatomical changes were recorded in the test animals, as shown in Table 2. These findings suggest that both cinnamic acid and its derivatives are non-toxic at doses of 4, 20, and 40 mg/kg in adult zebrafish. This absence of acute toxic effects or apparent anatomical defects represents an important finding, suggesting a promising safety profile for the therapeutic use of these compounds.

**Table 2.** Results of acute toxicity tests.

| Mortality |    |    |    |    | 96h                     |
|-----------|----|----|----|----|-------------------------|
| Sample    | CP | D1 | D2 | D3 | $LD_{50}$<br>(mg/kg)/IV |
| CiAc      | 0  | 0  | 0  | 0  | >40                     |
| CuAc      | 0  | 1  | 0  | 0  | >40                     |
| CaAc      | 0  | 1  | 0  | 0  | >40                     |
| FeAc      | 0  | 0  | 1  | 0  | >40                     |
| SiAc      | 0  | 0  | 0  | 1  | >40                     |

### Assessment of locomotor activity (open field test - OFT)

The Open Field Test (OFT) is widely used in zebrafish studies to investigate behaviors related to exploration, anxiety, and reactions to environmental or pharmacological stimuli. Derived from traditional models applied in rodents, the OFT can be performed in Petri dishes, offering a practical and low-cost alternative to assess locomotor and exploratory behaviors, and responses to anxiolytic stimuli [20].

CiAc, a natural compound present in several plants, has been studied for its neuroprotective effects and impact on animal locomotion, especially in models of neurodegenerative diseases [8]. Locomotion is an important behavioral parameter in zebrafish, widely used as an indicator of neural activity, anxiety, stress, or toxicity [20]. The graphs presented (Fig. 3) represent the number of line crossings in a 5-minute period, an indication of the level of locomotor activity of the animals.

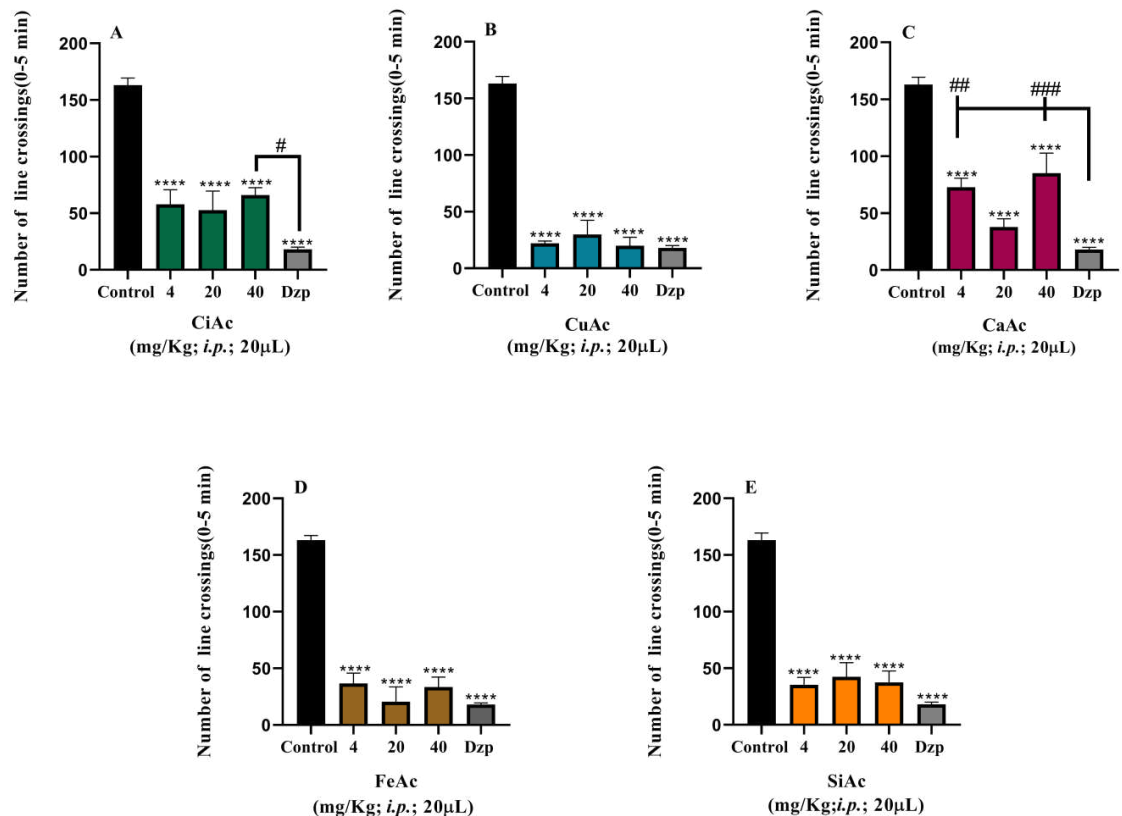


**Figure 3.** Influence of each treatment on locomotion parameters. During the open field test, it was observed that all three doses of CiAc (Fig. 3A), CaAc (Fig. 3B), CuAc (Fig. 3C), SiAc (Fig. 3D) and FeAc (Fig. 3E) significantly reduced the number of line crossings by the animals (\*\*\*\*p<0.0001 vs control). These effects were comparable to the positive control diazepam (40 mg/kg; \*\*\*\*p<0.0001, with a significant difference from the negative control DMSO 3%). The

doses of 40 mg/kg CiAc (Fig. 3A) and 4 and 40 mg/kg CaAc (Fig. 3B) also showed significant differences with DZP (CiAc: # $p < 0.05$ ; CaAc: ## $p < 0.01$  and ###  $p < 0.001$ ).

These data suggest that all groups treated with CiAc showed a significant reduction in the number of line crossings compared to the control group (\*\*\*\* $p < 0.0001$ ). Despite this significant reduction in locomotion, the 40 mg/kg dose had a different effect than the reference drug, DZP (# $p < 0.05$ ) (Fig. 3A). It is noted that there was a decrease in locomotor activity in the animals, but without the motor impairment typical of the sedative state induced by DZP. A similar response was observed for the 4 and 40 mg/kg doses of CaAc (## $p < 0.01$  and ### $p < 0.001$ ) (Fig. 3B). The results for CiAc are in agreement with the findings of <sup>[21]</sup>, which reported a significant improvement in the locomotor activity of mice after oral administration of CiAc in a Parkinson's disease model.

CaAc also influenced the locomotion of zebrafish. Specifically, the 4 and 40 mg/kg doses showed statistical relevance when compared to the group treated with DZP (## $p < 0.01$  and ### $p < 0.001$ ). This result suggests that, although the compound alters locomotion, it does not induce the motor impairment typical of the sedation state promoted by DZP, indicating a viable therapeutic projection, possibly with fewer side effects. On the other hand, the three doses (4, 20 and 40 mg/kg) of CuAc, SiAc and FeAc caused significant effects on the animal's locomotion (\*\*\*\* $p < 0.0001$ ) compared to the control group, Fig. 3C, 3D and 3E). These results suggest a more pronounced hypolocomotor effect, like that observed with DZP (\*\*\*\* $p < 0.0001$  compared to the control group), evidenced by the sharp reduction in the number of line crossings in the Petri dishes and, consequently, in locomotor activity. These data point to a possible depressant action of these molecules on the central nervous system (CNS).

SiAc and FeAc showed similar behavior to CuAc in the locomotor analysis, in that all doses caused a significant reduction in locomotor activity. Like CuAc, these molecules demonstrate a strong depressant action on the CNS in zebrafish. These findings suggest that such compounds have anxiolytic, sedative or neuromodulatory potential, and may have therapeutic applications in disorders of hyperactivity, anxiety and excessive neuronal excitation. In the locomotor analysis with CuAc and FeAc, it was observed that the marked inhibitory effect on locomotor activity may be related to their antioxidant and anti-inflammatory properties already described by <sup>[22]</sup>. This mechanism is relevant, as oxidative stress can trigger cellular and tissue damage <sup>[23]</sup>, resulting in neurochemical imbalance that compromises the

locomotor system. Furthermore, the consistency of the results at all doses evaluated indicates that even low doses are sufficient to trigger the observed effect.

### Anxiolytic Assessment (Light/dark test - LDT)

Tests of preference between light and dark are widely used in various animal models. In zebrafish, this methodology is recognized as an effective approach for assessing anxiety levels in zebrafish, and is widely used in drug screenings and neurobehavioral research [24].

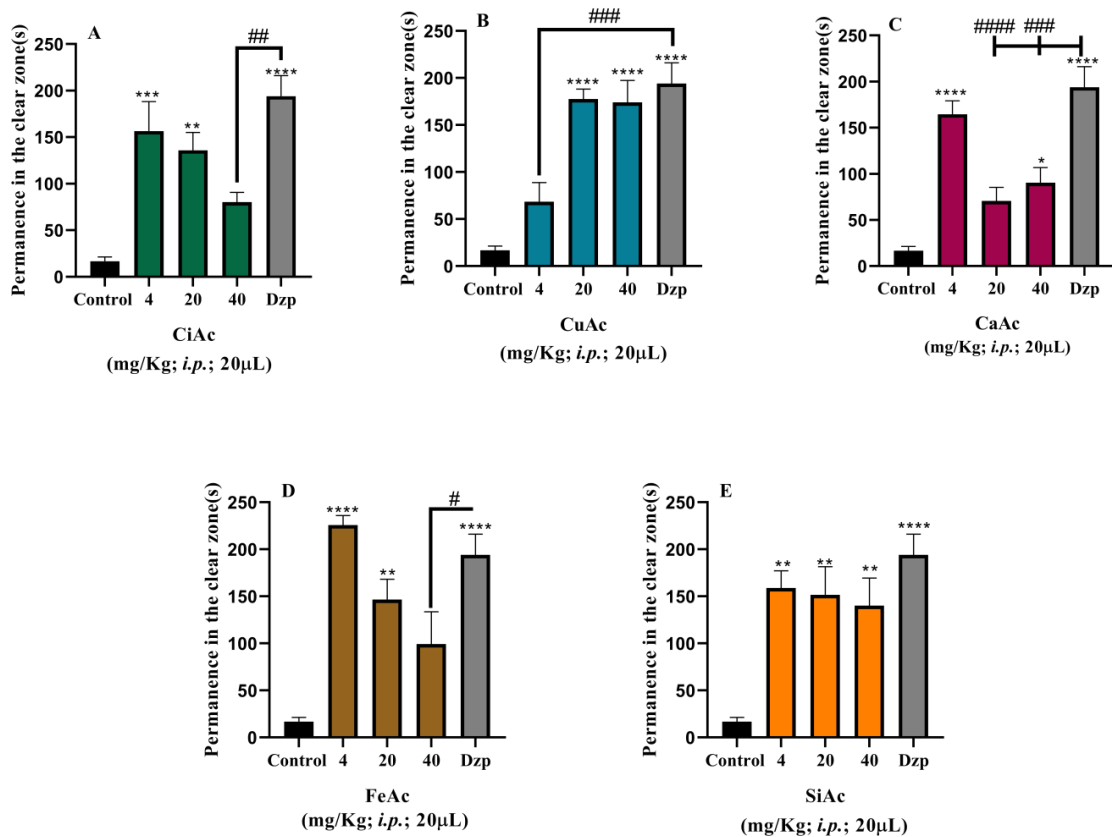


**Figure 4.** Anxiolytic effect of cinnamic acid derivatives in adult zebrafish in the light and dark test (0–5 min). DZP (40 mg/kg; 20  $\mu$ L; i.p.). Vehicle (DMSO 3%) (20  $\mu$ L; i.p.). Values represent the mean  $\pm$  standard error of the mean for 6 animals/group; ANOVA followed by Tukey's test. CiAc: (\*\*\*)  $p < 0.001$ , (\*\*)  $p < 0.01$  vs control; (##)  $p < 0.01$  vs DZP); CaAc (\*\*\*\*)  $p < 0.0001$ , (\*)  $p < 0.05$  vs control; (####)  $p < 0.0001$ , (###)  $p < 0.001$  vs DZP ); CuAc (\*\*\*\*)  $p < 0.0001$  vs control; (###)  $p < 0.001$  vs DZP); SiAc (\*\*)  $p < 0.01$  vs control); FeAc (\*\*\*\*)  $p < 0.0001$ , (\*\*)  $p < 0.01$  vs control; (#)  $p < 0.5$  vs DZP).

Although CiAc and its derivatives show theoretical potential as agents for neurobehavioral research, there is still a scarcity of direct and consistent evidence in animal

models. Investigations, especially with an emphasis on behavioral tests focused on anxiety, are essential to confirm this therapeutic possibility <sup>[25]</sup>.

In the obtained results, it was observed that CiAc (Fig. 4A) significantly increased the permanence in the clear zone at doses of 4 and 20 mg/kg, while the dose of 40 mg/kg presented a distinct response from the anxiolytic effect observed with DZP, used as a positive control. Regarding CaAc, only the lowest dose promoted an increase in permanence in the clear zone, while the intermediate dose (20 mg/kg) had a response inferior to the highest dose (40 mg/kg). Statistical analyses between these doses indicated significant variation, demonstrating a non-linear pattern (Fig. 4B and 4C). Similarly, the highest dose of FeAc (Fig. 4E) did not show a significant difference in comparison ( $*p<0.05$ ). These results suggest that the anxiolytic effect of the compounds may be associated with an ideal therapeutic window (hormesis), in which very high or intermediate doses reduce efficacy.

In the remaining results, the data show that the lowest dose (4mg/kg) of CiAc, CaAc, SiAc, and FeAc, induced anxiolytic behavior, evidenced by the greater time the animals spent in the clear zone of the aquarium, with significant differences in relation to the control group ( $***p<0.001$ ;  $****p<0.0001$ ;  $**p<0.001$ ;  $****p<0.0001$ ; Fig. 4A,4B,4D,4E). CuAc, however, presented a distinct profile, where the lowest dose did not differ significantly from the control, but showed a difference in relation to DZP ( $###p<0.001$ ). At doses of 20 and 40 mg/kg, there was a significant increase in the time spent in the clear zone, with robust statistical differences and results similar to the group treated with DZP, suggesting potential as a bioactive compound with activity in the CNS, possibly mediated by receptors related to anxiety.

