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Effect of Cinnamaldehyde Chalcone on Behavior in Adult *Zebrafish* (Danio rerio): *In Silico* Approach

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The study focuses on the anxiolytic potential of chalcone (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one (CHALCNM) in adult *zebrafish*. Successfully synthesized in 58% yield, CHALCNM demonstrated no toxicity after 96 h of exposure. In behavioral tests, CHALCNM (40 mg/kg) reduced locomotor activity and promoted less anxious behavior in *zebrafish*, confirmed by increased permanence in the light zone of the aquarium. Flumazenil reversed its anxiolytic effect, indicating interaction with GABAA receptors. Furthermore, CHALCNM (4 and 20 mg/kg) preserved *zebrafish* memory in inhibitory avoidance tests. Virtual screening and ADMET profile studies suggest high oral bioavailability, access to the CNS, favored by low topological polarity (TPSA $\leq$ 75 Å^2) and low incidence of hepatotoxicity, standing out as a promising pharmacological agent against the GABAergic system. In molecular coupling, CHALCNM demonstrated superior affinity to diazepam for the GABAA receptor. These results reinforce the therapeutic potential of CHALCNM in the treatment of anxiety, highlighting its possible future clinical application.

Introduction

■■Please provide academic titles (Prof., Dr.) for all authors■■

Anxiety is the most prevalent brain disorder and a common cause of human disability; being treated as an emotional state in which the individual feels distressed and/or euphoric about an event that may or may not happen. It is a complex disease with great clinical relevance, where studies with animal models are fundamental to understanding the pathogenesis of anxiety and its pharmacotherapy.[1,2] Several studies have been carried out to find new drugs that can solve this problem.[3,4,5] There are several drugs for the treatment of anxiety; among them are benzodiazepines, drugs considered anxiolytics, anticonvulsants and hypnotics. It is one of the most used classes of drugs in the world. However, it can cause health problems in the case of prolonged and disoriented use, generating dependence. Thus, despite the existence of these drugs, it is essential to develop more effective drugs with fewer side effects than existing drugs.[1]

Cinnamaldehyde (cinnamic aldehyde or 3-phenyl-2-propenal), a cyclic terpene alcohol, is the main active component of cinnamon essential oil (60–75%) and has been widely used in biological and pharmacological activities, and antimicrobial, antioxidant and antidiabetic activities have been reported.[6] Chalcones, belonging to the flavonoid family, appear as a possibility for treating anxiety. They are compounds that play a significant role in medicinal chemistry, whether in natural or synthetic form, exhibiting a wide range of pharmaceutical activities, including anxiolytic, antioxidant and antimicrobial.[4,7] In the search, the synthesis of a chalcone derived from cinnamaldehyde may be important for the development more specific drugs with fewer side effects.

In recent years, some works by the research group LQPN-UECE have shown the anxiolytic potential present in different synthetic chalcones to explore which structural characteristics are important to exert this effect. Thus, XAVIER[8] and MENDES[9] observed that hydroxyl and methoxyl substituent groups influenced anxiolytic activity. Furthermore, Ferreira[10] identified that the chlorine substituent (Cl) in the R1 position potentiated this effect. So, it was possible to observe that this relationship between structure and substituents is significant in determining pharmacological effects. In addition, in silico studies such as molecular docking and ADMET study are very important as they play essential roles in the drug discovery and development process, helping to understand molecular interactions, predict

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pharmacokinetic properties and identify promising drug candidates.[11]

Among the animal models used for this study is the *zebrafish*, which has gained prominence because of its great genetic and physiological homology compared to mammals, its ease and low maintenance cost and for being highly sensitive to various genetic, epigenetic and neuropharmacological factors.[2] Furthermore, they are widely used in toxicity studies,[12] as well as for learning and memory studies.[13,14] Therefore, the chalcone (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenyl-penta-2,4-dien-1-on, derived from cinnamaldehyde, was synthesized to verify its anxiolytic potential in *zebrafish*, as proposed for a possible drug.

Results and Discussion

In this study, we aimed to perform a comprehensive structure-activity analysis of a cinnamaldehyde derivative, called CHALCNM, and its properties involving acute toxicity and behavioral tests in different contexts. Our objective is to evaluate the impact of this substance on aspects such as anxiety, environmental exploration and learning and memory, using adult *zebrafish* as an animal model.

Structural Determination of Chalcone (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one

The CHALCNM ^{1}H-NMR spectrum (Figure S1, supplementary material) revealed the presence of four signals corresponding to olefinic hydrogens at δH 7.70 (d, Hα, J = 14.7 Hz), 7.37 (d, H7, J = 15.6 Hz), 7.72 (d, Hβ, J = 14.1 Hz) and 7.40 (d, H8, J = 14.8 Hz), whose coupling constant value (J) obtained confirms the E stereochemistry of the double bond. The signal at 12.90 (s, OH) refers to the hydroxyl of Ring A. The signals at 7.83–7.86 (m, H6′), 7.04 (d, H3′, J = 8, 5 Hz), 7.46–7.51 (m, H4′) and 6.93 (t, H5′, J = 8.1 Hz,) refer to the aromatic hydrogens of ring A. While the signals at 7.38 (d, H3/5, J = 7.4 Hz,), 7.51 (d, H2/6, J = 7.5 Hz) and 7.43 (m, H4) are associated with the aromatic hydrogens of the B ring.

The signals found for ^{1}H-NMR of CHALCNM are in agreement with the values found by SILVA[15] for olefinic hydrogens: 7.22 (d, Hα, J = 14.7 Hz), 7.05 (m, H7), 7.67–7.76 (m, Hβ) and 7.07 (m, H8) and for the phenolic hydroxyl (δ 12.88 s, OH). ■■Dear Author, Figure 1 is not mentioned in the text■■

The ^{13}C NMR spectrum (Figure S2) showed a total of 15 signals, one signal associated with the unsaturated α,β carbonyl at 193.5. Absorptions at 127.6; 127.0; 145.7; 143.1 are compatible with the olefinic carbons α (Cα; C7) and β (Cβ; C8), respectively. At 163.7 (C-2′), 136.1 (C-4′), 129.6 (C-6′), 120.2 (C-1′), 118.7 (C-3′) and 118.9 (C-5′) have the signs referring to the carbons present in the A ring. While the signs at 129.7 (C-4), 136.4 (C-1), 127.6 (C −2/6) and 129.6 (C-3/5) refer to the B ring carbons.

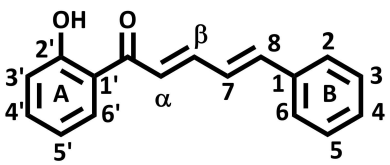


Figure 1. Enumerated structure of CHALCNM with demarcation of AB rings.

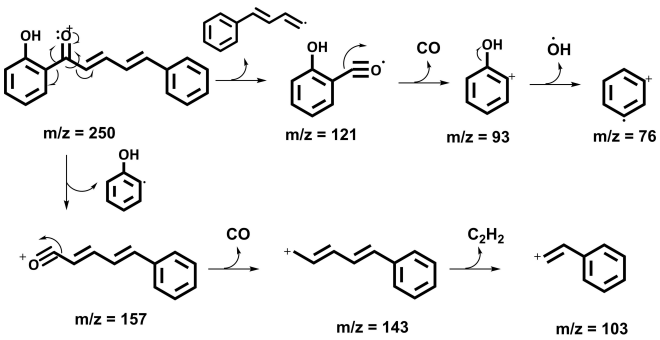
Similar values were found by SILVA[15] for the unsaturated α,β carbonyl at 194.0 and for the olefinic carbons at 123.5 (Cα), 126.7 (C7), 145.4 (Cβ), 142.9 (C8). ■■Dear Author, Table 1 is not mentioned in the text■■

From the ATR-FTIR spectrum (Figure S3), it was possible to verify the presence of the main functional groups present in the chalcone, such as the band at 1633 cm^{-1} referring to carbonyl (C=O). The values of the bands found corroborate the values reported in the study by XAVIER,[16] where the characteristic stretches –C=O and –C=C were at 1638–1689 cm^{-1}, 1444–1488 cm^{-1} and 1562 cm^{-1}, respectively. An overlap of the C=O stretching bands with the Cα–Cβ stretching bands is observed, with an increase in the intensity of the band with the lowest wave number. The C–O stretching of phenol is observed by the band at 1148 cm^{-1}. Out-of-plane C–H stretches for monosubstituted aromatics were evidenced by bands at 756–690 cm^{-1}. The hydroxyl group showed broad and shallow absorption at 3052 cm^{-1}.

