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Research report

Anxiolytic-like effect of chalcone N-{(4′-[(*E*)-3-(4-fluorophenyl)-1-(phenyl) prop-2-en-1-one]} acetamide on adult zebrafish (*Danio rerio*): Involvement of the GABAergic system

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ABSTRACT

Benzodiazepines are the standard drugs for the treatment of anxiety, but their undesirable side effects make it necessary to develop new anxiolytic drugs. The objective of this study was to evaluate the possible anxiolytic-simile effect of synthetic chalcone N-{(4′-[(*E*)-3-(4-fluorophenyl)-1-(phenyl) prop-2-en-1-one]} acetamide (PAAPFBA) on adult zebrafish (*Danio rerio*). PAAPFBA was synthesized with an 88.21% yield and its chemical structure was determined by ^{1}H and ^{13}C NMR. Initially, animals (n = 6/group) were treated (4 or 12 or 40 mg/kg, intraperitoneal) with PAAPFBA and were submitted to acute toxicity and open field tests. Then, other groups (n = 6/each) received PAAPFBA for the analysis of its effect on the Light & Dark Test. The participation of the GABAergic system was also assessed using the $GABA_A$ antagonist flumazenil. Molecular docking was performed using the $GABA_A$ receptor. The effect of PAAPFBA on anxiety induced by alcohol withdrawal was analyzed. PAAPFBA was non-toxic, reduced the locomotor activity, and showed an anxiolytic-like effect in both models. This effect was reduced by pre-treatment with the flumazenil. In agreement with *in vivo* studies, molecular docking indicated an interaction between chalcone and the $GABA_A$ receptor. The results suggest that PAAPFBA is an anxiolytic agent mediated *via* the GABAergic system.

1. Introduction

The chronic use of alcohol results in adaptations that can lead to tolerance and dependence, manifesting as physical and mental suffering when alcohol is withdrawn. Symptoms of ethanol abstinence include anxiety, insomnia, and autonomic hyperexcitation [1]. Therefore, alcoholism negatively affects not only dependency behaviors but also anxiety (stress and anxiety) and advanced cognitive behaviors, such as learning and memory [2].

Benzodiazepines are anxiolytics drugs used in the treatment of abstinence [3]. However, they have several side effects such as drowsiness, sedation and decreased motor coordination and at high doses these drugs can be fatal [4]. Thus, it is necessary to search for new anxiolytic drugs without undesirable side effects.

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Animal models have been used to help understand the mechanisms of anxiety disorders and the development of drug therapies for this type of disorder [5]. Among the animal models used, the zebrafish (*Danio rerio*) is an example of an animal model that has been used for the discovery of new anxiolytic drugs since it has 70–80% of human genetic homology, exclusive for the neurotransmitter receptors for mammals [6–8].

Flavonoids have the potential for the treatment of several neurological and mental conditions [9]. Chalcones belong to a subclass of flavonoids, and it is possible that these bioactive compounds exert a protective action on the central nervous system, contributing to mental health maintenance [10]. Besides this, chalcone chemistry continues to create interest among researchers in the 21st century due to the large number of replaceable hydrogens, which allows the synthesis of several derivatives and a variety of promising biological activities [11].

Therefore, the aim of this study was to evaluate the anxiolytic-like effect of the synthetic chalcone N-{(4′-[(*E*)-3-(4-fluorophenyl)-1-(phenyl) prop-2-en-1-one]} acetamide (PAAPFBA) and its possible mechanism of action in adult zebrafish (*Danio rerio*).

2. Material and methods

2.1. Drugs and reagents

The following substances were used: Diazepam (DZP, Neo Química®, 40 mg/kg), Flumazenil (FMZ; Sandoz®, 4.0 mg/kg), Acetic acid (CH_3COOH); Dimethyl sulfoxide (DMSO; Dynamic®; 3%); Sodium acetate/acetic acid (P.A; Dynamic®).

2.2. Synthesis and chemical characterization of chalcone PAAPFBA

The chalcone N-{(4′-[(*E*)-3-(4-fluorophenyl)-1-(phenyl) prop-2-en-1-one]} acetamide (PAAPFBA) was synthesized through the aldol condensation reaction of Claisen-Schmidt in basic medium using p-aminoacetophenone and p-Fluorobenzaldehyde, followed by the acetylation reaction with acetic anhydride in buffered medium (acetic acid/sodium acetate) at pH 4.5 under magnetic stirring [12], scheme 1 . The chemical structure of PAAPFBA was determined by ^{1} H and ^{13}C NMR, FT-IR and CG-MS.