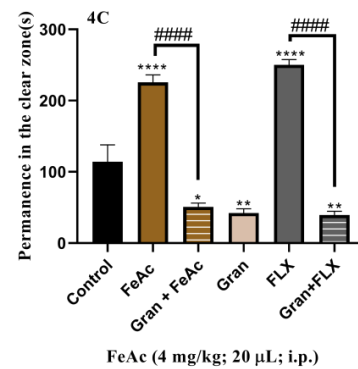
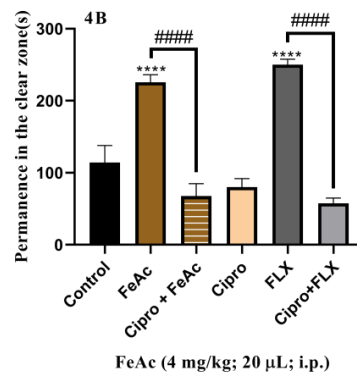
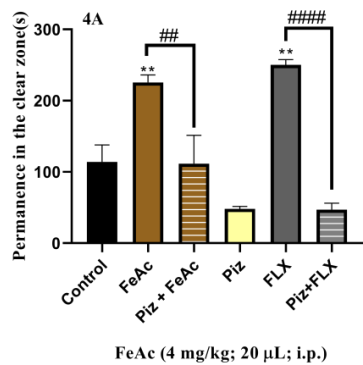
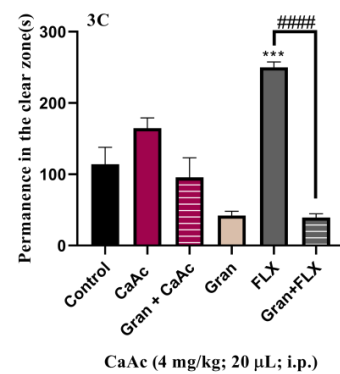
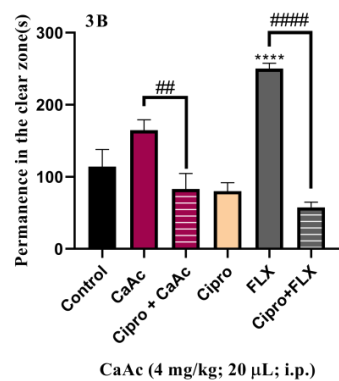
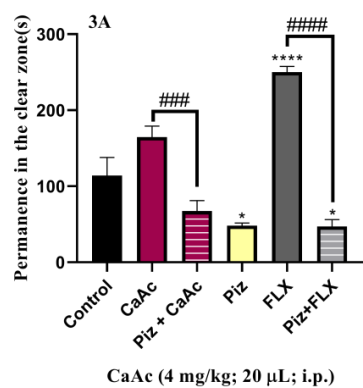
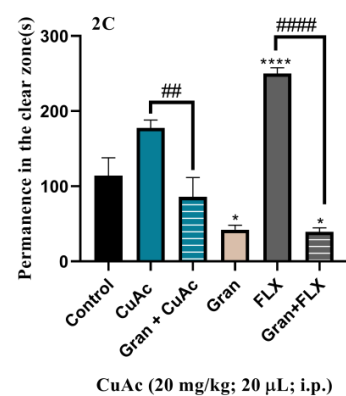
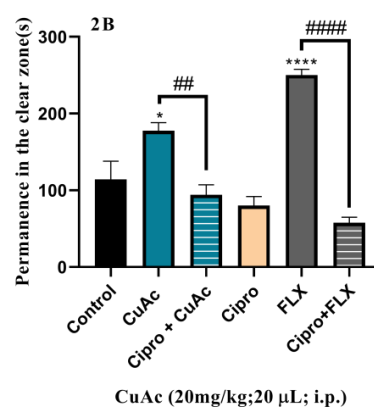
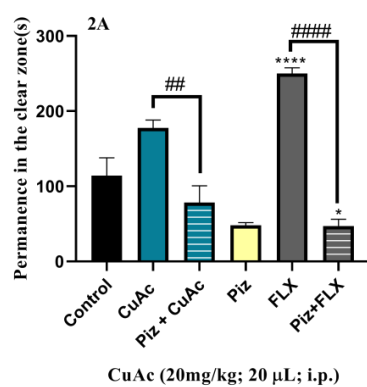
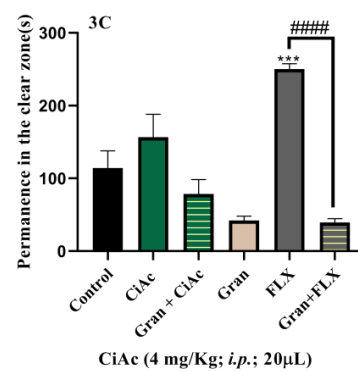
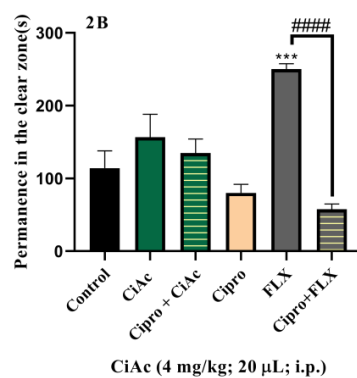
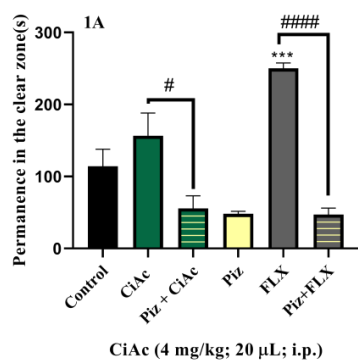
Among the compounds evaluated, SiAc stands out, which presented positive responses in all doses tested (4, 20, and 40 mg/kg), with a consistent effect ( $**p<0.01$ ) in relation to the control (Fig. 2D). This uniform and potent action suggests a high affinity for molecular targets. Furthermore, studies conducted by <sup>[26]</sup> indicate that this molecule also has antioxidant and anti-inflammatory properties, capable of modulating cytokines and redox systems in the CNS, which may contribute to its observed effects. FeAc also showed an anxiolytic effect at the lower doses (4 and 20 mg/kg), probably mediated by its antioxidant and neuroprotective properties, with possible involvement of dopaminergic and serotonergic modulation, as reported by [9]. It is worth noting that both FeAc and CaAc showed maximum statistical difference in relation to the control at the lowest dose, with results similar to the group treated

with DZP (\*\*\*\*  $p < 0.0001$ ) (Fig.4B and 4E), reinforced by the expressive increase in the time spent in the clear zone of the aquarium.

### **Assessment of SEROTONergic neuromodulation**

The mechanisms associated with the anxiolytic effect (Fig. 5) were identified through pre-treatment with serotonergic antagonists, selected based on their selectivity and pharmacological profile to block specific receptor subtypes. Pizotifen, an antagonist of 5-HT<sub>1</sub> and 5-HT<sub>2A/2C</sub> receptors, potentiated the inhibition of the serotonergic system. Granisetron, a selective antagonist of the 5-HT<sub>3</sub> receptor, may also be related to anxiety modulation [27], while cyproheptadine showed blockade of 5-HT<sub>3A/3B</sub> receptors [2].

The serotonergic system plays a fundamental role in the regulation of anxiety in zebrafish, being widely explored in behavioral studies and pharmacological interventions. The modulation of serotonin (5-HT) directly influences anxious responses, and an anxiolytic effect mediated by this pathway was observed, since each sample had its action attenuated by at least one of the antagonists tested.



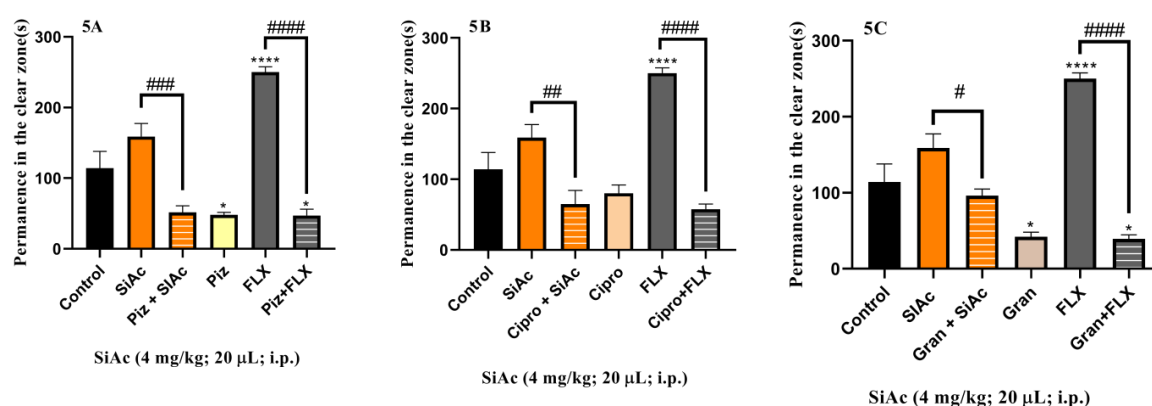


**Figure 5.** Effect of pizotifen (1A; 2A; 3A; 4A and 5A); cyproheptadine (1B; 2B; 3B; 4B and 5B) and granisetron (1C; 2C; 3C; 4C and 5C) (4 mg/kg; i.p.) on the anxiolytic mechanism of action of cinnamic acid and its derivatives in the light and dark test in adult zebrafish (*Danio rerio*). The results are expressed as mean values  $\pm$  S.E.M. ( $n = 6/\text{group}$ ). ANOVA followed by Tukey's test.

The mechanism of action analyses of CiAc in zebrafish showed that only pizotifen significantly blocked its anxiolytic effect (Fig. 5), while cyproheptadine (Fig. 5) and granisetron (Fig. 5) showed no blocking effect. For CaAc, both pizotifen (Fig. 5) and cyproheptadine (Fig. 5) significantly inhibited its anxiolytic action, a result not demonstrated by granisetron (Fig. 5).

SiAc showed a consistent anxiolytic profile, probably mediated by 5-HTR1, 5-HTR2, and 5-HTR3 receptors. This effect was strongly reduced by pizotifen (Fig. 3A), cyproheptadine (Fig. 3B), and granisetron (Fig. 3C), which significantly blocked its anxiolytic action (#### $p < 0.001$ ; ## $p < 0.01$ ; # $p < 0.05$ ). Similarly, FeAc also depends on the integrity of the serotonergic system, since all tested antagonists attenuated its anxiolytic effect. Pizotifen reduced its action via 5-HTR1A/5-HTR2A receptors (Fig. 4A), cyproheptadine reinforced the role of the serotonergic pathway (Fig. 4B), and granisetron suggested the involvement of the 5-HTR3 receptor (Fig. 5C).

Like SiAc and FeAc, CuAc demonstrated that it depends on the joint activation of multiple subtypes of serotonergic receptors (5-HTR1, 5-HTR2, 5-HTR3) to induce an anxiolytic effect, suggesting a multimodal serotonergic mechanism. These findings reinforce that AcCi derivatives act in an integrated manner on different 5-HT receptors, expanding their potential as candidates for natural anxiolytic agents.

Previous studies indicate that the possible therapeutic application demonstrated that CiAc and its derivatives have potential applicability as candidates for antidepressant drugs, through the modulation of serotonin and the participation of metabolites related to

inflammation, AA-COX-2/5-LOX and BDNF, in addition to presenting mechanisms similar to conventional antidepressant drugs<sup>[25]</sup>. Furthermore, their antioxidant and anti-inflammatory effects<sup>[28]</sup> can preserve the integrity and functionality of the serotonergic system<sup>[25]</sup>, favoring the activation of receptors such as 5-HT<sub>1A</sub>, directly related to the regulation of anxious behavior.

In the zebrafish model, these mechanisms resulted in greater exploration of the clear zones of the aquarium, a behavior consistent with an anxiolytic response, in agreement with the results observed in this study. Thus, the inhibition of the anxiolytic effect by the antagonists confirms the participation of the serotonergic system, suggesting that cinnamic acid derivatives can positively modulate 5-HT receptors and favor serotonergic neurotransmission, highlighting their pharmacological potential as natural anxiolytic agents.

### Molecular docking simulation and data output for anxiety analysis

Following in silico trials, RMSD values from 0.055 to 1.903 Å and affinity energy from -8.8 to -6.0 kcal/mol were observed (Table 3), values within the standard described in the literature for affinity energy (equal to or less than -6.0 kcal/mol)<sup>[29]</sup>. The analysis of the interactions showed that the evaluated compounds presented hydrogen bonds, hydrophobic interactions, and  $\pi$ -stacking with the 5-HTR1A receptor; hydrogen bonds and halogen bonds, hydrophobic interactions,  $\pi$ -stacking, and salt bridges with the 5-HTR2A receptor; hydrogen bonds, hydrophobic interactions, and  $\pi$ -stacking with the 5-HTR2C receptor; hydrogen bonds, hydrophobic interactions,  $\pi$ -cation,  $\pi$ -stacking, and salt bridges with the 5-HTR3A receptor (Table 3).