The structure of the synthesized chalcone were also confirmed through the analysis of the mass spectra (Figure S4) in a fragmentation proposal (Scheme 1), The mass spectra revealed peaks of the M $^{+}$ molecular ion at m/z 250 justifying the molecular formula $C_{17}H_{14}O_2$ of the synthesized chalcone, the base peak characteristic of the general process of fragmentation was observed at m/z 121, the peak at m/z 157 was obtained from an α segmentation and, the fragments at m/z 143 and m/z 93 were obtained by the loss of a CO molecule. In addition, peaks were observed at m/z 103 and m/z 43, the data were

Table 1. ^{1}H and ^{13}C NMR data of chalcone (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one in CDCI3. Chemical shifts in δC and δH are in ppm.

C	δ_C	δ_H
1'	120.2	
2'	163.7	12.90 (s, OH)
3'	118.7	7.04 (d, J = 8,5 Hz)
4'	136.1	7.46–7.51 (m)
5'	118.9	6.93 (t, J = 8.1 Hz)
6'	129.6	7.83–7.86 (m)
C=O	193.9	
1	136.4	
2/6	127.6	7.51 (d, J = 7.5 Hz)
3/5	129.6	7.38 (d, J = 7.4 Hz)
4	129.7	7.43 (m)
C_α	127.6	7.70 (d, J = 14.7 Hz)
C_β	145.7	7.72 (d, J = 14.1 Hz)
7	127.0	7.37 (d, J = 15.6 Hz)
8	143.1	7.40 (d, J = 14.8 Hz)



Scheme 1. Chalcone fragmentation proposal.

compared with the literature of chalcones of similar structures.[17,18]

Acute Toxicity 96h

Zebrafish is a promising animal model for neuroscientific research and behavioral studies. Among the several characteristics, there is the sensitivity to pharmaceutical compounds as an incentive for the use of *zebrafish* in the sense of modeling human diseases on a large scale. In this context, the use of *zebrafish* to study defensive and anxiety-related behaviors has gained prominence. It is noteworthy that adult *Zebrafish* is also used to assess the toxicity of pharmaceutical compounds,[19] as well as toxicological biomonitoring in drug development.[20] In this way, the acute toxicity was evaluated using the adult *Zebrafish* as an animal model of the chalcone CHALCNM and its starting material, cinnamaldehyde. As a result, it was found that the doses of 4 mg/kg, 20 mg/kg and 40 mg/kg of CHALCNM were not toxic to adult *zebrafish* (LD50 > 40 mg/kg, Table 2), as there was no death or anatomical change apparent in the animals during the 96 h of analysis ($p > 0.05$). However, the Cinnamaldehyde reagent was quite toxic at the concentrations tested.

The toxicity presented by chalcones is widely reported in the literature. And this characteristic has increasingly encouraged studies to discover new drugs using these compounds.[5,8,9,10,21]

Table 2. Results of the acute toxicity tests of CHALCNM and CINAMALDEHYDE.

Sample	Mortality				96h LD_{50} (mg/kg)/CI
	CN	D1	D2	D3	
CHALCNM	0	0	0	0	>40
CINAMALDEHYDE	0	6	6	6	Toxic

CN – Negative control group: DMSO 3%. D1 – Dose 1 (4 mg/kg). D2 – Dose 2 (20 mg/kg). D3 – Dose 3 (40 mg/kg). LD50-lethal dose to kill 50% of adult *Zebrafish*; CI – confidence interval; **Source**: Prepared by the author.

Assessment of Locomotor Activity (Open Field Test – OFT)

Considering the *zebrafish* as an animal model for the study of anxiety, one of the crucial parameters for behavioral research in larvae and adult animals is the open field test, which in the present work was used to evaluate the effect of chalcone on the locomotor activity of *zebrafish* adult. Through this test, it is possible to evaluate several parameters, such as hyperactivity and erratic movements, that can be indicators of anxiety.[22]

To explore which structural features of chalcones are important to exert their anxiolytic effect, some published works will be reported. Recently, it was demonstrated that during the open field test with *zebrafish*, chalcone with the substituents (R1 = H, R2 = Cl) (40 mg/kg), chalcone (R1 = H, R2 = F) (20 and 40 mg/kg) and all doses of chalcones (R1 = R2 = Cl) and (R1 = F, R2 = H) reduced animal locomotion and consequently reduced the number of crossings. These results about the synthesized chalcones were significantly similar to the Diazepam control and very different from the controls.[10]

In the present study, As a result of the open field (Figure 2), it was observed that CHALCNM (40 mg/kg) altered *zebrafish* locomotion (**** $p < 0.0001$ vs. control), statistically similar to Diazepam in animal locomotion. While the doses of 4 and 20 mg/kg had no difference with the control and were statistically different from the Dizepam group (#### $p < 0.0001$ vs DZP). It is worth mentioning that the chalcone in this study has the hydroxyl group as a substituent in its structure, which, as mentioned above, is a group present in compounds that may be associated with anxiolytic effects.

Novel Tank Test

When *zebrafish* are introduced to a new environment, it is common to observe a series of behavioral responses that resemble anxiety, such as remaining longer at the bottom of the aquarium and exhibiting freezing behavior.[23] These

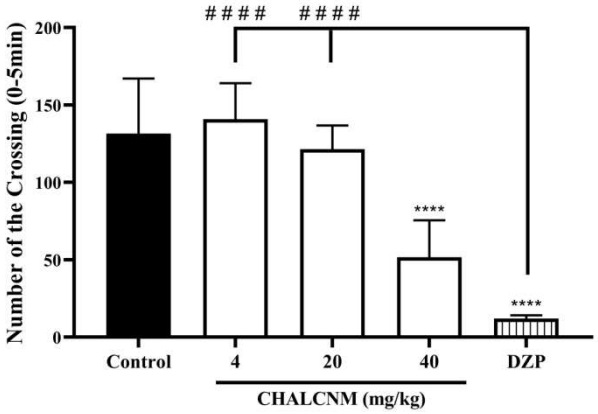


Figure 2. Effect of CHALCNM on locomotor behavior of adult *zebrafish* in the Open Field Test (0–5 min). Each column represents the mean ± standard error of the mean (n = 6 groups). Control: DMSO 3.0%; 20 µL, *i.p.* One-Way ANOVA followed by Tukey's test: (**** $p < 0.0001$ vs. Control; #### $p < 0.0001$ vs. DZP).

responses are indicative of adaptation to the new environment and reflect the emotional state of the fish. Previous research has demonstrated that compounds that influence anxiety can modulate these responses in adult *zebrafish*. For example, the anxiolytic effect of certain compounds has been associated with an increase in the time that fish spend in the upper region of the aquarium, indicating a reduction in anxious behaviors.[24] This suggests that *zebrafish* are a useful model for studying the effects of different compounds on regulating anxious behavior.

One-Way ANOVA showed statistical significance for mean speed, total distance traveled and immobility time (Figure 3 A, B and C, respectively). The dose of 20 mg/kg of CHALCNM affected the animals' locomotion when compared to the group treated with 3% DMSO (control), as it reduced the average speed, total distance covered and increased the animals' immobility (* $p < 0.05$; ** $p < 0.01$; Figure 3 A, B and C, respectively). The 40 mg/kg dose had a significant difference in average speed and total distance covered compared to DZP, showing that despite the anxiolytic effect caused by this dosage, it did not present as preponderant a sedative effect as DZP.