2.3. Animals

Adult Zebrafish (*Danio rerio*) (aZF), wild-type, of both sexes and aged 60–90 days, measuring 3.5 ± 0.5 cm in length and weighing 0.4 ± 0.1 g were obtained from Agroquímica: Comércio de Produtos Veterinários LTDA (Fortaleza, Brazil). Groups of 50 fish were acclimatized for 24 h in glass aquariums (30 × 15 × 20 cm) containing dechlorinated water (ProtecPlus®) and air pumps with submerged filters, at 25 °C and pH 7.0, with a circadian cycle of 14:10 h light/dark. Fish received *ad libitum* feed 24 h prior to the experiments. After the experiments, the animals were sacrificed by immersion in ice water (2–4 °C) for 10 min until loss of opercular movements occurred [13]. All experimental procedures were approved by the Animal Use Ethics Committee of the State University of Ceará (CEUA-UECE, #7210149/2016).

2.4. Acute toxicity (96 h)

The acute toxicity study was performed in adult zebrafish (*Danio rerio*) according to the Organization for Economic Cooperation and Development (OECD) guidelines [14] and was conducted according to the OECD Standard Method [15] to determine the LD_{50}-96 h. Adult zebrafish (n = 6/group) were treated (20 µL), intraperitoneally (*i.p.*), with PAAPFBA (4 or 12 or 40 mg/kg) or vehicle (control, 3% DMSO). The number of animals that died at each concentration from 24 to 96 h was recorded and LD_{50} was determined.

2.5. Open field test

Animals (n = 6/group) were pre-treated (20 µL; *i.p.*) with PAAPFBA (4 or 12 or 40 mg/kg), diazepam (DZP; 40 mg/kg) or vehicle (control; 3% DMSO). After 30 min of treatment, the animals were individually added to glass Petri dishes (10 × 15 cm), containing the same water as the aquarium and divided into quadrants [16]. A naive group was included. The number of line crosses was recorded during 0–5 min.

2.6. Preliminary anxiolytic evaluation

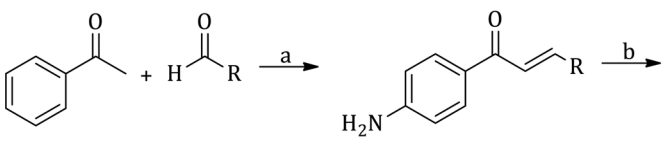
The animals (n = 6/group) were pre-treated with PAAPFBA, diazepam or vehicle or morphine (see 2.5) and submitted to the Light & Dark Test [6]. A glass aquarium (30 × 15 × 20 cm) with a light and a dark zone was filled with tap water pre-treated with antichlor until the height of 3 cm was achieved. After 30 min of the treatments, the animals were individually placed in the light zone of the aquarium and the anxiolytic-like effect was quantified as time (s) of permanence in the light zone during 5 min of analysis. A naive group was included.

To assess the involvement of the GABAergic system, other groups of animals (n = 6/each) received (4.0 mg/kg; *i.p.*) the $GABA_A$ antagonist flumazenil 15 min before PAAPFBA or diazepam and the test was performed in the same manner as described above.

2.7. Molecular docking

The interaction between PAAPFBA and the $GABA_A$ receptor was analyzed using molecular docking (Figs. 1B). Thus, it was performed based on the three-dimensional structure of the $GABA_A$ receptor (PDB ID: 4COF) (Fig. 1A), which was determined experimentally by crystallographic methods [17]. The interaction between PAAPFBA and the $GABA_A$ receptor (PDB ID: 4COF) was analyzed *in silico* using a molecular docking simulation. To validate the simulation, the $GABA_A$ receptor was also coupled to a classic agonist: Diazepam (PubChem CID: 2,733,484). The docking was performed using the software HEX 8.0.0, which makes the adjustments automatically, searching for all possible binding sites based on the energy association between PAAPFBA and the GABAA receptor in certain positions.