**Table 3.** Affinity Energy, RMSD and types of interactions formed between ligands and the 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub> and 5-HT<sub>3A</sub> receptors.

| 5-HT <sub>1A</sub> /Ligand | Affinity Energy<br>(kcal/mol) | RMSD (Å) | Interaction Type | Residue (Distance in Å)                           |
|----------------------------|-------------------------------|----------|------------------|---------------------------------------------------|
| CaAC                       | -6.2                          | 1.291    | Hydrophobic      | Ile189R (3.53), Phe361R (3.63)                    |
|                            |                               |          | Hydrogen Bonds   | Asn386R (2.10), Asn386R (3.43),<br>Tyr390R (2.05) |
| CiAC                       | -6.0                          | 0.565    | Hydrophobic      | Trp358R (3.46), Phe362R (3.61),<br>Tyr390R (3.55) |
|                            |                               |          | $\pi$ -Stacking  | Phe361R (3.93)                                    |

|                   |      |       |                 |                                                                                                      |
|-------------------|------|-------|-----------------|------------------------------------------------------------------------------------------------------|
| <b>CuAC</b>       | -6.2 | 0.187 | Hydrophobic     | Ile189R (3.70), Phe361R (3.53),<br>Tyr390R (3.89)                                                    |
|                   |      |       | Hydrogen Bonds  | Cys120R (3.56), Asn386R (2.18),<br>Asn386R (2.28)                                                    |
| <b>FeAC</b>       | -6.3 | 1.113 | Hydrophobic     | Trp358R (3.51), Phe362R (3.94)                                                                       |
|                   |      |       | Hydrogen Bonds  | Thr121R (3.78), Tyr390R (2.74)                                                                       |
|                   |      |       | $\pi$ -Stacking | Phe361R (3.99)                                                                                       |
| <b>SiAC</b>       | -6.1 | 0.525 | Hydrophobic     | Ile189R (3.90), Ile189R (3.95),<br>Ile189R (4.00), Ile189R (3.69),<br>Phe361R (3.78), Ala365R (3.98) |
|                   |      |       | Hydrogen Bonds  | Lys191R (2.01), Thr196R (2.32)                                                                       |
| <b>Pizotifen</b>  | -7.1 | 1.404 | Hydrophobic     | Tyr96R (3.56), Phe112R (3.87),<br>Ala383R (3.93), Asn386R (3.73)                                     |
|                   |      |       | Hydrogen Bond   | Gln97R (2.42)                                                                                        |
| <b>Fluoxetine</b> | -7.3 | 1.531 | Hydrophobic     | Ile189R (3.50), Ile189R (3.87),<br>Phe361R (3.64), Phe361R (3.56),<br>Phe362R (3.47), Ala365R (3.68) |
|                   |      |       | Hydrogen Bond   | Ala365R (2.41)                                                                                       |

| 5-HT2A/Ligand | Affinity Energy<br>(kcal/mol) | RMSD (Å) | Interaction Type | Residue (Distance in Å)                                                              |
|---------------|-------------------------------|----------|------------------|--------------------------------------------------------------------------------------|
| <b>CaAC</b>   | -6.6                          | 1.069    | Hydrophobic      | Phe339B (3.81)                                                                       |
|               |                               |          | Hydrogen Bond    | Ser242B (3.50)                                                                       |
|               |                               |          | $\pi$ -Stacking  | Trp336B (4.93), Trp336B (5.02),<br>Phe340B (4.76)                                    |
| <b>CiAC</b>   | -6.6                          | 0.088    | Hydrophobic      | Phe243B (3.63), Phe332B (3.68),<br>Trp336B (3.65), Phe339B (3.86),<br>Phe340B (3.92) |
|               |                               |          | $\pi$ -Stacking  | Trp336B (4.83), Phe340B (4.74)                                                       |

| <b>CuAC</b>           | -6.7                          | 0.388    | Hydrophobic      | Val156A (3.65), Trp336A (3.61),<br>Phe339A (3.54), Phe340A (3.77)                                                                                          |
|-----------------------|-------------------------------|----------|------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                       |                               |          | Hydrogen Bonds   | Ser159A (1.99), Asn343A (2.05),<br>Tyr370A (3.34)                                                                                                          |
| <b>FeAC</b>           | -6.4                          | 1.694    | Hydrophobic      | Trp336A (3.74), Phe340A (3.98)                                                                                                                             |
|                       |                               |          | Hydrogen Bond    | Tyr370A (2.77)                                                                                                                                             |
|                       |                               |          | $\pi$ -Stacking  | Phe339A (4.74)                                                                                                                                             |
| <b>SiAC</b>           | -6.6                          | 0.240    | Hydrophobic      | Phe339A (3.71), Phe340A (3.72)                                                                                                                             |
|                       |                               |          | Hydrogen Bonds   | Ser159A (2.24), Asn343A (2.29),<br>Tyr370A (2.84)                                                                                                          |
|                       |                               |          | $\pi$ -Stacking  | Phe339A (4.69)                                                                                                                                             |
| <b>Pizotifen</b>      | -8.3                          | 1.903    | Hydrophobic      | Trp151A (3.26), Ile152A (3.47),<br>Ile152A (3.51), Asp155A (3.85),<br>Val156A (3.43), Leu229A (3.44),<br>Phe339A (3.61), Phe339A (3.92)                    |
|                       |                               |          | Salt Bridges     | Asp155A (5.02)                                                                                                                                             |
| <b>Cyproheptadine</b> | -8.0                          | 1.871    | Hydrophobic      | Trp151A (3.46), Ile152A (3.97),<br>Val156A (3.11), Leu228A (4.00),<br>Leu229A (3.60), Leu229A (3.37),<br>Phe339A (3.61), Val366A (3.52),<br>Val366A (3.45) |
| <b>Fluoxetine</b>     | -8.4                          | 1.187    | Hydrophobic      | Val156A (3.70), Leu229A (3.75),<br>Phe339A (3.89), Phe339A (3.71)                                                                                          |
|                       |                               |          | Hydrogen Bonds   | Asp155A (2.67), Asp155A (2.68)                                                                                                                             |
|                       |                               |          | $\pi$ -Stacking  | Trp336A (4.80), Phe340A (4.77)                                                                                                                             |
|                       |                               |          | Halogen Bond     | Ser242A (3.77)                                                                                                                                             |
| 5-HT2C/Ligand         | Affinity Energy<br>(kcal/mol) | RMSD (Å) | Interaction Type | Residue (Distance in Å)                                                                                                                                    |
| <b>CaAC</b>           | -6.4                          | 0.841    | Hydrophobic      | Phe327A (3.82)                                                                                                                                             |
|                       |                               |          | Hydrogen Bonds   | Thr139A (2.56)                                                                                                                                             |

|                   |      |       |                 |                                                                                                      |
|-------------------|------|-------|-----------------|------------------------------------------------------------------------------------------------------|
|                   |      |       | $\pi$ -Stacking | Trp324A (4.90), Phe328A (4.95)                                                                       |
| <b>CiAC</b>       | -6.8 | 0.055 | Hydrophobic     | Ile142A (3.79), Ala222A (3.88),<br>Phe223A (3.55), Phe328A (3.80)                                    |
|                   |      |       | $\pi$ -Stacking | Trp324A (4.93), Phe328A (4.89)                                                                       |
| <b>CuAC</b>       | -6.8 | 0.071 | Hydrophobic     | Ile142A (3.71), Ala222A (3.77),<br>Phe223A (3.57), Phe328A (3.87)                                    |
|                   |      |       | $\pi$ -Stacking | Trp324A (5.07), Phe328A (4.92)                                                                       |
| <b>FeAC</b>       | -6.2 | 0.983 | Hydrophobic     | Trp130A (3.70), Val354A (3.84)                                                                       |
|                   |      |       | Hydrogen Bonds  | Asp134A (2.39), Leu209A<br>(2.12), Asn351A (2.39),<br>Asn351A (3.55)                                 |
| <b>SiAC</b>       | -6.6 | 0.226 | Hydrophobic     | Val208A (3.92)                                                                                       |
|                   |      |       | Hydrogen Bonds  | Asp134A (2.48), Leu209A<br>(1.99), Asn351A (2.46),<br>Asn351A (3.02)                                 |
| <b>Pizotifen</b>  | -8.4 | 1.178 | Hydrophobic     | Trp130A (3.39), Ile131A (3.84),<br>Val208A (3.74), Leu209A (3.67),<br>Phe327A (3.69), Val354A (3.89) |
| <b>Fluoxetine</b> | -8.8 | 0.981 | Hydrophobic     | Val135A (3.82), Val135A (3.71),<br>Val185A (3.68), Phe214A (3.21),<br>Phe214A (3.77), Ala222A (3.81) |
|                   |      |       | $\pi$ -Stacking | Trp324A (5.16), Phe328A (5.19)                                                                       |

| 5-HT3A/Ligand | Affinity Energy<br>(kcal/mol) | RMSD (Å) | Interaction Type | Residue (Distance in Å)                                                                         |
|---------------|-------------------------------|----------|------------------|-------------------------------------------------------------------------------------------------|
| <b>CaAC</b>   | -7.3                          | 0.964    | Hydrophobic      | Ile44E (3.74), Trp63E (3.96),<br>Arg65E (3.92), Tyr126E (3.89)                                  |
|               |                               |          | Hydrogen Bonds   | Asp42E (2.61), Ile44E (3.07),<br>Trp63E (2.35), Arg65E (3.51),<br>Arg65E (3.02), Arg169E (2.91) |
|               |                               |          | $\pi$ -Cation    | Arg65E (4.29)                                                                                   |

|                   |      |       |                 |                                                                                    |
|-------------------|------|-------|-----------------|------------------------------------------------------------------------------------|
| <b>CiAC</b>       | -7.2 | 0.106 | Hydrophobic     | Ile44D (3.75), Trp63D (3.76),<br>Trp63D (3.61), Arg65D (3.91),<br>Trp156E (3.48)   |
|                   |      |       | $\pi$ -Stacking | Tyr126D (5.17)                                                                     |
|                   |      |       | Salt Bridges    | Arg65D (4.07), Arg169D (3.57)                                                      |
| <b>CuAC</b>       | -7.0 | 0.929 | Hydrophobic     | Trp63E (3.66), Arg65E (3.60),<br>Tyr126E (3.79)                                    |
|                   |      |       | Hydrogen Bond   | Arg169E (2.63)                                                                     |
|                   |      |       | $\pi$ -Cation   | Arg65E (4.21)                                                                      |
| <b>FeAC</b>       | -7.3 | 1.074 | Hydrophobic     | Ile44E (3.95), Arg65E (3.63),<br>Tyr126E (3.93)                                    |
|                   |      |       | Hydrogen Bonds  | Val43E (3.22), Arg65E (3.43),<br>Arg169E (3.23)                                    |
| <b>SiAC</b>       | -6.8 | 0.299 | Hydrophobic     | Trp63E (3.78), Tyr126E (3.68)                                                      |
|                   |      |       | Hydrogen Bonds  | Tyr64E (2.34), Tyr64E (3.24),<br>Trp156A (2.40), Arg169E (2.98),<br>Arg169E (2.62) |
| <b>Fluoxetine</b> | -7.8 | 1.722 | Hydrophobic     | Thr154A (3.86), Phe199A (3.58),<br>Ile201A (3.75), Tyr207A (3.67)                  |
|                   |      |       | Hydrogen Bond   | Asn101A (2.45)                                                                     |
|                   |      |       | $\pi$ -Stacking | Trp63E (4.83), Trp156A (5.18),<br>Tyr207A (4.94)                                   |

The prediction of cavities that can facilitate interactions between ligands and receptors 5-HT1A, 5-HT2A, 5-HT2C, and 5-HT3A was performed, exhibiting values for volume ( $\text{\AA}^3$ ), surface ( $\text{\AA}^2$ ), and depth ( $\text{\AA}$ ). For the 5-HT1A receptor, the predicted cavity exhibits a volume of  $367.1 \text{ \AA}^3$ , surface of  $558.95 \text{ \AA}^2$ , depth of  $14.2 \text{ \AA}$ , and is formed by 26 residues: Asp116, Val117, Cys120, Thr121, Ile124, Ile167, Ile189, Ser190, Lys191, Tyr195, Thr196, Ser199, Thr200, Ala203, Trp358, Phe361, Phe362, Val364, Ala365, Leu368, Pro369, Gly382, Ile385, Asn386, Gly389, and Tyr390. These residues indicate possible routes of interaction between the ligands and the serotonergic receptors. Detailed analysis of the interactions showed that CaAc, CiAc, CuAc, FeAc,

and SiAc are inserted in the predicted cavity (Fig. 6) and interact with residues of the reference drug's binding site, indicating a similar action to fluoxetine (Fig. 6A and 6B). The data also showed that CaAc and CuAc presented interaction with the residue (Asn386) of the Pizotifen control's binding site (Table 3 and Fig. 6C).

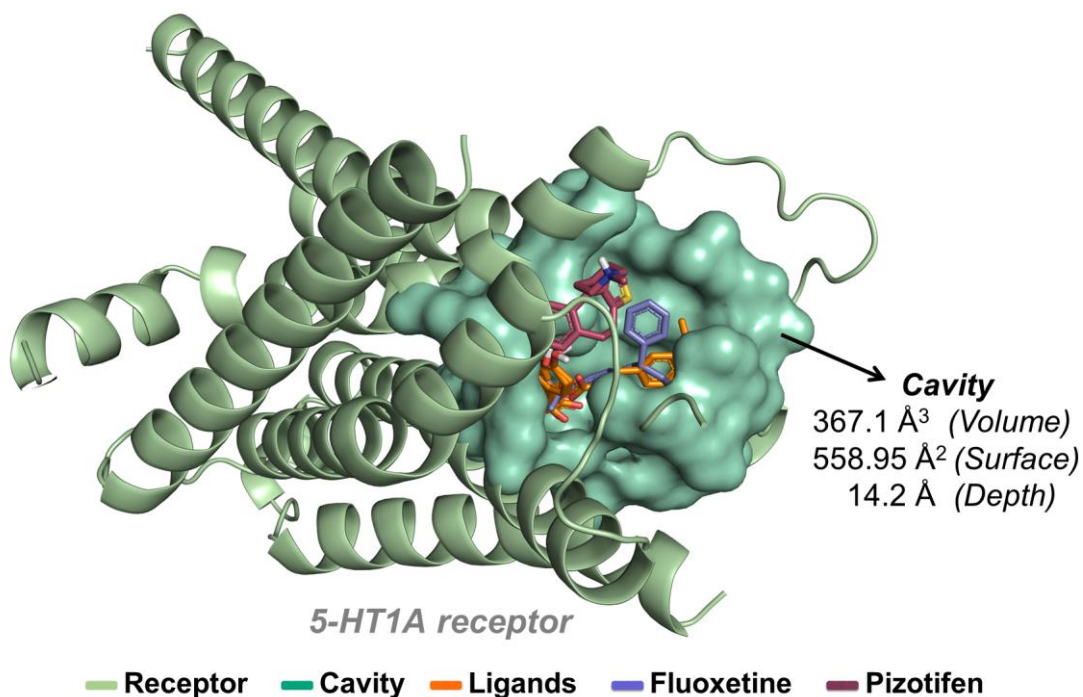


**Figure 6.** Three-dimensional image of the 5-HT1A receptor with the cavity predicted with volume (in Å<sup>3</sup>), surface (Å<sup>2</sup>) and depth (Å) values highlighted.