Regarding the animals' time at the top, we can observe that doses of 4 and 20 mg/kg of CHALCNM had a significant difference compared to DZP (## $p < 0.01$; #### $p < 0.0001$ vs DZP, respectively), showing that only the dose of 40 mg/kg had an anxiolytic effect similar to DZP (Figure 3 D). As for the distance covered at the top, it is possible to observe that the dose of 20 mg/kg of CHALCNM had a significant difference compared to DZP (# $p > 0.05$ vs DZP), indicating that at this dose the animals did not have much locomotion, presenting a behavior of anxiety and fear. The 40 mg/kg dose had an anxiolytic result similar to DZP (Figure 3 E).

Notably, fish treated with CHALCNM 40 mg/kg demonstrated greater permanence in the upper region of the aquarium during the novel tank test and the same dosage of CHALCNM (*** $p < 0.001$) also decreased anxiety in *zebrafish* in the light and dark test by increasing the time animals spend in the light area of the aquarium. These results suggest that CHALCNM may have anxiolytic properties in *zebrafish*, reducing anxiety-associated behaviors in different environmental contexts. These findings are consistent with previous studies that demonstrated the anxiolytic effects of different compounds in *zebrafish*, highlighting the potential of this model to investigate new therapeutic approaches for treating anxiety-related disorders.[10]

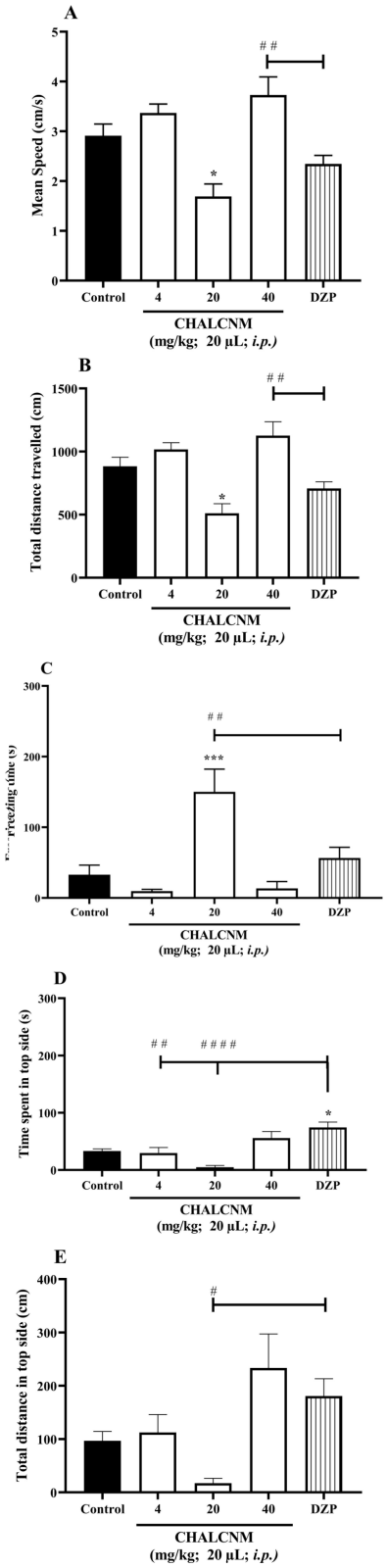


Figure 3. Effect of CHALCNM on *Zebrafish* locomotor activity and anxiety-like behavior in the Novel Tank test for 5 min. (n = 6 fish/group). a) average moving speed (cm/s), b) total distance traveled (cm), c) total time immobile (s), d) time at the top (s), e) distance traveled at the top (cm). Data are expressed as mean ± S.E.M. Each asterisk above the bars indicates significance compared to the negative control group – 3% DMSO (* $p \leq 0.05$; *** $p \leq 0.001$) hash indicates significance compared to control group – DZP (# $p \leq 0.05$; ## $p \leq 0.01$; #### $p \leq 0.0001$).

Anxiolytic Assessment (Light/Dark Test – LDT)

The light-dark test is a tool used to assess anxiety in *zebrafish*. This behavioral paradigm analyzes the animal's natural tendency to seek safe areas in relation to exposure to bright environments. Therefore, the analysis is based on the time the fish spends in each of these areas, and a prolonged stay in the dark region is generally interpreted as an indication of increased anxiety.[25] Based on this, we made a comparison with both tests (novel tank and light-dark) in the same study in order

to provide a more comprehensive assessment of fish behavior in different environmental contexts. By comparing the results of the two tests we were able to investigate how different experimental manipulations affect anxiety and environmental exploration in *zebrafish*.

In the present study, the highest dose of CHALCNM (*** $p < 0.001$ vs. Control) decreased anxiety in *zebrafish* in the light and dark test by increasing the time of permanence of animals in the light area of the aquarium similar to effect caused by Diazepam (*** $p < 0.001$ vs control) (Figure 4).

Xavier[21] explored the anxiolytic potential of the chalcone (2E, 4E)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-5-phenylpenta-2,4-dien-1-one, a chalcone also derived from cinnamaldehyde. Using the light and dark test, the researchers validated the anxiolytic effect of the substance, observing a maximum stay of the animals in the light zone of the protector, reducing the general anxiolytic potential of this class of compounds. When analyzing the structure of the chalcone in question, we can observe a unique configuration. The compound has a furan ring, which replaces what would traditionally be the B ring in a typical chalcone, while the A ring is composed of the –OCH3 and –OH groups. It is interesting to note that many of the anxiolytic compounds described in the literature present a common structural characteristic: the substitution of the hydroxyl group in the 2′ position of ring A. Furthermore, these compounds frequently present substitutions of the methyl, methoxy, nitro, dimethylamine and halogen groups in both rings, suggesting significant structural diversity associated with this class of compounds.[4]

These findings highlight the importance of chemical structure in the biological activity of compounds, offering important insights for the development of new pharmacological agents for the treatment of neuropsychiatric disorders. By better understanding the structure-activity relationships of this substance, we can improve its therapeutic efficacy and minimize potential adverse effects, thus contributing to advanced advances in neuropharmacology.

Assessment of GABAergic Neuromodulation (GABAA Receptor)

The mechanism of anxiolytic action via GABAergic neurotransmission was accomplished by pretreatment with flumazenil. The best dose of CHALCNM was analyzed. Pretreatment with flumazenil blocked the anxiolytic effect of CHALCNM (40 mg/kg) (#### $p < 0.0001$ vs FMZ + 40) and Diazepam (4 mg/kg) (## $p < 0.01$ vs FMZ + DZP) in animals (Figure 5), demonstrating that the anxiolytic effect of CHALCNM is related to the GABAA receptor in the binding region of benzodiazepines.

Determining the involvement of the GABAergic system in the action of substances in anxiety studies is often performed through the administration of flumazenil, a specific GABAA receptor antagonist that blocks the effects of benzodiazepines (BZDs).[25] Tests Behavioral behaviors that use diazepam and clonazepam as positive controls have been used to evaluate the anxiolytic efficacy of compounds. The results of these studies, extended by Benneh[25] Chaves,[26] Tabari and Tehrani[27] provide support for the validity of this method by demonstrating that pretreatment with flumazenil blocks the anxiolytic effects previously observed.

From this perspective, the findings of Mendes[9] corroborate these results, showing that the chalcone (E)-3-(furan-2-yl)-1-(2hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one exerts anxiolytic effects through the GABAergic system. The administration of flumazenil blocked the anxiolytic effects of both chalcone and diazepam, demonstrating the relationship between these compounds and the GABAA receptor. Meanwhile, the results of Xavier[10] had already reinforced this conclusion, demonstrating that the anxiolytic activity of chalcone derived from cinnamaldehyde is dependent on the GABAergic system. Flumazenil pretreatment significantly impairs the anxiolytic/sedative effects of both chalcone and diazepam in adult *zebrafish*.

These studies together provide consistent evidence for the role of the GABAergic system in mediating the anxiolytic effects of chalcones and benzodiazepines in *zebrafish*. Understanding these mechanisms of action is fundamental for the development of new treatments for anxiety disorders and may open the way to more effective and specific therapeutic approaches.