The data provided were analyzed using PyMol v1.4.7, which allows a detailed investigation of the complexes formed: association energy, chemical bonding, involved amino acid residues and conformational nuances. The parameters used in the software interface for the adaptation process were: Type of correlation: shape only; Calculation device - GPU (graphic process units); FFT mode - fast 3D life; Grid Dimension - 0.6; Receiver range - 180; Ligand Range - 180°; Twist range - 360° and distance range - 40.



R = 4-F-Ph

Scheme 1. Preparation of chalcone a) NaOH 50% w/v, ethanol, r.t., 48 h. b) $(CH_3CO)_2$, (AcOH /AcONa), r.t.

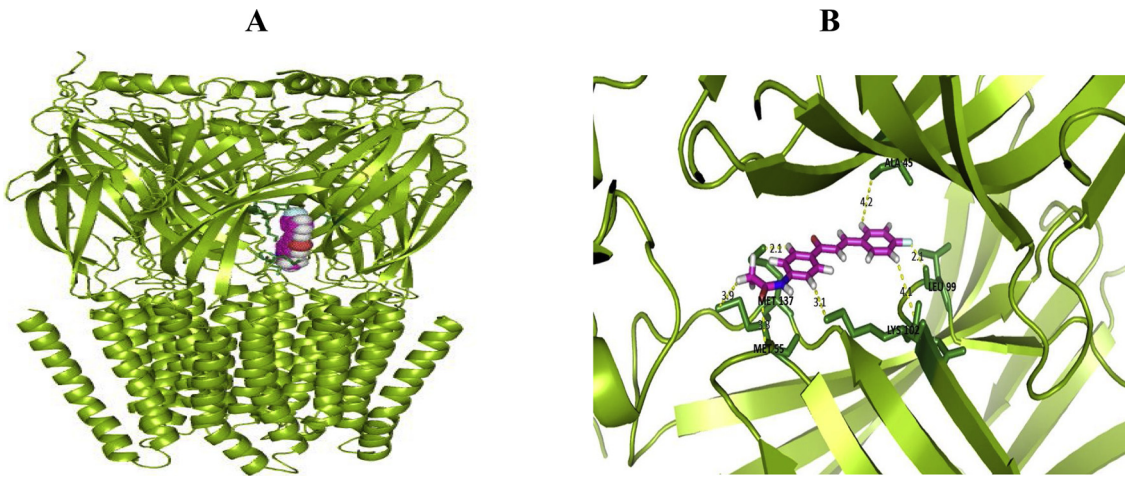


Fig. 1. (A) Three-dimensional structure of the $GABA_A$ receptor (green) with the most energetic clusters of PAAPFBA superimposed on a binding site in the β-leaf rich portion. **(B) -** Enlarged view of the interaction site, showing the binding distances between the $GABA_A$ (green) channel and PAAPFBA (purple), ranging from 2.1 to 4.1 Å, recruiting 5 amino acid residues, responsible for 7 chemical bonds.

2.8. Anxiety induced by alcohol withdrawal

Anxiety induced by alcohol withdrawal was investigated in adult zebrafish, according to the method described by Benneh [18], with adaptations. Yellow sugar cane brandy (ACAA) was used as the source of ethanol (38% EtOH). Initially, ACAA toxicity was investigated. Animals (n = 6/group) were treated orally (20 µL; by gavage), from day 1 to day 5, with ACAA (EtOH; 0.38 or 3.8 or 38%; v/v) or distilled water (control). Mortality was evaluated from day 1^{st} to day 11^{th}, as described in item 2.4. Then, the animals (n = 6/group) were divided into six groups receiving (20 µL) vehicle (*i.p.*), ACAA (gavage), PAAPFBA (*i.p.*) or diazepam (*i.p.*) and the Light & Dark Test was performed to characterize the behavior for 11 consecutive days. A naive group was also included.

Groups:

Group I – Naive (without treatments);
Group II – Vehicle (3% DMSO);
Group III – ACAA (1^{st} to 5^{th} day);
Group IV– ACAA (1^{st} to 5^{th} day) + PAAPFBA (4 mg/kg; 11^{th} day);
Group V– ACAA (1^{st} to 5^{th} day) + PAAPFBA (12 mg/kg; 11^{th} day);
Group VI – ACAA (1^{st} to 5^{th} day) + PAAPFBA (40 mg/kg; 11^{th} day);
Group VII– ACAA (1^{st} to 5^{th} day) + diazepam (40 mg/kg; 11^{th} day).

After 1 h of the oral treatments and 30 min of the intraperitoneal treatments, the Light & Dark Test was performed.