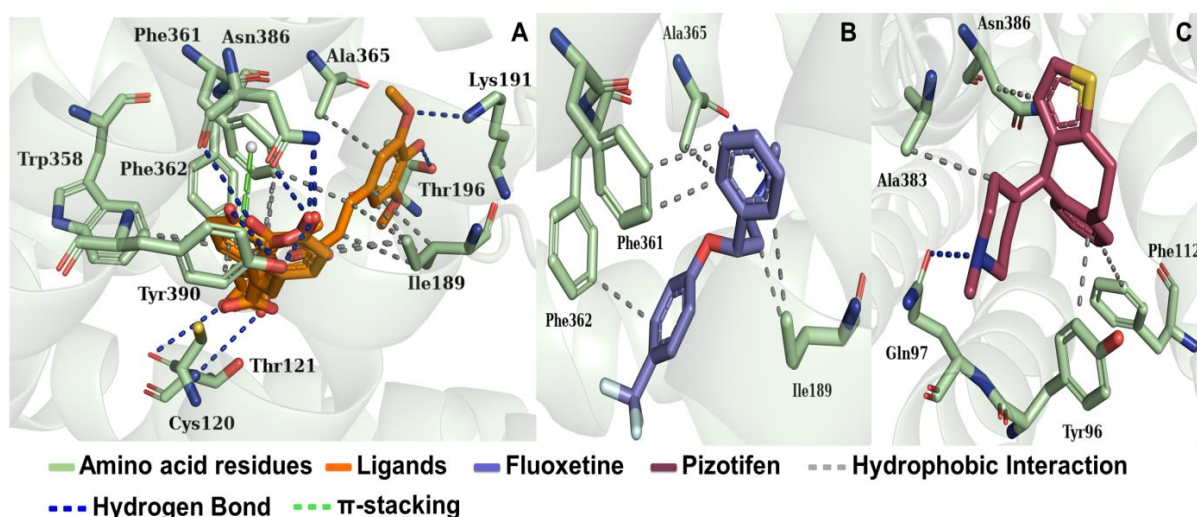


**Figure 7.** Binding site of the compounds against 5-HT1A receptor. (A) CaAc, CiAc, CuAc, FeAc and SiAc, (B) Fluoxetine and (C) Pizotifen.

The prediction for the 5-HT<sub>2A</sub> receptor showed the presence of two cavities, identified as cavity I: volume of 566.27 Å<sup>3</sup>, surface area of 793.71 Å<sup>2</sup>, depth of 16.49 Å and formed by 36 residues (Chain A): Ser131, Thr134, Ile135, Tyr139, Trp151, Ile152, Asp155, Val156, Ser159, Thr160, Ile163, Ile206, Ser207, Pro209, Ile210, Lys223, Cys227, Leu228, Leu229, Phe234, Val235, Ile237, Gly238, Ser239, Ser242, Phe243, Phe332, Trp336, Phe339, Phe340, Asn343, Leu362, Val366, Trp367 and Tyr370; cavity II: volume of 507.9 Å<sup>3</sup>, surface area of 680.98 Å<sup>2</sup>, depth of 20.77 Å and formed by 37 residues (Chain B): Ser131, Thr134, Ile135, Tyr139, Trp151, Ile152, Asp155, Val156, Ser159, Thr160, Ile163, Ile206, Ser207, Pro209, Ile210, Lys223, Cys227, Leu228, Leu229, Phe234, Val235, Ile237, Gly238, Ser239, Val241, Ser242, Phe243, Phe332, Trp336, Phe339, Phe340, Asn343, Leu362, Val366, Trp367 and Tyr370. Detailed analysis of the interactions showed that CuAc, FeAc, and SiAc are inserted into cavity I (chain A); CaAc and CiAc in cavity II (chain B) (Fig. 7). Furthermore, CuAc, FeAc, and SiAc (Fig. 8A) interact with residues of the reference drug binding site (Fig. 8C) and the controls Cyproheptadine (Fig. 8D) and Pizotifen (Fig. 8E), indicating a similar effect to the controls, thus they may act as competitive antagonists. With respect to CaAc and CiAc, it was observed that they do not compete for the same binding site as fluoxetine and the controls (Fig. 8B), indicating that they may act as non-competitive antagonists.

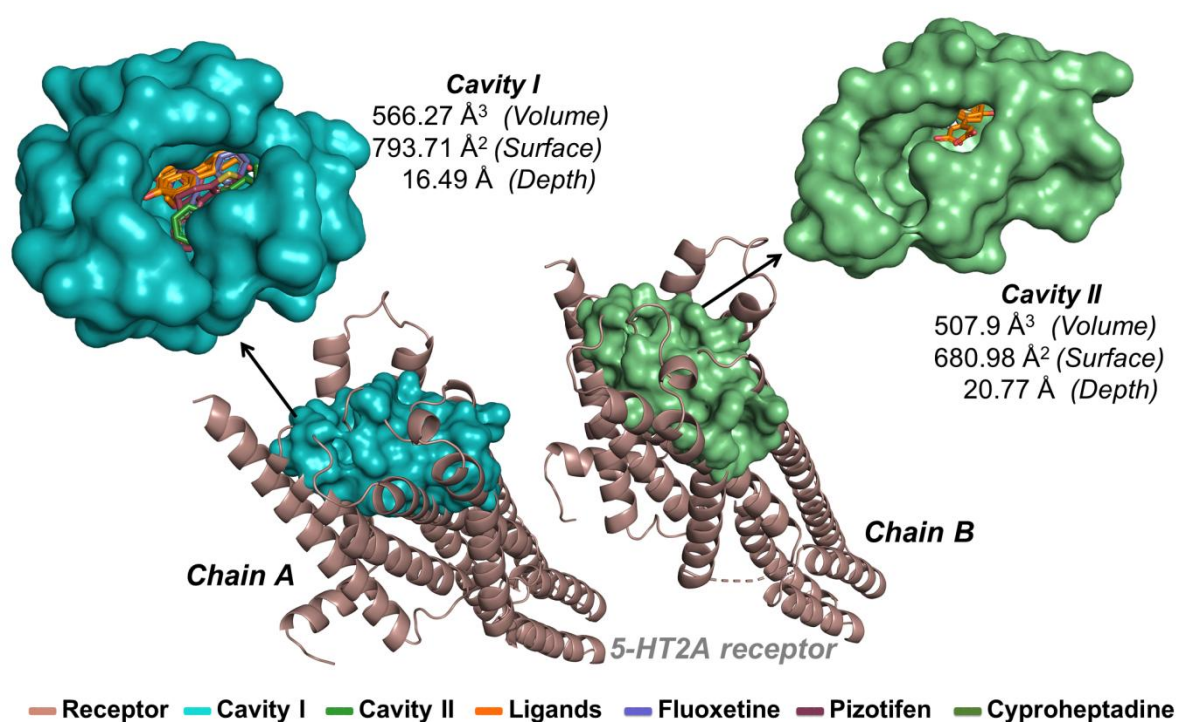


**Figure 8.** Three-dimensional image of the 5-HT<sub>2A</sub> receptor with the cavities predicted with volume (in Å<sup>3</sup>), surface (Å<sup>2</sup>) and depth (Å) values highlighted.

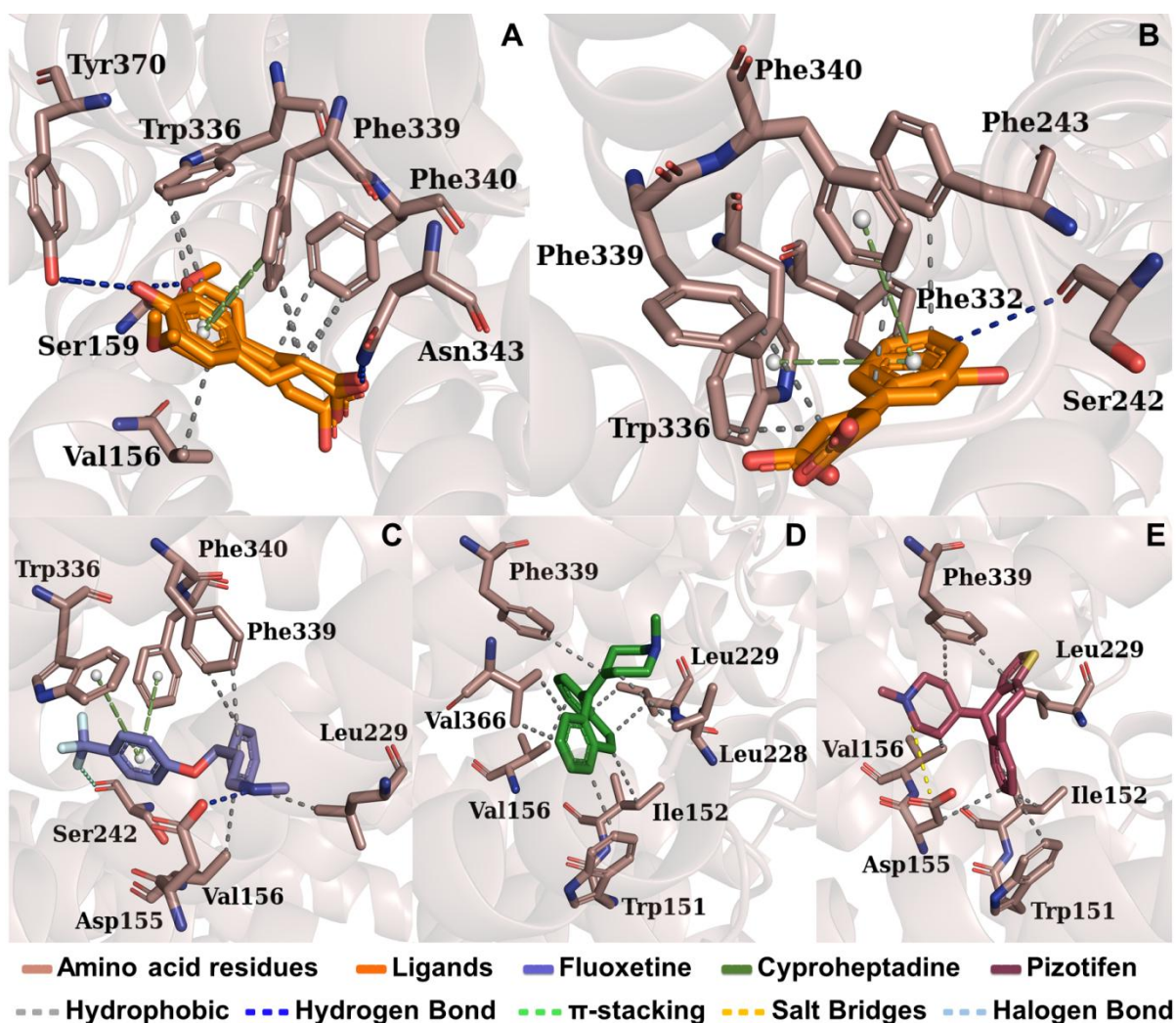


**Figure 9.** Binding site of the compounds against 5-HT<sub>2A</sub> receptor. (A) CuAc, FeAc and SiAc in chain A, (B) CaAc and CiAc in chain B, (C) Fluoxetine in chain A, (D) Cyproheptadine in chain A, (E) Pizotifen in chain A.

In the presence of the 5-HT<sub>2C</sub> receptor, the prediction showed the presence of two cavities, identified as cavity I: volume of 240.64 Å<sup>3</sup>, surface area of 310.18 Å<sup>2</sup>, depth of 11.0 Å and formed by 18 residues: Asp134, Val135, Ser138, Thr139, Ile142, Val185, Leu209, Phe214, Val215, Gly218, Ser219, Ala222, Phe223, Phe320, Trp324, Phe327, Phe328 and Tyr358; cavity II: volume of 152.06 Å<sup>3</sup>, surface area of 322.78 Å<sup>2</sup>, depth of 9.19 Å and formed by 15 residues: Ser110, Ala113, Ile114, Tyr118, Trp130, Ile131, Asp134, Cys207, Val208, Leu209, Phe327, Leu350, Asn351, Val354 and Tyr358. Detailed analysis of the interactions showed that CaAc, CiAc and CuAc are inserted in cavity I; FeAc and SiAc in cavity II (Fig. 9), furthermore, CaAc, CiAc and CuAc (Fig. 10A) interact with residues of the binding site of the reference drug, indicating a similar effect to Fluoxetine (Fig. 10B). The data also showed that

FeAc and SiAc (Fig. 10A) interact with residues of the binding site of the control, indicating a similar effect to Pizotifen (Fig. 10C).

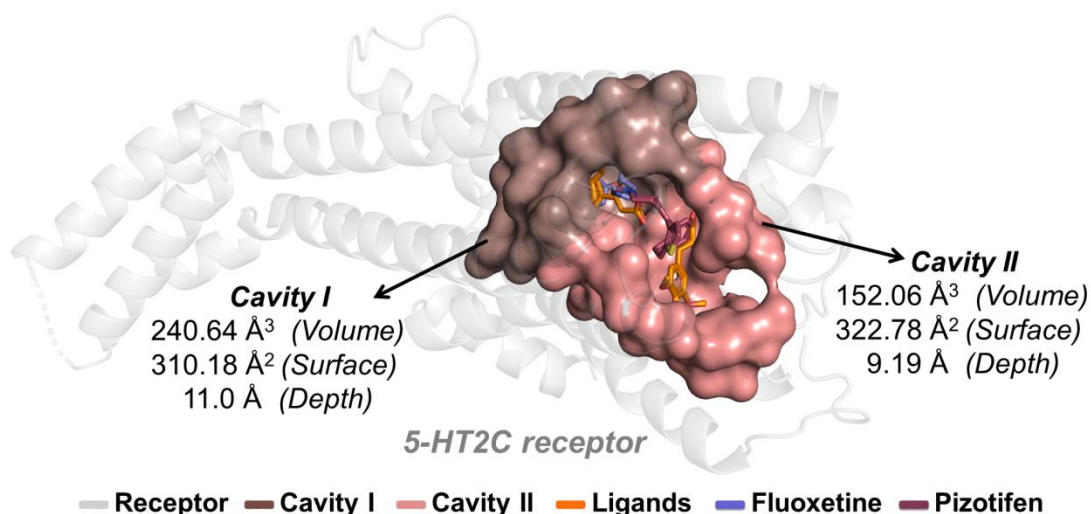


**Figure 10.** Three-dimensional image of the 5-HT2C receptor with the cavities predicted with volume (in Å<sup>3</sup>), surface (Å<sup>2</sup>) and depth (Å) values highlighted.