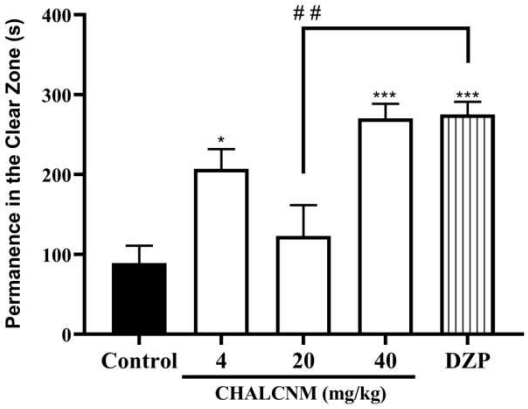


Figure 4. Effect of CHALCNM on the anxiety behavior of adult *zebrafish* in the light and dark test (0–5 min).

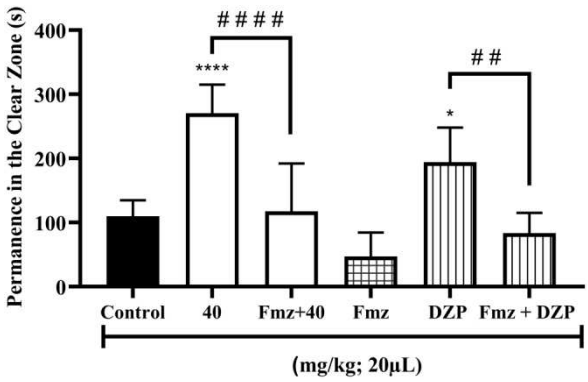


Figure 5. GABAergic mechanism of action of CHALCNM (40 mg/kg) on the anxiety behavior of adult *zebrafish* in the light and dark test (0–5 min).

Inhibitory Avoidance Test

To continue our investigation into the effects of CHALCNM on *zebrafish* behavior, we performed the inhibitory avoidance test. By performing the avoidance test, we sought to evaluate how CHALCNM influences memory retention in *zebrafish*, using a paradigm recognized for its effectiveness in investigating associative learning and memory in animal models. Studies of learning and memory in rodents and other model organisms over decades have demonstrated that simple learning paradigms based on associative conditioning of aversive stimuli are highly effective in inducing robust behavioral responses and observing stable memory traces. These approaches are essential for exploring the evolutionary mechanisms underlying conserved memory.[13] Thus, the latency time to completely enter the dark compartment in the inhibitory avoidance test was measured in both sessions and the test latencies are used as a retention index.[13]

The results obtained reveal that the administration of CHALCNM at doses of 4 and 20 mg/kg prevented memory in *zebrafish*, as indicated by the inhibitory avoidance caused by electroshock (Figure 6). In these groups, a statistically significant difference was observed between the latencies of the training session and the test session (** $p < 0.01$ and **** $p < 0.0001$, respectively). This suggests that fish treated with these doses of CHALCNM were able to remember and associate the aversive electroshock stimulus with the dark compartment, resulting in effective avoidance during the testing session. On the other hand, the group treated with Diazepam did not demonstrate a significant difference in latencies between the training and testing sessions. This indicates a failure in memory retention associated with the aversive stimulus, suggesting that Diazepam may have impaired the zebrafish's ability to remember the aversive experience during the inhibitory avoidance test.[28]

Furthermore, there was a significant difference in the retention rate between the CHALCNM group at a dose of 20 mg/Kg, compared with the retention rate of the group treated with DZP in the test session (Figure 7).

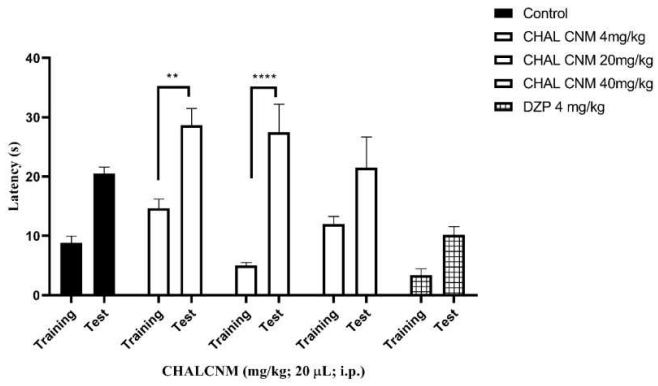


Figure 6. Effect of CHALCNM on adult *zebrafish* memory in the Inhibitory Avoidance Test (0–5 min). Values represent the mean ± standard error of the mean for 6 animals/group; Two-way ANOVA followed by Tukey's test.

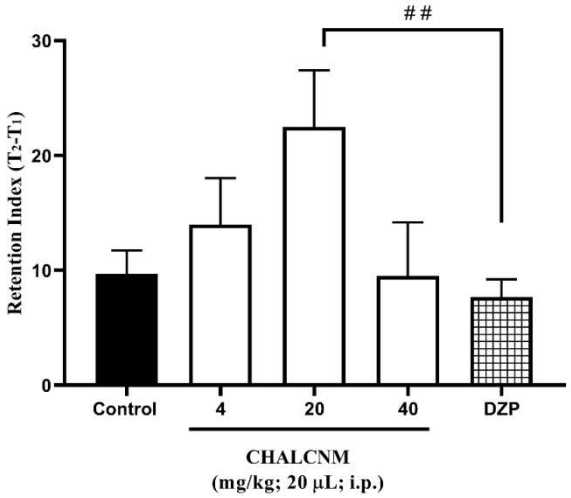


Figure 7. Effect of CHALCNM on memory retention index of adult *zebrafish* in the Inhibitory Avoidance Test (0–5 min). Values represent the mean ± standard error of the mean for 6 animals/group; One-way ANOVA followed by Tukey's test.

These results suggest that CHALCNM, at specific doses, is capable of promoting memory retention in *zebrafish*, while Diazepam may have an opposite effect in this regard.

Benzodiazepines, commonly used in human medicine as anxiolytics or sedatives, can cause amnesia at higher doses, as occurred in our study at a dose of 40 mg/kg. In the study by Wan[29] in rats demonstrated that lorazepam compromises both recognition memory and synaptic plastic processes (depression and long-term potentiation). Both deficiencies result from actions on the perirhinal cortex, thus providing insight into the mechanism by which benzodiazepines affect recognition memory.

Virtual Screening and Activity Spectra

This study used an initial prediction from a virtual screening based on chemical structure, a technique aided by machine learning functions to drive the biological activity or mechanism of action of new drug candidates. These functions are based on similarity tests of the input compound with ligands deposited in databases, whose biological activity is characterized and known.[30] This technique was supported by the prediction of the activity spectrum for substances (PASS),[31] where it was possible to notice that chalcone presents similarity with substances that interact with GABA-A channels, with a substantial contribution from its group hydroxyl and its rigid rings, estimated to be the main pharmacophores in interactions with the target.

The results of the structure-based virtual screening can be visualized in the graphical representations of Figure 8. Analysis shows that chalcone performs most of its biological interactions with enzymes (20%) and oxidoreductases (16.7%). In addition, however, the substance tends to interact with ion channels (6.7%), including GABA-A channels, in order of similarity with 4

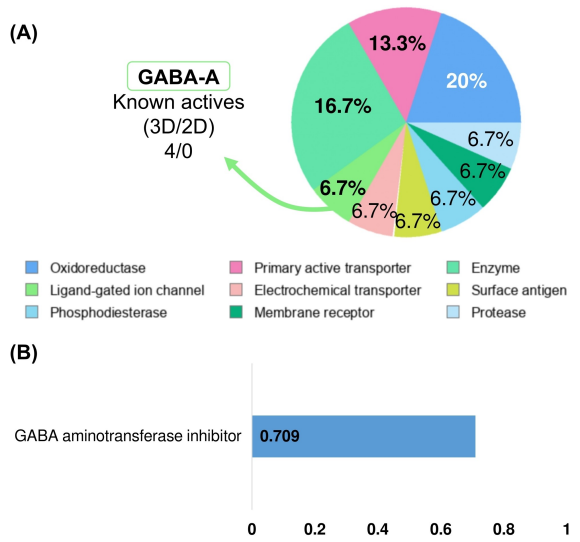


Figure 8. Results of chalcone biological activity prediction, including structure-based virtual screening (A) and activity spectra from PASS test (B).

known 3D structures deposited in the ChEMBL database (Figure 9a), including hydroxylated compounds with at least 2 rigid rings in its base frame. In addition, the activity spectrum prediction test for substances (PASS) estimated a probability of activity (Pa) of 0.709 of the chalcone acting as an inhibitor of GABA-A receptors (Figure 9b).