2.9. Statistical analysis

The results were expressed as mean ± standard error of the mean for each group of 6 animals. After confirmation of the normality of data distribution and data homogeneity, the differences between the groups were submitted to analysis of variance (one-way ANOVA), followed by Tukey test. All analyses were performed using GraphPad Prism, v. 6.01. The level of statistical significance was set at 5% ($p < 0.05$). In the toxicity test, LD_{50} was determined by the Trimmed Spearman-Karber mathematical method with 95% confidence interval [15].

3. Results

3.1. PAAPFBA chalcone synthesis and characterization

Chalcone N-{4′-[(*E*)-3-(4-Fluorophenyl)-1-(phenyl) prop-2-en-1-one]} acetamide (PAAPFBA) (Fig. 2) was synthesized and the yield was 88.21%. The following spectroscopic data were obtained for the chemical characterization (Supplementary material):

Yellow solid (Yield: 88.21%), m.p. 178.4–179 °C; FT-IR (KBr, $\nu_{cm^{-1}}$): 3430, 1650, 1615, 1500, 1300, 980. ^{1}H-NMR (CD_3OD, 300 MHz): δ 7.76 (m, H-2/H-6), 7.16 (t, H-3/H-5, J = 8.70 Hz), 8.07 (d, H-2′/H-6′, J = 8.67 Hz), 7.73 (m, H-3′/H-5`), 772 – 7.81 (s broad, H-α, H-β), 2.16 (s, CH_3). ^{13}C-NMR (CD_3OD, 75 MHz): δ C-1 133.1, C-2/C-6 131.1, C-3/C-5 116.9, C-4 164.0, C-1′ 132.1, C-2′/C-6′ 131.1, C-3′/C-5′ 122.9, C-4′ 144.9, C-α 120.4, C-β 144.5, C = O 190.8, N-C = O 172.1. MS (EI) m/z ($M^{+\cdot}$ 283), calcd for $C_{17}H_{14}NO_2F$/283.

3.2. 96-h acute toxicity

The chalcone PAAPFBA was found to be non-toxic against adult zebrafish in up to 96 h of analysis (LD_{50} < 40 mg/kg).

3.3. Open field test

Chalcone PAAPFBA (**$p < 0.05$ - ***$p < 0.001$) and Diazepam (****$p < 0.0001$) decreased the locomotor activity of adult zebrafish in comparison to the naive and vehicle groups ($F_{5,\ 30}$ = 12.17; Fig. 3).

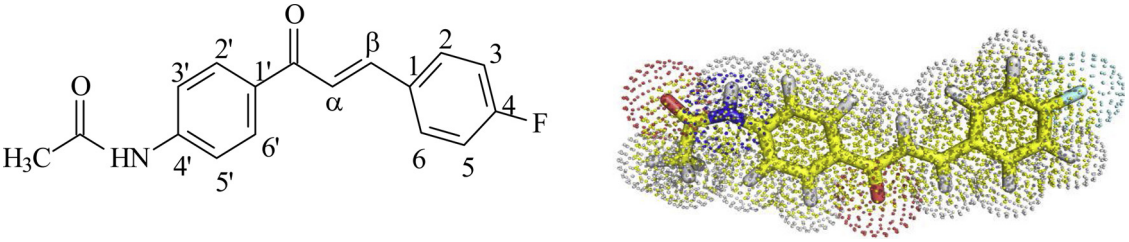


Fig. 2. Chemical structure of N- {4′- [(E) -3- (4-fluorophenyl) -1- (phenyl) prop-2-en-1-one] acetamide (PAAPFBA) and its dimensional structure obtained by molecular modeling [30].

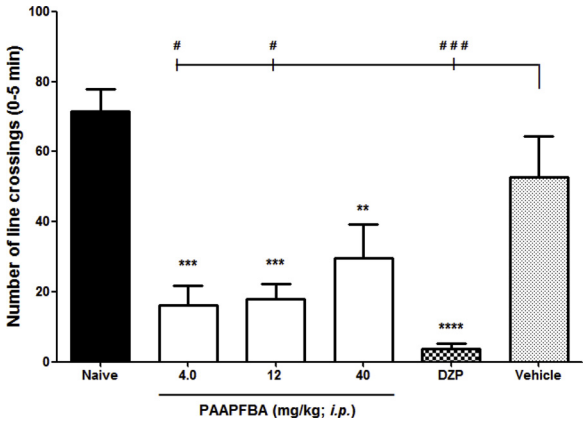


Fig. 3. Effect of PAAPFBA on the locomotor activity of adult zebrafish (*Danio rerio*) in the open field test (0–5 min). Naïve - untreated animals. DZP-diazepam (40 mg/kg; *i.p.*). Vehicle - 3% DMSO (20 µL; *i.p.*). Values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ *vs.* Naïve; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ *vs.* Vehicle).