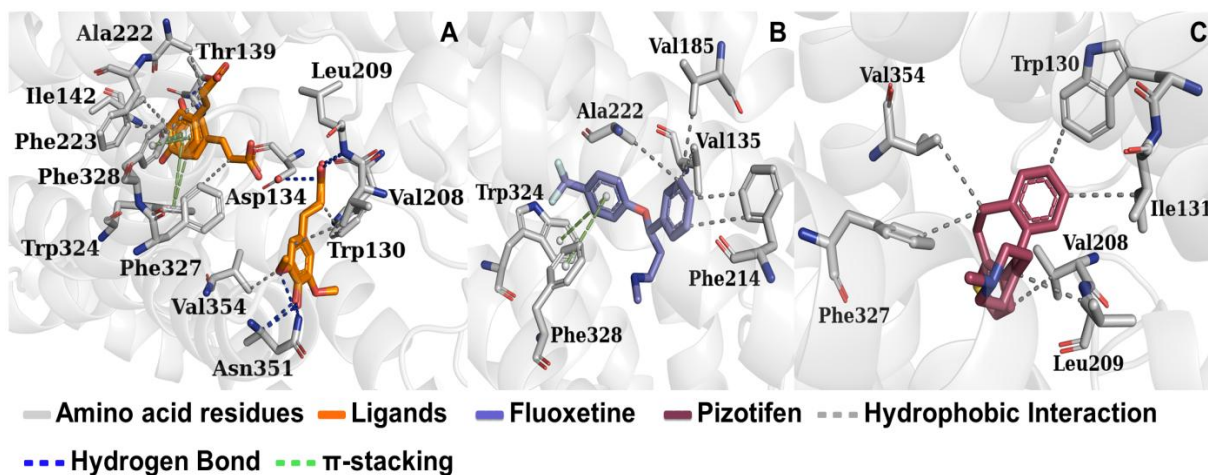


**Figure 11.** Binding site of the compounds against 5-HT2C receptor. (A) CaAc, CiAc, CuAc, FeAc and SiAc, (B) Fluoxetine and (C) Pizotifen.

The prediction for the 5-HT3A receptor also showed the presence of two cavities (Fig. 11A), identified as cavity I (Fig. 11C): volume of 213.5 Å<sup>3</sup>, surface of 397.01 Å<sup>2</sup>, depth of 10.76 Å and formed by 15 residues: Asp42, Val43, Ile44, Trp63, Tyr64, Arg65, Tyr126 and Arg169 (Chain D), Asn101, Thr154, Trp156, Phe199, Ile201, Tyr207 and Glu209 (Chain E); cavity II (Fig. 11B): volume of 209.92 Å<sup>3</sup>, surface of 381.93 Å<sup>2</sup>, depth of 10.7 Å and formed by 16 residues: Asn101, Thr154, Ser155, Trp156, Phe199, Ile201, Tyr207 and Glu209 (Chain A), Asp42, Val43, Ile44, Trp63, Tyr64, Arg65, Tyr126 and Arg169 (Chain E). The redocking

of granisetron, co-crystallized against the 5-HT<sub>3A</sub> receptor, showed an RMSD value of 1.679 Å and an affinity energy of -10.9 kcal/mol. The binding site of granisetron is formed by the residues Asp42, Val43, Ile44, Trp63, Tyr64, Arg65, Asn101, Tyr126, Thr154, Ser155, Trp156, Arg169, Asp177, Phe199, Ile201, Tyr207 and Glu209<sup>[28,30,31]</sup>. After analysis of the interactions, it was possible to observe that the ligands interact in the same region of the binding site of the co-crystallized granisetron in chains D and E, indicating a similar action to granisetron (Fig. 11D and 11E). We also observed that CaAc, CuAc, FeAc, SiAc are inserted in cavity II (Fig. 11B) and interact with residues of the binding site of the reference drug, indicating an effect like Fluoxetine (Fig. 11D).

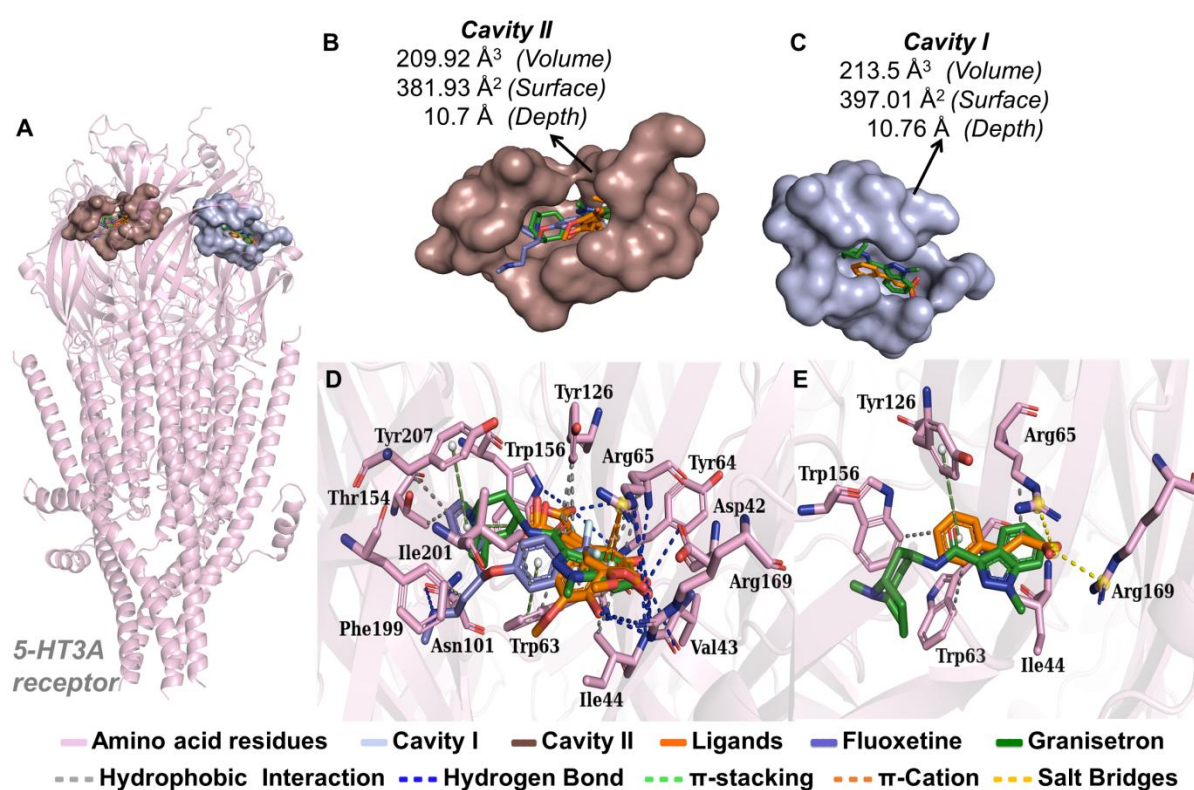


**Figure 12.** Three-dimensional image of the 5-HT<sub>3A</sub> receptor (A); cavities predicted with volume (in Å<sup>3</sup>), surface (Å<sup>2</sup>) and depth (Å) values highlighted (B and C); CaAc, CuAc, FeAc and SiAc, Fluoxetine and Granisetron (D); CiAc and Granisetron (E).

### Learning and memory analysis

The impact of prolonged antidepressant use on neuroplasticity has garnered increasing interest, especially considering the implications for the long-term treatment of depressive disorders. Neuroplasticity refers to the brain's ability to reorganize and adapt its neural connections in response to experiences, medications, and other external stimuli <sup>[32]</sup>. Although

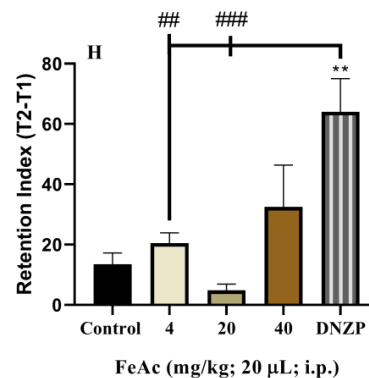
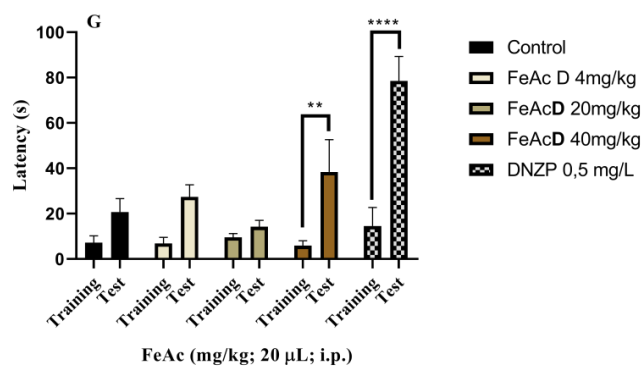
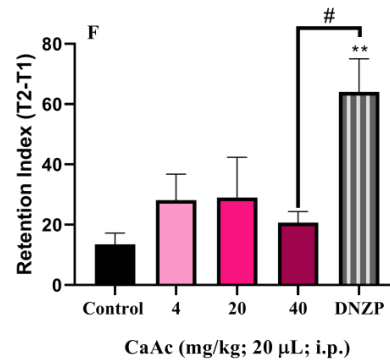
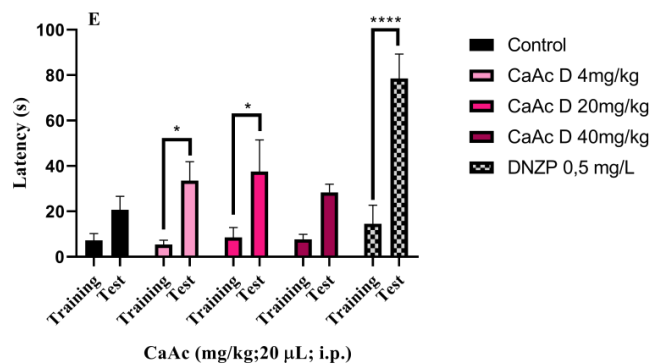
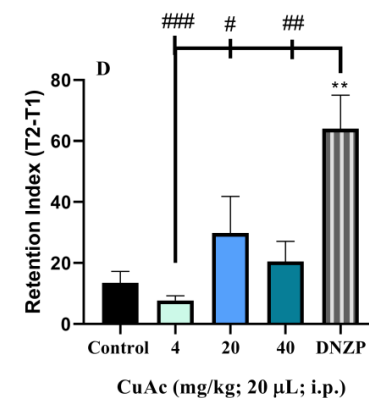
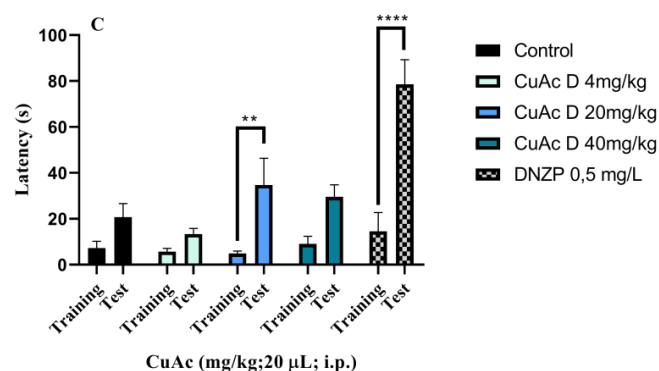
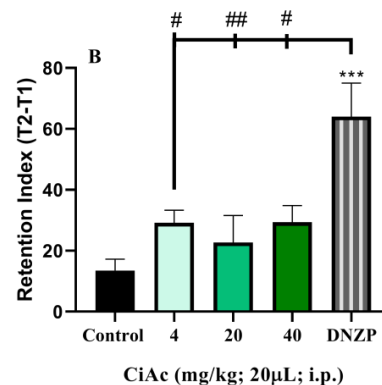
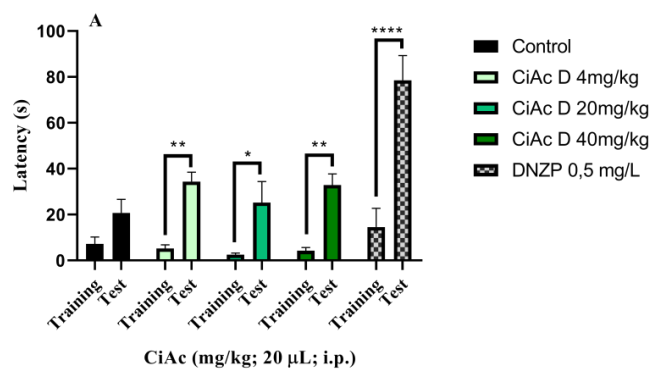
antidepressants may initially stimulate synaptic plasticity, there is evidence that prolonged use can induce excessive stabilization of neural networks, reducing the ability to adapt to new experiences or therapies. This phenomenon may be related to the development of tolerance, in which the effectiveness of treatment decreases over time, requiring adjustments in dosage or the adoption of new therapeutic strategies [33].

Additionally, studies report that prolonged exposure to these drugs can induce structural changes in the brain, such as a reduction in hippocampal volume, which could have negative implications for cognition and emotional resilience [4]. Cognitive difficulties, such as memory complaints, may be related not only to structural changes but also to psychological factors such as anxiety, depression, stress, self-esteem, quality of life, self-efficacy, metamemory, and level of education [34, 35, 36].

Cognition comprises mental processes linked to learning, memorization, and perception [4]. Thus, given the positive responses of phenolic acids in anxiolytic activity, the ability of these compounds to preserve memory was also investigated in the adult zebrafish animal model, where donepezil was used as a reference drug because it acts as an acetylcholinesterase inhibitor, helping to maintain acetylcholine levels in the brain and promoting cognitive function.

The results showed that all doses (4, 20 and 40 mg/kg) of CiAc, the doses of 4 and 20 mg/kg of CaAc, the intermediate dose (20 mg/kg) of CuAc, the highest dose (40 mg/kg) of FeAc, and the doses of 20 and 40 mg/kg of SiAc preserved memory, in a pattern similar to that of donepezil. These compounds showed a significant difference between the training day and the test day, evidenced by the greater latency on the second day, indicating that the animals remained alert (Fig. 13).

It is relevant to note that, unlike donepezil, which induced signs of sedation and resulted in low latency in the test, the phenolic compounds did not present this side effect. In the retention time graphs, this behavior is especially evident at doses of 20 and 40 mg/kg of SiAc, which showed significant differences compared to the control group (\* $p < 0.05$ ; Fig. 13J). In contrast, the absence of memory preservation was observed at all doses of CiAc (Fig. 12B), at the highest dose of CaAc (Fig. 13D), at all doses of CuAc (Fig. 13F), at the lowest doses of FeAc (4 and 20 mg/kg; Fig. 13H), and at the lowest dose of SiAc (4 mg/kg; Fig. 13J).