However, the substance must have drug-likeness properties to carry out its GABAergic mechanism of action. Recently, a chalcone derived from cinnamaldehyde, containing a 2-OH group, was characterized against GABAergic activity and showed to be very promising for the mechanism of action.[8]

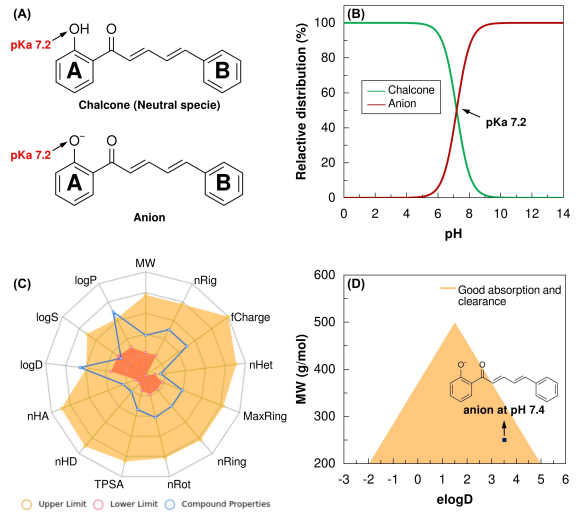


Figure 9. pKa claculation to the hydroxyl group from chalcone (A) and distribution of microspeces pH dependent (B), druglikeness radar (C) and golden triangle method to estimate absorption (or permeability) and clearance (D).

Physicochemical Properties and Drug-Likeness

The distribution of chalcone microspecies, as a function of pH, can be seen in the graph of Figure 9. The calculated value of pKa in the order of 7.2 (Figure 9a) for the phenolic hydroxyl (H-bond donor) suggests a chemical equilibrium between the neutral species (ph–OH) and the ionized species (ph–O^-) at pH 7.2, a balance that shifts towards the formation of the conjugate base at physiological pH (pH of approximately 7.4), reaching an estimated majority of 61.5 % (Figure 9b).

With the analysis of the calculated physicochemical properties listed in Table 3, it is possible to notice that MW values in the order of 250.1 g/mol and estimated logP of 3.71 guarantees an ideal molecular size and lipophilicity for good absorption or permeability within Lipinski's "rule of five" spectrum (MW < 500 g/mol and logP < 5).[32]

In a visual inspection of the radar of Figure 9c, it is possible to notice that the chalcone is more lipophilic than the ideal predicted by the Pfizer filter (logP > 3), suggesting that the substance can bind strongly with plasma proteins, limiting its pharmacokinetics. When aligned with the low polar surface, evaluated at 37.3 $Å^2$, it is possible to notice an alert associated with adverse effects, such as penetration of the blood brain barrier (BBB), within the Pfizer database[33] (Table 3).

In addition, the total of 2 aromatic rings is within the ideal for a balance between solubility and lipophilicity, according to the GSK filter,[34] agreeing with the solubility coefficient (logS) in the order of −4.44, indicating a moderate solubility, in addition

Table 3. Calculated physicochemical properties and evaluation of drug-likeness of this chalcone.

Property	Value	Drug-likeness filter
Molecular Weight (MW)	250.1 g/mol	100~600 g/mol
logS	−4.44	−4~0.5
logP	3.71	0~3
logD	3.51	1~3
nHA	2	0~12
nHD	1	0~7
TPSA	37.3 $Å^2$	0~140 $Å^2$
nRot	4	0~11
nRing	2	0~3
MaxRing	6	0~18
nHet	2	1~15
fChar	0	−4~4
nRig	15	0~30
Lipinski rule		Accepted
GSK rule		Accepted
Pfizer rule		Alert
Golden Triangle		Accepted

Note: nHA (number of H-bond Acceptors); nHD (number of H-bond Donors); TPSA (Topological Polar Surface Area); nRot (number of Rotatable Bonds); nRing (number of Rings); MaxRing (number of atoms in the biggest ring); nHet (number of Heteroatoms); fChar (formal charge); nRig (number of Rigid bonds).

to contributing strongly to the 15 rigid bonds of the chalcone (Table 3).

To evaluate the pharmacological efficacy, it is necessary to estimate oral bioavailability since it is related to the molecular fraction of the drug absorbed and present in the systemic circulation.[35] In experimental observations made by Martin,[36] it is possible to notice that the polar surface area influences the oral bioavailability, which is more incident in compounds with TPSA $\leq$ 75 Å^2. For Hughes,[33] from Pfizer, Inc., compounds within the lipophilicity range with logP > 3 that have TPSA $\leq$ 75 Å^2 are more likely to penetrate the BBB, considered an adverse effect in their dataset. In this study, it was possible to notice that chalcone occupies a physicochemical space where its polarity estimates a ready penetrability in the CNS to carry out its pharmacological effect as an anxiolytic drug.

Estimate of the Pharmacokinetics

The results of the pharmacokinetic prediction regarding absorption, distribution, metabolism and excretion (ADME) can be consulted in Table 4 and visually observed by the graph of Figure 9d. The analysis shows that the alignment between the calculated MW of 250.1 g/mol and the buffer lipophilicity (logD) in the order of 3.51 is directly associated with the predominant anion at pH 7.4, which occupies a physicochemical space favorable to good permeability and low debugging, related to a great ADME profile.

Table 4. Predicted pharmacokinetic descriptors by the consensual test between ADMETlab 2.0 and SwissADME web tools.

Property	Value	Source
P_{app} MDCK	1.2×10^{-5} cm/s	ADMETlab 2.0
P-gp substrate	No	Consensual
HIA	High	Consensual
Vd	0.478 L/kg	ADMETlab 2.0
$CL_{int,u}$	3.95 mL/min/kg	ADMETlab 2.0
F	0.85	SwissADME
BBB penetration	Yes	Consensual
HLM	CYP2 C9 (0.97)	ADMETlab 2.0

Note: P_{app} MDCK (Passive permeability predicted by the Madin-Darby Canine Kidney cells model); P-gp (P-glycoprotein); Vd (Volume of distribution); $CL_{int,u}$ (Clearance); *F* (Fraction of bioavailability); BBB (Blood-brain barrier); and HLM (Human liver microsome).

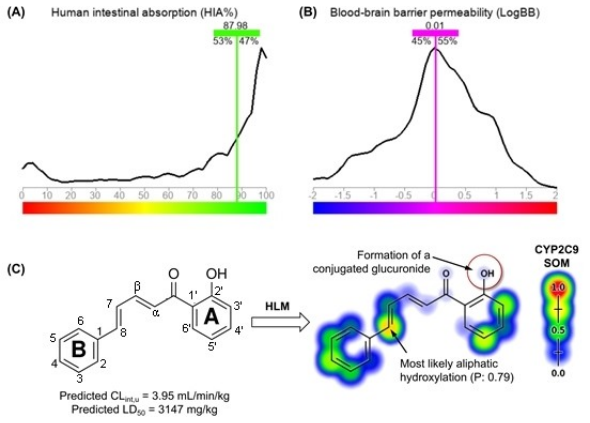


Figure 10. Prediction of the intestinal absorption (a) and BBB penetration (b) by the structure-based prediction model of the ADMET–LMC web tool, and structural contributions of the chalcone metabolism in the HLM system (c).

This empirical decision corroborates the pharmacokinetic descriptors obtained, where the Papp value (MDCK cell model) in the order of 1.2×10^{-5} cm/s guarantees a high passive permeability, in addition to being little susceptible to efflux by P-gp (Table 4), resulting in high human intestinal absorption (HIA) estimated at 87.98% (Figure 10a). Furthermore, when combined with the low rate of CLint,u evaluated at 3.95 mL/min/kg indicates that the low hepatic clearance of chalcone justifies the high oral bioavailability (F) estimated at 0.85 (Table 4).