3.4. Preliminary anxiolytic evaluation

PAAPFBA (40 mg/kg; *i.p.*) and diazepam (40 mg/kg; *i.p.*) increased (****$p < 0.0001$ *vs* naive or vehicle) the permanence in the light zone of the Light & Dark Test ($F_{5,\ 30} = 20.26$; Fig. 4). Flumazenil reduced (**$p < 0.01$) the anxiolytic-like effect of PAAPFBA (40 mg/kg; *i.p.*) and Diazepam (40 mg/kg; *i.p.*) ($F_{6,\ 35} = 64.72$; Fig. 5). In the molecular docking between the $GABA_A$ channel and PAAPFBA, the most strongly interacting amino acids are: Ala45, Met55, Leu99, Lys102 and Met137. The $GABA_A$ channel interacts with the ligand, with bonds varying from 2.1 to 4.1 Å).

3.5. Anxiety induced by alcohol withdrawal

ACAA (all concentrations tested) was nontoxic to adult zebrafish during the 11 days of analysis; therefore, ACAA was used as the source of ethanol (38% EtOH). Continuous exposure of adult zebrafish (Groups III-VII; see 2.3) to ACAA (38% EtOH; v/v) resulted in an anxiolytic-like effect (Fig. 6). The time spent by the animals in the light zone of the aquarium (4th and 5th days of treatment), as well as on the 6th day of ACAA abstinence was (4th to 6th days – ***$p < 0.001$; **$p < 0.01$; $F_{6,\ 385} = 40.98$) different when compared to the control groups (naive and vehicle).

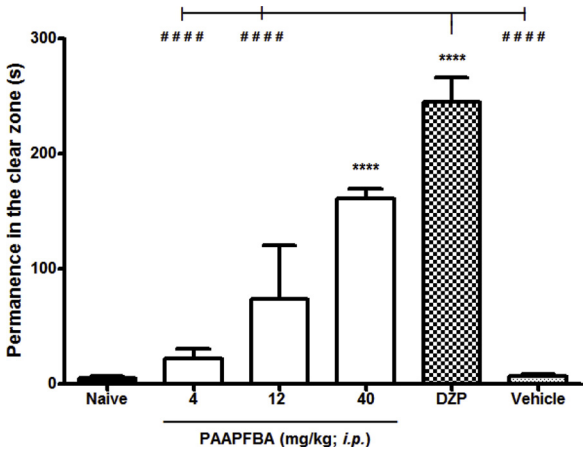


Fig. 4. Anxiolytic-like effect of PAAPFBA in the Light & Dark Test (0–5 min). Naïve - untreated animals. DZP - Diazepam (40 mg/kg; *i.p.*). Vehicle – 3% DMSO (20 µL; *i.p.*). Values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey (****$p < 0.0001$ *vs.* Naïve and vehicle; $^{\#\#\#\#}p < 0.0001$ *vs.* DZP).

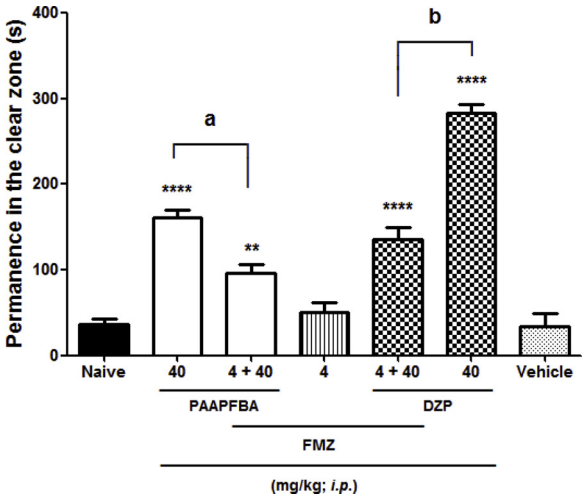


Fig. 5. Effect of flumazenil (FMZ; 4 mg/kg; *i.p.*) on the anxiolytic-like effect of PAAPFBA in the Light & Dark test. Values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. ANOVA followed by Tukey (**$p < 0.01$; ****$p < 0.0001$ *vs.* Naive or Vehicle; $^{a}p < 0.01$ *vs.* PAAPFBA (40mg/kg; *i.p*); $^{b}p < 0.0001$ *vs* DZP.; DZP – Diazepam (40mg/kg; *i.p*) . Vehicle – 3% DMSO (20 µL; *i.p.*).