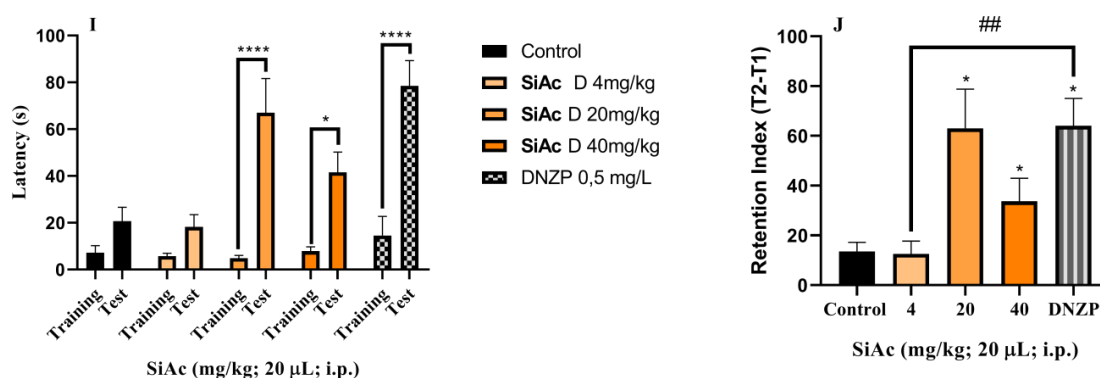


**Figure 13.** Effect of cinnamic acid derivatives on the memory of adult zebrafish in A; C; E; G and I. Effect of cinnamic acid derivatives on the retention index on the memory of adult zebrafish (B; D; F; H and J). Donepezil (DNZP) 0.5 mg/ml used as reference drug demonstrates a significant difference (\*\*\*\*  $p < 0.0001$ ) in the inhibitory avoidance test between the training day and the test day, and demonstrates a significant difference (\*\* $p < 0.01$ ) for memory retention. DMSO 3% (20µl; i.p.) used as a positive control. Values represent the mean  $\pm$  standard error of the mean for 6 animals/group. ANOVA followed by Tukey.

Analysis of the memory retention graphs reveals a consistent pattern: as functional groups are introduced into the reference molecule (CiAc), a progressive improvement in the animals' memory capacity is observed. This effect may be related to the increased antioxidant character of the derivatives, molecules, which intensifies according to the oxidation level of the compound, suggesting that structural modifications exert a direct influence on neurobehavioral performance.

### Molecular docking analysis (memory)

Learning is the process of acquiring new information through the formation of memory, while memory is the process of consolidating, storing, and retrieving acquired information<sup>[37]</sup>. Acetylcholinesterase inhibitors help improve cognitive processes such as learning and memory because they increase the amount of acetylcholine (ACh), one of the most important neurotransmitters in the central cholinergic system<sup>[38]</sup>, involved in signal transduction related to memory and learning ability<sup>[66]</sup>. From this perspective, molecular docking simulations were performed to investigate the possible mechanism of action of AcCi and its derivatives: AcCu, AcCa, AcFe, and AcSi against AChE. The in silico assays showed RMSD values from 0.064 Å to 0.997 Å and affinity energy from -7.3 kcal/mol to -6.8 kcal/mol (Table 3). Analysis of the interactions showed that the evaluated compounds presented hydrogen bonds, hydrophobic interactions, and  $\pi$ -stacking (Table 3).

**Table 4.** Affinity Energy, RMSD and types of interactions formed between ligands and the AChE.

| Ligand | Affinity Energy<br>(kcal/mol) | RMSD (Å) | Interaction Type | Residue (Distance in Å)                                           |
|--------|-------------------------------|----------|------------------|-------------------------------------------------------------------|
| CaAC   | -7.1                          | 0.942    | Hydrophobic      | Trp86A (3.66)                                                     |
|        |                               |          | Hydrogen Bonds   | Ser125A (2.20), Glu202A (2.32)                                    |
|        |                               |          | $\pi$ -Stacking  | Trp86A (3.78), Trp86A (4.01)                                      |
| CiAC   | -6.8                          | 0.064    | Hydrophobic      | Trp86A (3.71), Tyr337A (3.96)                                     |
|        |                               |          | Hydrogen Bonds   | Trp86A (3.16), Ser125A (2.11)                                     |
|        |                               |          | $\pi$ -Stacking  | Trp86A (3.74), Trp86A (3.84)                                      |
| CuAC   | -6.9                          | 0.097    | Hydrophobic      | Trp86B (3.78), Tyr337B (3.91)                                     |
|        |                               |          | Hydrogen Bonds   | Tyr124B (2.32), Ser125B (3.09),<br>Tyr341B (3.46)                 |
|        |                               |          | $\pi$ -Stacking  | Trp86B (3.74), Trp86B (3.92)                                      |
| FeAC   | -7.0                          | 0.997    | Hydrophobic      | Trp86A (3.74), Tyr337A (3.87)                                     |
|        |                               |          | Hydrogen Bonds   | Ser125A (3.20)                                                    |
|        |                               |          | $\pi$ -Stacking  | Trp86A (3.74), Trp86A (3.97)                                      |
| SiAC   | -7.3                          | 0.692    | Hydrophobic      | Phe297A (3.72), Phe297A (3.77),<br>Phe338A (3.83)                 |
|        |                               |          | Hydrogen Bonds   | Gly122A (2.54), Tyr124A (2.34),<br>Ser203A (2.27), His447A (2.76) |
|        |                               |          | $\pi$ -Stacking  | Phe338A (4.96)                                                    |

The redocking of the inhibitor Galantamine exhibited an RMSD of 1.917 Å and a binding energy of -7.6 kcal/mol. The binding site of co-crystallized Galantamine in chains A and B of the AChE receptor is formed by the residues Trp86, Gly120, Gly121, Gly122, Tyr124, Ser125, Tyr133, Glu202, Ser203, Ala204, Phe295, Phe297, Tyr337, Phe338, and His447[40]. Regarding the cavity prediction, two binding sites were observed (Fig. 12), identified as cavity I: volume of 336.38 Å<sup>3</sup>, surface area of 451.25 Å<sup>2</sup>, depth of 14.06 Å, formed by 30 residues

(chain B): Gln71, Tyr72, Val73, Asp74, Thr83, Trp86, Asn87, Pro88, Gly120, Gly121, Gly122, Tyr124, Ser125, Gly126, Tyr133, Glu202, Ser203, Trp286, Ser293, Val294, Phe295, Arg296, Phe297, Tyr337, Phe338, Tyr341, His447, Gly448, Tyr449, and Ile451; cavity II: volume of 329.22 Å<sup>3</sup>, surface area of 441.24 Å<sup>2</sup>, depth of 13.79 Å, formed by 28 residues (chain A): Gln71, Tyr72, Val73, Asp74, Thr83, Trp86, Asn87, Pro88, Gly120, Gly121, Gly122, Tyr124, Ser125, Tyr133, Glu202, Ser203, Trp286, Ser293, Val294, Phe295, Arg296, Phe297, Tyr337, Phe338, Tyr341, His447, Gly448, and Ile451. Detailed analysis of the interactions showed that CuAc is inserted in cavity I; CaAc, CiAc, FeAc, and SiAc are inserted in cavity II. Furthermore, the data also showed that the analyzed compounds interact with residues of the co-crystallized inhibitor's binding site, indicating a similar effect to Galantamine (Fig. 13A and Fig. 13B).

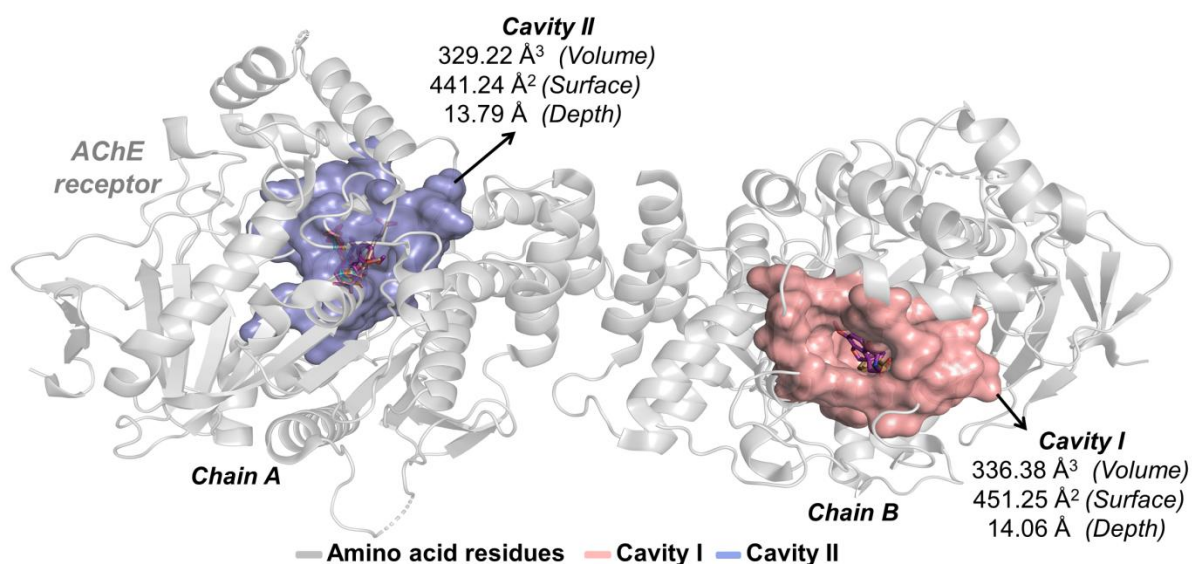


**Figure 14.** Three-dimensional image of the AChE receptor with the cavities predicted with volume (in Å<sup>3</sup>), surface (Å<sup>2</sup>) and depth (Å) values highlighted.

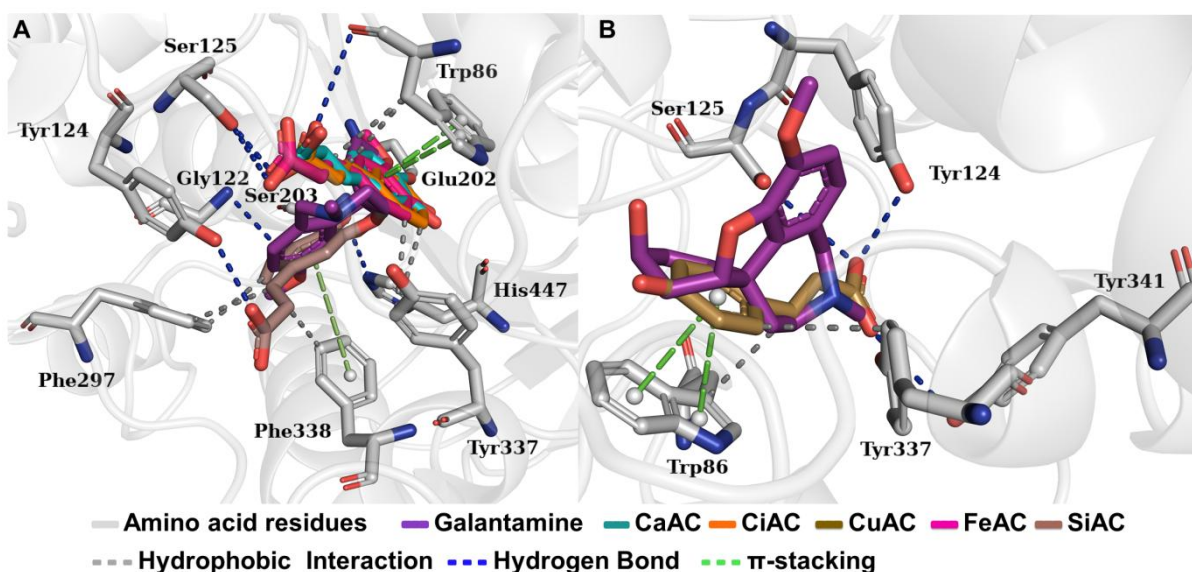


**Figure 15.** Binding site of the compounds against AChE receptor. (A) Galantamine co-crystallized in the Chain A, and CaAc, CiAc, FeAc, SiAc, (B) Galantamine co-crystallized in the Chain B and CuAc.

### **Relationship of the structure-activity**

The results obtained highlight the crucial role of the chemical structure of phenolic compounds in the anxiolytic and memory-preserving activity observed in adult zebrafish. To elucidate the differences in the potential of these molecules, a chemical analysis was performed relating each structure to the biological activities investigated.

All the investigated compounds share a basic CiAc structure but differ in the functional groups substituting the aromatic ring, which can influence anxiolytic activity, interaction with neurotransmitter receptors, and memory preservation, thus affecting therapeutic efficacy. CiAc ( $C_9H_8O_2$ ), a basic compound with a phenyl group, double bond, and carboxylic acid, without hydroxyls or methoxyls, showed good anxiolytic activity, especially at a dose of 40 mg/kg. Despite the absence of hydroxyl/methoxyl groups, its  $\pi$ -conjugated structure allows it to act as a redox agent, capable of donating electrons to neutralize reactive oxygen species (ROS)<sup>[41]</sup>, promoting an antioxidant environment in the central nervous system. This action may contribute to the protection of neural receptors sensitive to oxidative stress, such as 5-HT1A, favoring inhibitory neurotransmission and reducing neuronal excitability, which is related to a decrease in anxious behavior.

Although CiAc did not promote significant memory retention in inhibitory avoidance tests, its antioxidant character may have contributed to the preservation observed in some test groups (Fig. 12A), aligning with the findings of <sup>[42]</sup>, which demonstrated that CiAc improves memory by reducing oxidative stress and cholinergic dysfunction in diabetic mice.

Comparatively, DNPZ and FLX, although structurally distinct from CiAc and its derivatives, share lipophilic aromatic characteristics that facilitate passive diffusion through the blood-brain barrier and allow  $\pi$ - $\pi$  interactions with enzymes such as AChE<sup>[43]</sup>. CuAc has a hydroxyl in the para position on the aromatic ring, which seems to give the compound stable anxiolytic activity across the tested doses, favoring serotonergic neurotransmission and contributing to the reduction of anxious behavior. In tests performed on zebrafish, the highest doses of CuAc showed significant effects, like DZP, showing superior results to those observed with CiAc. In addition, the hydroxyl acts as a donor of protons ( $H^+$ ) and electrons ( $e^-$ ), neutralizing reactive oxygen species (ROS) and preventing oxidative stress in the central nervous system, protecting neurons and receptors involved in the regulation of anxiety and memory<sup>[41]</sup>. In the memory test,

only the intermediate dose showed efficacy (\*\* $p < 0.01$ ; Fig. 12E), suggesting that the antioxidant and anti-inflammatory effects of CuAc<sup>[41]</sup> support cognitive function, in agreement with <sup>[44]</sup>. CaAc showed an anxiolytic effect only for the lowest dose (4mg/kg), despite having two hydroxyl groups (-OH) in the ortho position on the aromatic ring (catechol group), which confer greater polarity and antioxidant potential, favoring interactions with serotonergic receptors. However, this same functional group presents hydrophilic properties for this molecule, making it difficult to penetrate the CNS<sup>[45]</sup>, justifying the limited response observed.