Properties such as MW and lipophilicity estimated by logD can easily replace other physicochemical properties commonly used in drug discovery, such as H-bond and TPSA acceptors and donors, as they are directly related to the ionization state of the molecule. Small compounds (MW < 500 g/mol) that have low relative lipophilicity (logD < 5) occupy a physical-chemical space where a high passive cell permeability (Papp > 10×10^{-6} cm/s) and low clearance rate in the HLM system (CLint, u < 100 mL/min/kg) are more likely.[37] The results explored here reveal that chalcone aligns with physicochemical properties and pharmacokinetic descriptors that allow its preparation as an oral drug.

In addition, the permeability coefficient BBB (logBB) in the order of 0.01 suggests that the low topological polarity, associated with carbonyl and hydroxyl fragments, favors the penetration of at least 10% of the chalcone molecular fraction into the central nervous system (CNS),[38] to carry out the GABAergic activity (Figure 10b).

Site of Metabolism (SOM) and Toxic Effects

The prediction of a substance's sites of metabolism is a structure-based estimate that can provide information about the oral dose control of a drug candidate.[39] In addition, it allows avoiding the formation of reactive, secondary metabolites from CYP450 isoform substrates and relating the metabolic clearance rate with the drug's half-life.[40,41]

Structural contributions to chalcone metabolism can be seen in the heatmap in Figure 10c. With the results, it wabbs possible to notice that the substance tends to act as a substrate of the CYP450 isoform of the 2C9 subfamily in the human liver microsome (HLM), in an estimated probability of 0.97, being able to form secondary metabolites from the hydroxylation of the most unprotected unsaturated carbon of the aliphatic chain (atom 8).

Furthermore, the hydroxyl group constitutes a conjugation site in phase II (post-systemic) metabolism, forming a glucuronide conjugate mediated by UGT, making the substance more susceptible to excretion. The formation of hydroxylated metab-

olites does not present a severe toxic response to the host since the estimated value of LD50 in the order of 3147 mg/kg suggests a low toxic incidence by ingestion (toxicity class 5).[42]

Furthermore, the volume of distribution evaluated at 0.478 L/kg (Table 4), combined with the low rate of hepatic clearance, suggests a pharmacokinetics of distribution more confined to blood plasma and with a longer half-life.[43]

In this study, it was possible to note that the aliphatic hydroxylation undergone by chalcone (atom 8) does not tend to form hepatotoxins, ensuring better control of the daily oral dose administered.

Evaluation of the hERG blocker effect

Another prediction that can be made by reading the toxic fragments is associated with the cardiotoxicity model. A database was used to identify substructures susceptible to blocking hERG channels responsible for the flow of K^+ cations in the cardiovascular system,[44] provided they were present in the chalcone structure. With the results, it was possible to notice that the hydrophobic contributions of chalcone are the main responsible for the low effectiveness in hERG channels, substantially reducing the probability of the substance showing a cardiotoxic response and constituting a promising pharmacological model.

From the statistical regressions of the ADMET–LMC database, it is possible to observe that the estimated pKi value of 5.55 suggests that the structural contributions of chalcone effectively decrease the chances of interaction with hERG, resulting in a low probability of a cardiotoxic response. (Figure 11a). In the 2D probability map (Figure 11b), it can be observed that the groups with the polar surface, that is, the carbonyl and hydroxyl groups, constitute negative contributions (magenta color) as sites of interaction with hERG, an activity that is reduced by the hydrophobic contributions of the aromatic rings and aliphatic alkenes (green color).

Molecular Docking

Root-Mean-Square Deviation (RMSD) and affinity energy were used for statistical validation of complex formation simulation results and best pose selection. The RMSD is calculated using the average distance between the atoms of the two ligands, having as validation criteria values close to 2 Å.[45] Affinity energy, on the other hand, must present values lower than −6.0 kcal/mol.[46,47] The chalcone/GABAA and diazepam/GABAA complexes presented RMSD in the order of 0.893 Å and 1.02 Å, respectively. That is, they were below the reference value. And the affinity energy in the order of −8.2 kcal/mol and −7.4 kcal/mol, respectively, indicating that CHALCNM has a higher affinity for the GABAA receptor when compared to diazepam. Analyzing the interaction patterns against the GABAA receptor, it was possible to identify that the chalcone/GABAA complex is formed by twelve hydrophobic interactions, nine with the nonpolar side chain of residues Trp 246D (3.41 and 3.67 Å), Leu 297E (3.52 Å), Phe 301E (3.49, 3.54 and 3.68 Å), Ile 423E (3.68 Å), Trp 426E (3.76 Å), Phe 431E (3.36 Å), one with the uncharged polar side chain of residue Tyr 304E (3.66 Å), two with the acidic side chain residue Glu 298E (3.77 and 3.79 Å) and a p-Stacking interaction with the nonpolar side chain of the aromatic residue Phe 301E (5.01 Å).

The co-crystallized diazepam binding site between the D and E chains of the GABAA receptor is formed by residues Ile 228D, Leu 232D, Pro 233D, Met 236D, Thr 237D, Met 261E, Thr 262E, Thr 265D, Asn 265E, Leu 269D, Asp 282E, Leu 285E, Met 286E and Phe 289E (MASIULIS et al., 2019; ROSE et al., 2018). Chalcone also complexes between the D and E chains. However, it interacts with different residues of the diazepam binding site (DZP), suggesting a possible synergistic effect (Figure 12).

Recently, Xavier[8] performed molecular docking of chalcone (2E, 4E)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)-5-phenylpenta-2,4-dien-1-one with the GABAA receptor and obtained as a result a high affinity energy, of the order of −8.1 Kcal/mol and the chalcone also presented three hydrophobic interactions, coupling in a different region of the inhibitor Diazepam, thus being an indicator of potential candidate for synergism studies. These data corroborate those obtained in the present study.

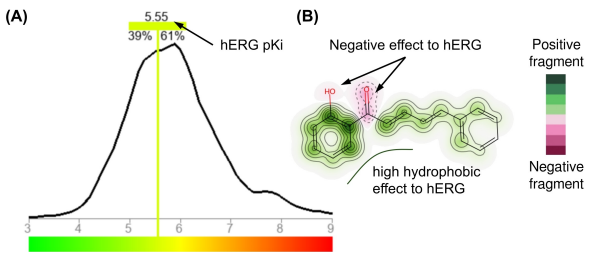


Figure 11. Estimated pKi against the hERG target (a) and structural contributions to the hERG blockage of the chalcone (b).

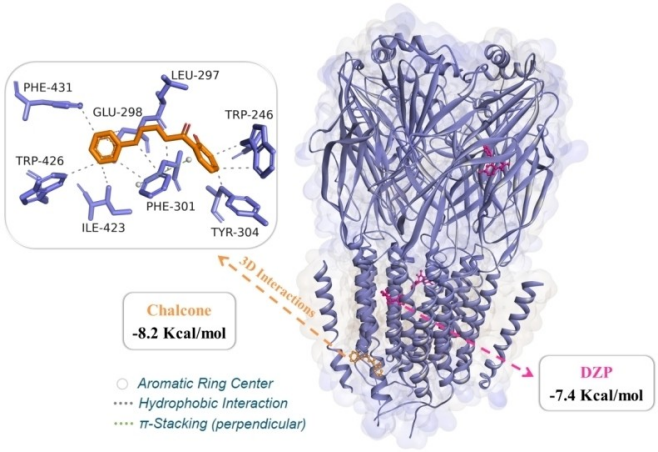


Figure 12. Interaction complex between the GABAA receptor, chalcone and the co-crystallized inhibitor diazepam (DZP).

Experimental Section

Drugs and Reagents

The following substances were used: Diazepam (DZP, Neo Química®), Flumazenil (Fmz; Sandoz®), Dimethylsulfoxide (3% DMSO; Dynamic®), Cinnamaldehyde (Sigma-Aldrich®), 2-hydroxyacetophenone (Sigma-Aldrich®), Sodium Hydroxide (Sigma-Aldrich®), Hydrochloric Acid (Sigma-Aldrich®), Ethanol (Sigma-Aldrich®).