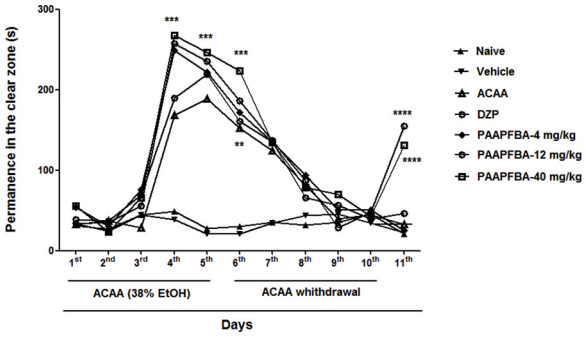


Fig. 6. Effect of PAAPFBA (mg/kg; *i.p.*) on alcohol withdrawal induced-anxiety in the Light & Dark Test. Values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. Naïve - untreated animals. ACCA - yellow sugar cane brandy (38% EtOH); Vehicle – 3% DMSO (20 µL; *i.p.*). DZP - Diazepam (40 mg/kg; *i.p.*). ANOVA followed by Tukey (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ *vs.* Naive or Vehicle).

On the 11th day of ACAA withdrawal, the anxiety prevention in adult zebrafish with PAAPFBA or DZP (40 mg/kg, *i.p.*) was performed. According to the preliminary anxiolytic-simile test, only PAAPFBA (40 mg/kg; *i.p.*) significantly ($F_{6,\ 35} = 27.23$; ****$p < 0.0001$ *vs* naive or vehicle or ACAA) prevented the anxyolitic-like effect in adult zebrafish. This effect was significantly like DZP (40 mg/kg; *i.p.*), Fig. 7.

4. Discussion

Although there are authors who have investigated the anxiolytic potential of chalcones [19], this is the first study to evaluate the ability of a synthesized chalcone (PAAPFBA) to attenuate anxiety using the adult zebrafish (*Danio rerio*) as animal model.

Infrared data revealed the presence of conjugated ketone carbonyl and amide, in addition to bands that characterized the presence of the -NH group. The ^{13}C spectrum showed a set of signals for aromatic carbon and characteristic signals of the conjugated system of enones, consisting of a carbonyl group and an alpha carbon and a beta carbon. Revealed the presence of amide carbonyl, confirming the formation of chalcone and its acetylation. The ^{1}H spectrum showed signals for the

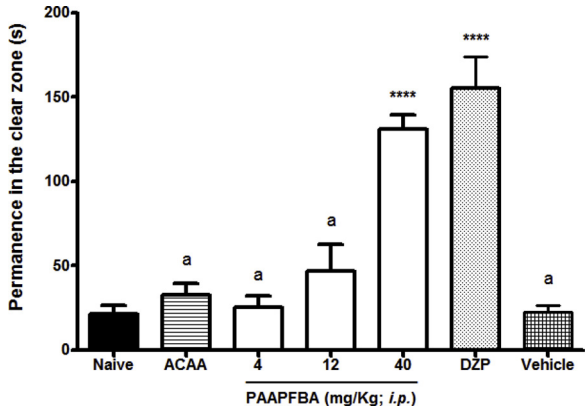


Fig. 7. Prevention of the alcohol withdrawal induced-anxiety (11th day) by PAAPFBA in the Light & Dark Test (0–5 min). Values represent the mean ± standard error of the mean (E.P.M.) for 6 animals/group. Naïve - untreated animals. ACCA - yellow sugar cane brandy (38% EtOH); DZP - Diazepam (40 mg/kg; *i.p.*). Vehicle – 3% DMSO (20 µL; *i.p.*). ANOVA followed by Tukey (****$p < 0.0001$ *vs.* Naïve or ACAA or Vehicle; [a]$p < 0.0001$ *vs.* DZP).

alpha and beta hydrogens present in the enone system, but expressed in a displacement range, due to the overlap of these signals with hydrogen signals from the aromatic ring. Correlation data analysis (HMBC) reveals the presence of the hydrogen signal of the -NH group of amide in 7.21 ppm, however, it is not a prominent signal in the spectrum, due to its overlap with signals of hydrogen from the aromatic ring. Mass value found in the spectrum CG—MS corresponds to the calculated mass value. The analysis of this data set confirmed the structure of chalcone (see the Supplementary material).