CaAc presents a dose-dependent anxiolytic potential, since high doses can have a pro-oxidant effect, compromising its efficacy. In the inhibitory avoidance test, the lowest doses were able to preserve the animals' memory (Fig. 12C). Similar findings were described by <sup>[23]</sup>, who demonstrated beneficial effects of CaAc on learning deficits in a model of Alzheimer's disease, attributed to the suppression of oxidative stress and inflammation via the p38 MAPK signaling pathway. FeAc, containing a methoxyl (R-O-CH<sub>3</sub>) and a hydroxyl (-OH), has greater oxidation than CaAc, conferring antioxidant and lipophilic properties that facilitate its penetration into the CNS<sup>[41]</sup>. Methoxylation (-OCH<sub>3</sub>) represents a higher level of oxidation than hydroxylation (-OH), as oxygen is linked to an additional carbon. Therefore, even though CaAc and FeAc have 2 oxygenated groups, FeAc is more oxidized due to the presence of methoxyl. This compound demonstrated anxiolytic effect, with greater prominence at lower doses. The combination of methoxyl and hydroxyl provides superior activity to that of CaAc, although less potent than that observed for SiAc, which has two methoxyl groups). This pattern suggests that the presence of more methoxyl substituents may potentiate the anxiolytic action. However, the anxiolytic effect observed in FeAc may be influenced by the dose, since at high doses it can exert a pro-oxidant action, a similar behavior to that observed for CaAc in anxiety tests in zebrafish.

Analyses conducted by <sup>[42]</sup> demonstrated the relationship between the antioxidant activity of FeAc and its effects on the CNS, evidenced by increased activities of superoxide dismutase (SOD) and catalase (CAT), as well as reduced levels of thiobarbituric acid-reactive substances (TBARS) in the hippocampus of male Swiss mice treated with ferulic acid <sup>[9]</sup>. A similar study conducted by <sup>[46]</sup> further showed an association between the antidepressant effect and the anti-inflammatory activity of ferulic acid in rats subjected to prenatal stress. In this context, it was found that the antidepressant activity of ferulic acid is partly related to its anti-inflammatory capacity and to the regulation of the hypothalamic–pituitary–adrenal (HPA) axis <sup>[46]</sup>.

In the learning and memory test with FeAc, a distinct effect was observed: only the highest dose showed significant efficacy on the second day of evaluation (Fig. 12G). This finding may be related to the antioxidant properties of this molecule <sup>[41]</sup>, corroborating the results of <sup>[47]</sup>, who reported inhibition of AChE activity and reduced oxidative stress following FeAc treatment, reinforcing its neuroprotective action. The structure of SiAc contains two methoxy (-OCH<sub>3</sub>) groups and one hydroxyl (-OH) group. This derivative exhibited a consistent anxiolytic effect at all doses, suggesting good interaction with anxiety-related targets. However, its structural modification reduces its antioxidant capacity compared to CaAc, since the methoxy groups block reactive positions and do not efficiently donate hydrogen, limiting the neutralization of reactive oxygen species. On the other hand, these groups increase the molecule's lipophilic properties, favoring its penetration into the central nervous system <sup>[48]</sup>. Thus, SiAc may exert anxiolytic effects through indirect modulation of serotonergic systems, although it has lower antioxidant potency compared to CaAc. This profile also suggests potential anti-inflammatory properties, as previously described for other phenolic derivatives <sup>[45, 42]</sup>.

In the inhibitory avoidance test in adult zebrafish, SiAc showed positive results (Fig. 12I), which may be explained by its effect on antioxidant enzyme levels or by decreasing malondialdehyde levels in the hippocampus. These findings are consistent with those reported by <sup>[26]</sup>, demonstrating the efficacy of SiAc in preventing memory loss in Alzheimer's models, even with lower antioxidant capacity compared to CaAc.

Overall, the data support the therapeutic potential of these compounds in modulating behavioral disorders such as anxiety, making them promising prototypes for the development of new antidepressant agents with fewer adverse effects. Although CiAc did not show high affinity for direct AChE inhibition, its derivatives demonstrated moderate inhibitory activity, reinforcing the idea that the phenylpropanoid core may be a useful structural basis for the synthesis of new inhibitors. The oxidation degree of the compounds was a determining factor for their anxiolytic effects and memory preservation. In this regard, following the order of increasing oxidation, SiAc (sinapic acid)—the most oxidized among the compounds analyzed—showed the most consistent anxiolytic effects at all tested doses, in addition to best preserving memory.

## CONCLUSION

CiAc and its derivatives showed no apparent anatomical alterations and were non-toxic up to 96 hours of analysis, indicating a promising safety profile for therapeutic applications. All evaluated CiAc derivatives promoted a significant reduction in locomotion in adult zebrafish, with CuAc, FeAc, and SiAc standing out for their strong and consistent effects at all doses. Although CaAc and CiAc also reduced locomotor activity, they did not produce the motor impairment characteristic of the sedative state induced by diazepam. The compounds exhibited anxiolytic activity mediated by the modulation of 5-HT receptors. The data obtained indicate the involvement of multiple receptors, including 5-HT<sub>1R1</sub> and 5-HT<sub>3A/3B</sub> (blocked by Pizotifen and Ciprofloxacin) and 5-HT<sub>3</sub> (blocked by Granisetron), confirming the relevance of the serotonergic pathway to the observed effects. In inhibitory avoidance analyses, a consistent pattern of memory retention was observed: as functional groups were introduced into the reference molecule (CiAc), there was a progressive improvement in animal performance. In this context, SiAc was particularly noteworthy, showing the highest memory retention. The relationship between anxiolytic activity and effects on memory proved to be dependent on structural modifications to the aromatic ring. Hydroxyl (-OH) groups, especially in the para position, enhanced activity; methoxy (-OCH<sub>3</sub>) groups increased lipophilicity and CNS penetration while maintaining efficacy; and the combination of hydroxyl and methoxy groups in higher numbers, as in SiAc, resulted in a stable and potent anxiolytic effect in adult zebrafish. Molecular docking results were consistent with the *in vivo* tests, showing that the compounds interact favorably with serotonergic receptors—particularly 5-HT<sub>3A</sub>—displaying significant affinity values. In addition to their anxiolytic potential, the derivatives demonstrated the ability to inhibit AChE activity, which may contribute to improved cognitive performance.

## Author Contributions

Thaís Rocha Cavalcante and Jéssica Bezerra Maciel: Supervision, writing, and review and editing; Joilna Alves da Silva, Amanda Maria Barros Alves, and Tainá de Sousa Silva: Investigation, formal analysis, and writing – original draft. Emmanuel Silva Marinho, Márcia Machado Marinho, and Jacilene Silva: Conceptualization, methodology, and conducted computational analysis of molecular structures. José Roberval Cândido Júnior, Bruno Poti e Silva e Andreia Ferreira de Castro Gomes and Cleia Rocha de Sousa Feitosa: Writing – original

draft and assisted in structure and activity analysis. Jane Eire Silva Alencar de Menezes and Hécio Silva dos Santos: Project administration and writing – review and editing.

## ACKNOWLEDGMENT

Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and scholarship. Helcio Silva dos Santos acknowledges financial support from CNPq (Grant 306008/2022-0) and FUNCAP-INTERNACIONALIZAÇÃO (Grant ITR-0214-00060.01.00/23). Emmanuel Silva Marinho acknowledges financial support from CNPq PQ [Grant#: 309349/2025-7]. The authors thanks to Prof. Dr. Tiago Pinheiro Braga from Physical-Chemistry Department and High Computing Data Center (NPAD/UFRN) at Universidade Federal do Rio Grande do Norte for computational resources

## REFERENCES

- [1] E.G. Ribeiro, E. L. Souza, J.D.O Nogueira & R. Eler. Saúde mental na perspectiva do enfrentamento à COVID-19: manejo das consequências relacionadas ao isolamento social. *Rev Enfermagem e Saúde Coletiva*, **2020**, 5(1).
- [2] F.J Farach, L.D. Pruitt, J.J Jun, A.B. Jerud, L.A Zoellner, P. P & Roy-Byrne. Tratamento farmacológico dos transtornos de ansiedade: tratamentos atuais e direções futuras. *Jornal de transtornos de ansiedade*, **2012**, 26(8).
- [3] V. R. Fávero, M. D. O Sato & R. M. Santiago. Uso de ansiolíticos: abuso ou necessidade?. *Visão acadêmica*, **2017**, 18(4).
- [4] J. Bennett, M. Carter & N. Allen. Structural brain changes associated with prolonged anti-depressant use: Implications for cognitive function. *Neuroscience Letters*, **2023**, 781.
- [5] C. López-Rubalcava, E. Estrada-Camarena. Plantas medicinais mexicanas com atividade ansiolítica ou antidepressiva: Foco na pesquisa pré-clínica. *J. Etnofarmacol*, **2016**, 20.
- [6] W.X. Zhang, H. Wang, H. R. Cui, W.B. Oliveira, F. Zhou, D. S. Cai, B. Xu, X. H. Jia, X. M. Huang, Y. Q. Yang. Projeto, Síntese e Avaliação Biológica de Derivados

- de Ácido Cinâmico com Neuroproteção Sinérgica e Efeito de Angiogênese. *Eur. J. Med. Chem.* **2019**, 183.
- [7] G. D. A. De Lima, M.P. Rodrigues, T. A. O. de Mendes, G. A. Oliveira, R. P. Oliveira, A. M.da Silva, B. G. Vaz, J. L. R. Fietto, G. C Bressan, M. Machado-Neves. Avaliação da síntese e atividade antimetastática de derivados do ácido cinâmico contendo porções de 1,2,3-triazólicos. *Toxicol. In vitro*, **2018**, 53.
- [8] J. S. Lan, J. W. Hou, Y. Liu, Y. Ding, Y. Zhang, L. Li, T. Zhang. Projeto, síntese e avaliação de novos derivados de ácido cinâmico contendo fração N-benzil piridínio como inibidores multifuncionais da colinesterase para a doença de Alzheimer. *J. Enzima Inibida. Med. Chem.* **2017**, 32.
- [9] J. M. Oliveira, A. Oliveira, S. Hoenke, R. Csuk. Em busca de novos sabores e fragrâncias derivados do ácido cinâmico. *Resultados Chem.* **2019**, 1.
- [10] G. Mancilla-Montelongo, G. S. Castañeda-Ramírez, J. F. de Jesús Torres-Acosta, C. A. Sandoval-Castro, R. Borges-Argáez. Avaliação do ácido cinâmico e seis análogos contra ovos e larvas de *Haemonchus Contortus*. *Veterinário. Parasitol.* **2019**, 270.
- [11] S. Hu, X. Yang, J. Xue, X. Chen, X. H. Bai, Z. H. Yu. Microextração de solidificação flutuante de grafeno / dodecanol para a pré-concentração de níveis vestigiais de derivados de ácido cinâmico em medicamentos tradicionais chineses. *J. Sep. Sci.* **2017**, 40.
- [12] G. Mancilla-Montelongo, G. S. Castañeda-Ramírez, J. F. de Jesús Torres-Acosta, C. A. Sandoval-Castro, R. Borges-Argáez. Avaliação do ácido cinâmico e seis análogos contra ovos e larvas de *Haemonchus Contortus*. *Veterinário. Parasitol.* **2019**, 270.
- [13] M. Kumazaki, H. Shinohara, K. Taniguchi, N. Yamada, S. Ohta, K. Ichihara, Y. Akao Y. Propolis cinnamic acid derivatives induce apoptosis through both extrinsic and intrinsic apoptosis signaling pathways and modulate of miRNA expression. *Phytomedicine*, **2014**, 8.
- [14] L. S. Feng, Z. Xu, L. Chang, C. Li, X. F. Yan, C. Gao, C. Ding, F. Zhao, F. Shi, X. Wu, *Med. Res. Rev.* **2020**, 40(3).
- [15] L. S. Feng, Z. Xu, L. Chang, C. Li, C., X. F. Yan, C. Gao, & X. Wu. Hybrid molecules with potential in vitro antiplasmodial and in vivo antimalarial activity against drug-resistant *Plasmodium falciparum*. *Medicinal Research Reviews*, **2020**, 40(3).