Synthesis and Characterization of Cinnamaldehyde-Derived Chalcone

Synthesis (2E,4E)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one (CHALCNM)

Chalcone was synthesized by a Claisen-Schmidt condensation reaction in a basic medium (Scheme 2). An ethanolic solution of 2-hydroxyacetophenone (2 mmol) was added to a solution of cinnamaldehyde (2 mmol), followed by adding 10 drops of 50% w/v aqueous NaOH with stirring for 48 h at room temperature. After 48 h, the reaction mixture was neutralized with dilute HCl (10%), and ice water was added. The product was obtained as a yellow solid (yield: 58%). The solid formed was filtered under reduced pressure, washed with cold water and analyzed by TLC. The chemical structure of CHALCNM was determined and confirmed by ^{1}H and ^{13}C NMR.

Infrared Analysis (ATR-FTIR)

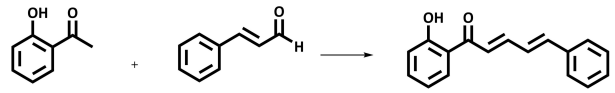
The infrared spectra were determined by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) using a Bruker vacuum spectrometer, model VERTEX 70 V with a HeNe laser source with a wavelength of 633 nm according to XAVIER.[16].

Nuclear Magnetic Resonance Analysis of Hydrogen (NMR^{1}H) and Carbon (NMR^{13}C)

The ^{1}H-NMR spectra were determined in Bruker DRX 500 MHz spectrometer equipment, operating at a frequency of 300 MHz for hydrogen and 125 MHz for carbon, respectively. Spectra were measured in *CDCl3* solvent, and chemical shifts are reported as δ values in parts per million (ppm) according to XAVIER.[16]

Zebrafish

Zebrafish (Danio rerio) (age 90 to 120 days; 0.4 ± 0.1 g, 3.5 ± 0.5 cm), wild, of both sexes, was purchased at a local store (Fortaleza, CE). The animals were kept in a 10 L (n = 3/L) glass aquarium (30×15×20 cm), at a temperature of 25 ± 2 °C, in 24 h light-dark cycles with chlorinated water (ProtecPlus®) and air pump with submerged filters, under a temperature of 25 °C and pH 7.0, circadian cycle of 10–14 h (light/dark). The fish received chow (Spirulina®) 24 h before the experiments. Before the treatments, the animals were anesthetized in ice water. After the experiments, they were euthanized by immersion in ice water (2 and 4 °C) for 1 min until the loss of opercular movements. The work was approved by the Ethics Committee on the Use of Animals of the State University of Ceará (CEUA-UECE; n° 04983945/2021) in accordance with the Ethical Principles of Animal Experimentation.



Scheme 2. CHALCNM synthesis reaction

General Protocol

Zebrafish (Zfa) of both sexes were randomly selected in the experiments, anesthetized in ice water and transferred to a wet sponge, treated with 20 μL of CHALCNM and CINAMALDEHYDE solutions (4, 20 and 40 mg/kg; each), Diazepam (4 mg/kg) and 3% DMSO (control group – drug diluent) intraperitoneally (i.p). Then, to mimic social isolation, an anxiety-inducing factor, the animals were individually placed in plastic cups (500 mL) containing 350 mL of aquarium water and kept at rest. In intraperitoneal (*i.p.*) treatments, insulin syringes (0.5 mL; UltraFine® BD) with a 30G needle were used.

Acute Toxicity 96 h

After the open field test, the fish (n = 6/group) that were treated intraperitoneally (*i.p.*) with the evaluated samples (4, 20 and 40 mg/kg; 20 μL) and with the control (vehicle: DMSO at 3%; 20 μL; *i.p*), were left to rest for analysis of the mortality rate for a period of 96 h, recording every 24 h the number of dead fish in each group,[48] with the lethal dose capable of to kill 50% of the animals (LD50) determined by the mathematical method Trimmed Spearman-Karber with a confidence interval of 95%.

Assessment of Locomotor Activity (Open Field Test – OFT)

The open field test was performed to evaluate possible side effects and/or presence or absence of changes in motor coordination in animals.[10] Initially, fish (n = 6/group) were treated intraperitoneally (i.p.) with CHALCNM (4, 20, and 40 mg/kg; each), DZP (4 mg/kg), or vehicle (Control; 3% DMSO). After 30 min of treatment, the animals were added to glass Petri dishes (10×15 cm) containing the same aquarium water, marked with four quadrants, and analyzed for locomotor activity by counting the number of lines of crossing (CL) performed by the animals during five minutes of analysis.

Novel Tank Test

The fish (n = 6, for each treatment) from each group, after 60 minutes of treatments with CHALCN (4, 20 and 40 mg/kg), DZP (2 mg/kg) and 3% DMSO (Control), were transferred individually into a rectangular tank (20 cm long×15 cm high×12 cm wide) with up to 2.8 L, the sides of which were covered with white paper, except the front wall to allow camera recording. The behavior of the fish was immediately recorded for 5 minutes. The tank was divided into three equal horizontal areas (lower, middle and upper) of 4 cm height each to investigate locomotor activity and anxious behavior. The following parameters were considered: total distance covered, average moving speed, total immobility time, time and distance covered in the upper area and latency to enter the upper area.[49]

Anxiolytic Assessment (Light/Dark Test – LDT)

The experiment was carried out in a glass aquarium (30 cm×15 cm×20 cm) divided into light and dark areas. The aquarium was filled up to 3 cm with tap water without chlorine and heavy metals, which simulated a new shallow environment different from the conventional aquarium and capable of inducing anxiety behaviors.[50]

In animals (n = 6/group), 20 μL of the CHALCNM solutions was administered i.p. at doses of 4 mg/kg, 20 mg/kg and 40 mg/kg. Negative and positive control groups consisted of 3% DMSO and 4 mg/kg Diazepam solution, respectively. After 30 min of treatment, the animals were placed individually in the clear zone. The anxiolytic effect was measured based on the time spent in the clear zone of the aquarium within 5 minutes of observation.[50]

Assessment of GABAergic Neuromodulation

The receptor involved in the anxiolytic effect of CHALCNM was identified by pretreatment with flumazenil (a benzodiazepine antagonist at the GABAA receptor).[25] *Zebrafish* (n = 6/group) were pretreated intraperitoneally (i.p.) with flumazenil (4 mg/kg; 20 μL; i.p.). After 15 min, the best anxiolytic dose of CHALCNM (40 mg/kg, found in the previous section) was administered intraperitoneally. 3% DMSO (Vehicle; 20 μL; i.p.) was used as a negative control, and Diazepam (DZP; 4 mg/kg, 20 μL;) as a positive control. After 30 min of the treatments, the animals were submitted to the light/dark test described in the previous section.

Inhibitory Avoidance Test

Assessment of the CHALCNM on inhibitory avoidance was performed as described by Bertoncello.[14] The equipment consisted of a glass tank (28 cm long×14.7 cm wide×19 cm high) that contained 1.3 L of non-chlorinated water. The tank was divided into two identical compartments (black and white), separated by a guillotine-type partition, which could be operated manually (10×10 cm). In the black compartment there were three sets of metal bars (1 cm in diameter) spaced 3 cm apart, all connected to an electrostimulation device. The objective was to apply an aversive stimulus, for which the fish were subjected to pulsed shocks at a frequency of 100 Hz for 5 seconds. Each group of animals (n = 6 animals per group), previously isolated individually in 500 mL jars and identified, underwent a training session. During this session, each fish was placed in the white compartment of the device. After one minute of adaptation, the guillotine door was raised and the time it took for each animal to enter the black area was recorded as latency time. Once the fish passed through the dark compartment, the door was lowered and a mild electric shock (125 mA, 3 ± 0.2 V) was administered. Subsequently, the fish was removed from the tank and treated i.p. (n = 6 fish per group) with CHALCNM, in three different doses (4; 20 or 40 mg/kg, one dose for each group). An additional group was treated with DZP (4 mg/kg, i.p.), while another group received 3% DMSO (negative control; drug diluent). After 24 hours, the test session was carried out, following a similar procedure to the training session, but without the administration of an electric shock.

ADMET Profile

Virtual Screening and Activity Spectrums

To estimate the biological activity of chalcone, virtual screening techniques based on the structure of the SwissTargetPrediction online server (http://www.swisstargetprediction.ch/) and the prediction of the spectrum of biological activity for substances (PASS) from the PASS Online tool, Way2Drug (http://way2drug.com/passonline/), were combined, where the consensual predictive response constitutes the distribution of biological interactions, through the test of similarity with known compounds from the ChEMBL dataset, as well as the activity probability (Pa) of a specific mechanism of action.

Physical-Chemical Properties and Drug-Likeness

The two-dimensional structure of the chalcone was designed for the calculation of the acid ionization constant (pKa) in the academic license software MarvinSketch version 22.5, ChemAxon (https://chemaxon.com/products/marvin), to evaluate the distribution of microspecies as a function of of the pH, including the selection of the dominant microspecies in physiological pH (≈7.4). Subsequently, the structure was loaded in linear SMILES notation on the online server ADMETlab 2.0 (https://admetmesh.scbdd.com/), where the physicochemical properties of molecular weight (MW), solubility coefficients (logS) and lipophilicity (logP and logD), number of H-bond acceptors and donors (nHA and nHD) and Topological Polar Surface Area (TPSA) were applied to the drug-likeness criteria of the Pfizer, Inc. including Lipinski's rule[32] and Golden Triangle,[37] while the topological descriptors of number of rotatable bonds (nRot), number of rings (nRing), number of atoms in the biggest ring (MaxRing), number of heteroatoms (nHet), formal charge (fChar) and number of rigid bonds (nRig) were applied to the GSK criteria,[34] which relate aromaticity and lipid solubility.

Pharmacokinetic Estimation

The empirical decisions of the drug-likeness test were supported by the prediction of absorption, distribution, metabolism and excretion (ADME) attributes, including the pharmacokinetic descriptors of passive permeability in the Madin-Darby canine renal cell model (Papp MDCK), P- glycoprotein (P-gp), human intestinal absorption (HIA), volume of distribution (Vd), hepatic clearance rate (CLint,u), oral bioavailability (F) and blood-brain barrier (BBB) penetration that combines the use of the tools available online ADMETlab 2.0 (https://admetmesh.scbdd.com/), SwissADME (http://www.swissadme.ch/) and ADMET–LMC (http://qsar.chem.msu.ru/admet/).

Metabolism Site (SOM) and Toxic Effects

The two-dimensional structure of the chalcone was submitted to the similarity test with substructures susceptible to biotransformation in the hepatic metabolism present in the XenoSite server (https://swami.wustl.edu/xenosite), where the graphic response in heatmap was supported by the substrate prediction of Cytochrome P450 (CYP450) isoforms, drug metabolizers in the human liver microsome (HLM), from the ADMETlab 2.0 online server (https://admetmesh.scbdd.com/) and by the lethal dose estimate (LD50) from the GUSAR Online server (http://www.way2drug.com/gusar/), as a method of predicting toxic response by ingestion.

Evaluation of the Effect of the hERG Blocker

Statistical regressions from the ADMET–LMC server (http://qsar.chem.msu.ru/admet/) related the chemical structure of chalcone with pharmacophores present in interactions with hERG channels (human Ether-a-go-go-Related Gene), estimating the affinity coefficient (pKi) towards the target. The prediction was supported by the 2D probability map from the online server Pred-hERG 4.2 (http://predherg.labmol.com.br/), evidencing the positive and negative structural contributions to the cardiotoxicity model.

Molecular Docking

Computational Details

To perform the simulations, the following codes were used: MarvinSketch™ 19.12.0 (http://www.chemaxon.com),[55] Avogadro™

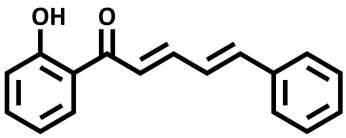


Figure 13. Chemical structure of chalcone at physiological pH.

(http://avogadro.cc/),[56] Autodocktools™, AutoDockVina ™,[51] UCSF Chimera ™,[52] Discovery studio visualizer ™ viewer[53] and Pymol.[54]

Ligand Design and Optimization

The chemical structure of the chalcone was designed using the MarvinSketch code,[55] saved at physiological pH (Figure 13), the lowest energy conformer was optimized using the Avogadro code,[56] configured to use the steepest descent with cycles of 50 iterations, applying force field MMFF94 (Merck Molecular Force Field).[57,58]

General docking Procedures

To evaluate the in silico mechanism of action of chalcone against the GABAA receptor, molecular docking simulations were performed using the methodology proposed by Mendes,[9] where the structure of the GABAA receptor was obtained from the Protein Data Bank repository (https://www.rcsb.org/), identified as "CryoEM structure of human full-length alpha1beta3gamma2 L GABA(A)R in complex with diazepam (Valium), GABA and megabody Mb38" (PDB 6HUP).[59] To select the best pose, the statistical parameter RMSD (Root Mean Square Deviation) with values up to 2.0 Å[60] and the affinity energy with values lower than −6.0 kcal/mol were used as criteria.[46,47]

Statistical Analysis

Results were expressed as mean values ± standard error for each group of 6 animals. After confirming the normality of distribution and homogeneity of the data, the differences between the groups were identified through the one-way ANOVA one-way analysis of variance in the preliminary tests (OFT and LDT) and two-way ANOVA for the mechanisms of action via GABA, followed by the Tukey test. All analyzes were performed using GraphPad Prism v. 8.0. The level of statistical significance was set at 5% ($p < 0.05$).

Conclusions

This research demonstrated that the chalcone (2*E*,4*E*)-1-(2-hydroxyphenyl)-5-phenylpenta-2,4-dien-1-one, derived from Cinnamaldehyde, did not present toxicity during the 96 hours of analysis. Furthermore, CHALCNM demonstrated an anxiolytic effect through the GABAA pathway, as evidenced in the Novel tank and Light Dark tests. In the avoidance and learning test, chalcone preserved memory formation after the aversive electroshock stimulus.

Molecular docking analysis was performed to corroborate the anxiolytic pathway of the molecule. The results indicated that chalcone has significant affinity for the GABAA receptor, suggesting a distinct interaction with the drug diazepam. The interaction in a different region of the diazepam binding site, cocrystallized between the D and E chains, suggests a possible synergistic effect between chalcone and diazepam, since they do not compete for the same binding site.

The computer-assisted predictive models used in this study allow the formulation of a promising pharmacokinetic model, characterized by high oral bioavailability and access to the central nervous system. The low topological polarity of chalcone favors its penetration into the CNS, while the biotransformation pharmacokinetics suggests a low incidence of hepatotoxicity. These results consolidate chalcone as a promising pharmacological tool in the context of the GABAergic system, with potential for the development of effective and safe therapies against anxiety-related disorders.

Author Contributions

Ivana Carneiro Romão: Supervision, writing e review and editing; Walber Henrique Ferreira Ribeiro: Investigation, formal analysis, and writing – original draft. Antônio Wlisses Da Silva and Maria Kuerislene Amâncio Ferreira: Formal analysis, softwares, validation, and reviewed the manuscript Hélcio Silva dos Santos: Conceptualization, methodology, and determined the molecular structures. Andreia Ferreira de Castro Gome and Sônia Maria Costa Siqueira: Writing – original draft and aided in the analysis of the spectra. Jane Eire Silva Alencar de Menezes and Marcia Machado Marinho: Project administration and writing – review and editing.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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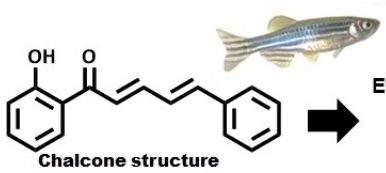

Effect of chalcone derived from cinnamaldehyde on behavior, memory and anxiety in adult zebrafish (Danio rerio)

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Effect of Cinnamaldehyde Chalcone on Behavior in Adult *Zebrafish* (Danio rerio): *In Silico* Approach

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