- [16] S.Y. B. de Carvalho, R. R. Almeida, N. A. R. Pinto, C. de Mayrinck, S. S. Vieira, J. F. Haddad, & L. G. D. L. Guimarães. Encapsulamento de óleos essenciais utilizando nanogel de quitosana enxertado com ácido cinâmico: Preparação, caracterização e atividade antifúngica. *Jornal Internacional de Macromoléculas Biológicas*, **2021**, 166.
- [17] S. Oliveira e R. R. Kannan. Zebrafish: Um sistema modelo emergente em tempo real para estudar a doença de Alzheimer e a descoberta de medicamentos neuroespecíficos. *Morte Celular Discov.* **2018**, 4, 45.
- [18] F. E., Williams, D. Branco, W. S. Messer. Uma tarefa simples de alternância espacial para avaliar a função de memória em peixe-zebra. *Comportar-se. Processo*, **2002**, 58, 1.
- [19] T. Canção, Z. Yu, Q. Shen, Y. Xu, H. Hu, J. Liu, K. Oliveira, J. Lei, L. Y. Estudos farmacodinâmicos e de toxicidade de análogos de 6-isopropilditio-2'-guanosina na leucemia linfoblástica T aguda. *Cânceres*, **2024**, 16(9).
- [20] D. L. Champagne, C.C. Hoefnagels, R. E. De Kloet, & M. K. Richardson. Traduzindo o repertório comportamental de roedores para o peixe-zebra (Danio rerio): relevância para a pesquisa do estresse. *Pesquisa comportamental do cérebro*, **2010**, 214(2).
- [21] T. Prorok, M. Jana, D. Patel, K. Pahan. O ácido cinâmico protege o nigrostriatum em um modelo de camundongo da doença de Parkinson por meio do receptor ativado por proliferador de peroxissomoα. *Neuroquímica Res*, **2019**, 44.
- [22] R. D. P. Osorio. Análise da relação entre o potencial antioxidante dos ácidos ferúlico e p-cumárico e a enzima catalase de *saccharomyces cerevisiae*, **2021**.
- [23] Y. Wang, Y. Wang, J. Li, L. Hua, B. Han, Y. Zhang, X. Yang, Z. Zeng, H. Bai, H. Yin, & J. Lou. Efeitos do ácido cafeico nos déficits de aprendizagem em um modelo de doença de Alzheimer. *Revista internacional de medicina molecular*, **2016**, 38(3).
- [24] L. R. L. Diniz, M. T. D. S. Souza, J. N. Barboza, R. N. D. Almeida, & D. P. D. Sousa. Antidepressant potential of cinnamic acids: mechanisms of action and perspectives in drug development. *Molecules*, **2019**, 24(24).
- [25] L. R. L. Diniz, M. T. D. S. Souza, J. N. Barboza, R. N. D. Almeida, & D. P. D. Sousa. Antidepressant potential of cinnamic acids: mechanisms of action and perspectives in drug development. *Molecules*, **2019**, 24(24).
- [26] P. Shahmohamady, A. Eidi, P. Mortazavi, N. Panahi, & D. Minai-Tehrani. Efeito do ácido sináptico nos déficits de memória e degeneração neuronal induzidos pela

- administração intracerebroventricular de estreptozotocina em ratos. *Jornal polonês de patologia: jornal oficial da Sociedade Polonesa de Patologistas*, **2018**, 69(3).
- [27] M. A. Nowicki, D. J. Wald, M. W. Hamburger, M. Hearne, & E. M. Thompson. Desenvolvimento de um modelo globalmente aplicável para previsão quase em tempo real de deslizamentos de terra induzidos por terremoto. *Geologia de engenharia*, **2014**, 173.
- [28] M. A. Alam, N. Subhan, H. Hossain, M. Hossain, H. M. Oliveira, M. M.; Rahman, M. O. Ullah. Derivados do ácido hidroxicinâmico: Uma classe potencial de compostos naturais para o tratamento do metabolismo lipídico e da obesidade. *Nutr. Metab.* **2016**, 13, 27.
- [29] S. Shityakov and C. Förster. “In silico predictive model to determine vector-mediated transport properties for the blood-brain barrier choline transporter,” *Adv. Appl. Bioinforma. Chem.*, **2014**, (7).
- [30] S. Basak, Y. Gicheru, A. Kapoor, M. L. Mayer, M. Filizola, and S. Chakrapani, “Molecular mechanism of setron-mediated inhibition of full-length 5-HT<sub>3A</sub> receptor,” *Nat. Commun.*, **2019**, 10(1).
- [31] A. S. Rose, A. R. Bradley, Y. Valasatava, J. M. Duarte, A. Prlić, and P. W. Rose, “NGL viewer: web-based molecular graphics for large complexes,” *Bioinformatics*, **2018**, 34(21).
- [32] Z. Liu, Z., Y. Wang, & Q. Li. Long-term antidepressant use and its impact on hippocampal neurogenesis: A review. *Journal of Affective Disorders*, 2023, 334.
- [33] M. Thompson, & P. Fox, P. Antidepressant tolerance and the role of synaptic plasticity in long-term treatment outcomes. *Brain Research Bulletin*, **2022**, 178.
- [34] M. Nakamura, Y. Kawata, M. Hirose, T. Ota, & N. Shibata. Efeitos diferenciais do exercício agudo na memória emocional em homens e mulheres. *Fronteiras em Esportes e Vida Ativa*, **2023**, 5.
- [35] M. Nakamura, Y. Kawata, M. Hirose, T. Ota, & N. Shibata. Efeitos diferenciais do exercício agudo na memória emocional em homens e mulheres. *Fronteiras em Esportes e Vida Ativa*, **2023**, 5.
- [36] E. V. Filatova, M. I. Shadrina, & P. A. Slominsky. Depressão maior: um cérebro, uma doença, um conjunto de processos entrelaçados. *Células*, **2021**, 10(6).
- [37] R. Gerlai, “Learning and memory in zebrafish (*Danio rerio*),” in *Methods in Cell Biology*, **2016**, 134. 36

- [38] Q. Huang, C. Liao, F. Ge, J. Ao, and T. Liu, "Acetylcholine bidirectionally regulates learning and memory," *J. Neurorestoratology*, **2022**, 10(2).
- [39] Z.-R. Chen, J.-B. Huang, S.-L. Yang, and F.-F. Hong, "Role of Cholinergic Signaling in Alzheimer's Disease," *Molecules*, **2022**, 27(6).
- [40] J. Cheung, M. J. Rudolph, F. Burshteyn, M. S. Cassidy, E. N. Gary, J. Love, & J. J. Height. "Structures of human acetylcholinesterase in complex with pharmacologically important ligands," *J. Med. Chem.*, **2012**,. 55 (22).
- [41] S.E. Soares. Ácidos fenólicos como antioxidantes. *Revista de nutrição*, **2002**, 15.
- [42] A. A. Hemmati. S. Alboghobeish, & A. Ahangarpour. Efeitos do ácido cinâmico nos déficits de memória e estresse oxidativo cerebral em camundongos diabéticos induzidos por estreptozotocina. *O jornal coreano de fisiologia e farmacologia: jornal oficial da Sociedade Coreana de Fisiologia e da Sociedade Coreana de Farmacologia*, **2018**, 22 (3).
- [43] P. Honorio, S. Hannongbua, & P. Saparpakorn. Papéis dos andaimes híbridos de donepezil como potentes inibidores da acetilcolinesterase humana usando análise de interação in silico, semelhança com drogas e previsão farmacocinética. *Interações químico-biológicas*, **2022**, 368.
- [44] S. Ghaderi, P. Gholipour, A. Komaki, I. Salehi, K. Rashidi, S. Esmacil Khoshnam, & M. Rashno. O ácido p-cumárico melhora os distúrbios cognitivos e não cognitivos em um modelo de rato da doença de Alzheimer: o papel do estresse oxidativo e da inflamação. *Imunofarmacologia internacional*, **2022**, 112.
- [45] J. Lenzi, R. De Santis, V. De Turre, M. Morlando, P. Laneve, A. Calvo, & I. Bozzoni. ALS mutant FUS proteins are recruited into stress granules in induced pluripotent stem cell-derived motoneurons. *Disease models & mechanisms*, **2015**, 8(7).
- [46] X. Zheng, Y. Cheng, Y., Y. Chen, Y. Yue, Y. Li, S. Xia, Y. Li, H. Deng, J. Zang, Y. Cao. Ferulic acid improves depressive-like behavior in prenatally-stressed offspring rats via anti-inflammatory activity and HPA axis. *International Journal of Molecular Sciences*, **2019**, 20(3).
- [47] H. Yang, Z. Qu, J. Zhang, L. Huo, J. Gao, & W. Gao. Ferulic acid improves memory impairment in d-galactose. *International Journal of Food Science and Nutrition*, **2016**, 67(7).

- [48] S. Lee, H. B. Kim, E. S. Hwang, E. S. Kim, S. S. Kim, T. D. Jeon, & J. H. Parque. Efeitos semelhantes aos antidepressivos do ácido p-cumárico nas alterações depressivas e inflamatórias induzidas por LPS em ratos. *Exp. Neurobiol.* **2018**, 27(3).
- [49] O. Arellano-Aguilar, S. Solis-Angeles, L. Serrano-García, E. Morales-Sierra, A. Mendez-Serrano, R. Montero-Montoya. Uso do Teste de Toxicidade de Embriões de Peixe-Zebra para Avaliação de Risco: Estudo de Caso. *Jornal de Fisheriessciences.com*, **2015**, 9.
- [50] C.K. Benneh, R.P. Biney, P.K. Mante, A. Tandoh, D.W. Adongo, E. Woode. Maerua angolensis stem bark extract reverses anxiety and related behaviours in zebrafish Involvement of GABAergic and 5-HT systems. *J. Ethnopharmacol.*, **2017**, 207.
- [51] R.P. Biney, C.K. Benneh, J.O. Kyekyeku, E.O. Ameyaw, E. Boakye-Gyasi, E. Woode. Attenuation of anxiety behaviours by xylopic acid in mice and zebrafish models of anxiety disorder. *Pharm. and Bio. J.*, **2018**, 6.
- [52] K. T. Bertencello, T. E. Müller, B. D. Fontana, F. Franscescon, G. LB Filho, & D. B. Rosemberg. Taurine prevents memory consolidation deficits in a novel alcohol-induced blackout model in zebrafish. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, **2019**, 93.
- [53] T. A. Halgren, “Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94,” *Jornal de química computacional*, **1996**, 17(5–6).
- [54] H. Berman, K. Henrick, and H. Nakamura, “Announcing the worldwide Protein Data Bank,” *Nature structural & molecular biology.*, **2003**,. 10 (12). , no. 12, 2003.
- [55] P. Xu, S. Huang, H. Zhang, C. Mao, X. E. Zhou, X. Cheng & H. E. Xu. “Structural insights into the lipid and ligand regulation of serotonin receptors,” *Nature*, **2021**, 592(7854).
- [56] K. T. Kimura, H. Asada, A. Inoue, F. M. N. Kadji, D. Im, C. Mori, & T. Shimamura. “Structures of the 5-HT<sub>2A</sub> receptor in complex with the antipsychotics risperidone and zotepine,” *Nat. Struct. Mol. Biol.*, **2019**, 26(2).
- [57] Y. Peng, J. D. McCorvy, K. Harpsøe, K. Lansu, S. Yuan, P. Popov, & Z. J. Liu. “5-HT<sub>2C</sub> Receptor Structures Reveal the Structural Basis of GPCR Polypharmacology,” **2018**, 172(4).
- [58] J. Yan, G. Zhang, J. Pan, and Y. Wang, “ $\alpha$ -Glucosidase inhibition by luteolin: kinetics, interaction and molecular docking,” *Int. J. Biol. Macromol.*, **2014** (64).

- [59] R. Huey, G. M. Morris, and S. Forli, "Using autodock 4 and autodock vina with autodocktools : a tutorial." *The Scripps Research Institute Molecular Graphics Laboratory*, **2012**, 92037.
- [60] R. Huey, G. M. Morris, and S. Forli, "Using autodock 4 and autodock vina with autodocktools : a tutorial." *The Scripps Research Institute Molecular Graphics Laboratory*, **2012**, 92037.
- [61] O. Trott and A. J. Olson. "AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading," *J. Comput. Chem.*, **2010**, 31(2).
- [62] E. M. Marinho, J. B. de Andrade Neto, J. Silva, C. R. da Silva, B. C. Cavalcanti, E. S. Marinho, & H. V. N. Júnior. "Virtual screening based on molecular docking of possible inhibitors of Covid-19 main protease," *Microb. Pathog.*, **2020**, (148).
- [63] D. Yusuf, A. M. Davis, G. J. Kleywegt, and S. Schmitt, "An Alternative Method for the Evaluation of Docking Performance: RSR vs RMSD," *J. Chem. Inf. Model.*, **2008**, (48).
- [64] J. Graef, C. Ehrt, and M. Rarey, "Binding Site Detection Remastered: Enabling Fast, Robust, and Reliable Binding Site Detection and Descriptor Calculation with DoGSite3," *J. Chem. Inf. Model.*, **2023**, 63(10).
- [65] E. M. Marinho, J. B. de Andrade Neto, J. Silva, C. R. da Silva, B. C. Cavalcanti, E. S. Marinho, & H. V.N. Júnior. "Virtual screening based on molecular docking of possible inhibitors of Covid-19 main protease," *Microb. Pathog.*, **2020**, 148.
- [66] D. Yusuf, A. M. Davis, G. J. Kleywegt, and S. Schmitt, "An Alternative Method for the Evaluation of Docking Performance: RSR vs RMSD," *J. Chem. Inf. Model.*, **2008**, 48(7).
- [67] Koopmans, T. (1934). Über die Zuordnung von Wellenfunktionen und Eigenwerten zu den Einzelnen Elektronen Eines Atoms. *Physica*, 1(1–6), 104–113. [https://doi.org/10.1016/S0031-8914\(34\)90011-2](https://doi.org/10.1016/S0031-8914(34)90011-2)
- [68] Krishnan, R., Binkley, J. S., Seeger, R., & Pople, J. A. (1980). Self-consistent molecular orbital methods. XX. A basis set for correlated wave functions. *The Journal of Chemical Physics*, 72(1), 650–654. <https://doi.org/10.1063/1.438955>
- [69] Lu, T. (2024). A comprehensive electron wavefunction analysis toolbox for chemists, Multiwfn. *The Journal of Chemical Physics*, 161(8). <https://doi.org/10.1063/5.0216272>

- [70] Lu, T., & Chen, F. (2012). Multiwfn: A multifunctional wavefunction analyzer. *Journal of Computational Chemistry*, 33(5), 580–592. <https://doi.org/10.1002/jcc.22885>
- [71] M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. V. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, ... D. J. Fox. (2019). *Gaussian 16, Revision C.01*. Gaussian, Inc.
- [72] Morell, C., Grand, A., & Toro-Labbé, A. (2005). New Dual Descriptor for Chemical Reactivity. *The Journal of Physical Chemistry A*, 109(1), 205–212. <https://doi.org/10.1021/jp046577a>
- [73] Roy Dennington, Todd A. Keith, & John M. Millam. (2016). *GaussView* (6.0). Semichem Inc. Shawnee Mission, KS.
- [74] Wang, L., Ding, J., Pan, L., Cao, D., Jiang, H., & Ding, X. (2021). Quantum chemical descriptors in quantitative structure–activity relationship models and their applications. *Chemometrics and Intelligent Laboratory Systems*, 217, 104384. <https://doi.org/10.1016/j.chemolab.2021.104384>
- [75] Zhao, Y., & Truhlar, D. G. (2008). The M06 suite of density functionals for main group thermochemistry, thermochemical kinetics, noncovalent interactions, excited states, and transition elements: two new functionals and systematic testing of four M06-class functionals and 12 other functionals. *Theoretical Chemistry Accounts*, 120(1–3), 215–241. <https://doi.org/10.1007/s00214-007-0310-x>



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