PAAPFBA promoted no deaths of fish during the 96 h of exposure. The PAAPFBA decreased (**$p < 0.01$ – ***$p < 0.001$ *vs* vehicle) the locomotor behavior of the animals, like that of anxiolytic compound diazepam (****$p < 0.0001$ *vs* vehicle). In this context, the possible anxiolytic-like effect of PAAPFBA was investigated.

The Light & Dark Test is indicated to assess new benzodiazepine-type anxiolytic agents [6] and it has been validated for use in both rats and mice [20]. In the test with adult zebrafish, anxiety is characterized by the innate aversion of zebrafish to illuminated [6]. Similar studies have shown that adult zebrafish that were not treated with anxiolytic drugs exhibit the same behavior in mice [6,20,21]. Benneh et al. [18] carried out a comparative analysis of anxiety-like behavior in adult zebrafish using the Light & Dark and Novel Tank Tests, and suggested that the Light & Dark Test is more sensitive to pharmacological treatments in comparison with the Novel Tank Test (anxiety in this test is reflected in reduced exploration [22].

PAAPFBA (40 mg/kg; *i.p.*) and diazepam (40 mg/kg; *i.p.*) increased (****$p < 0.0001$ *vs* vehicle) the time spent by the fish on the light zone, suggesting an anxiolytic-like effect of the chalcone (Fig. 4). It is noteworthy that flavone derivatives, another subclass of flavonoids, called 6,3′-dinitroflavone also showed anxiolytic effect in mice. This derivative was considered an anxiolytic drug 30 to 100-fold more potent than DZP at doses of 3 and 30 µg/kg [23].

To investigate the anxiolytic action mechanism of PAAPFBA, flumazenil, a specific $GABA_A$ antagonist was used. Flumazenil acts as a competitive inhibitor in $GABA_A$ channel, and is considered to be an excellent tool for studies of $GABA_A$ receptors, because it antagonizes the effects of benzodiazepines, including the anxiolytic, sedative and hypnotic effects [24]. Reduction of anxiolysis by pretreatment with flumazenil suggests a possible involvement of the $GABA_A$ receptor in the anxiolytic effects of PAAPFBA (Fig. 5).

The results obtained in molecular docking suggest that there is chemical, spatial and energetic compatibility between the $GABA_A$ receptor and PAAPFBA, with Van der Waals and hydrogen bonding interactions. It can be observed that a three-dimensional protein structure is not static, due to the vibration and movement caused by the different atoms and, thus, the molecule has an intrinsic energy, which stabilizes and shows energy loss when it binds to PAAPFBA. The interaction of PAAPFBA with the $GABA_A$ receptor occurs in a stable structural domain, without steric hindrance and strong electrostatic repulsions, while promoting a possible agonist action, since the same interaction evidence shows this site is a favorable one for stabilization by external molecules, such as diazepam [17]. Therefore, the formation of the $GABA_A$/PAAPFBA complex is demonstrated *in silico*, from energetic, spatial and chemical perspectives, and this stability conferred on the receptor by the interaction with the chalcone molecule is directly related to the anxiolytic activity confirmed *in vivo*.

Alcohol withdrawal anxiety models have been established in rodents [25,26], as well as in adult zebrafish [18,27]. Therefore, zebrafish has become an important model for the study of the neurobehavioral effects of chronic alcohol use. Here we show that the repeated yellow sugar cane brandy (ACAA) consumption induced anxiolytic-like behavior in adult zebrafish followed by an anxiety-like behavior after alcohol its withdrawal (Fig. 6).

The chronic anxiety test was performed in this study because acute alcohol consumption at relatively low concentrations may result in "pleasurable" effects, such as relaxation and relief from stress or anxiety [28]. In addition to anxiety, chronic alcohol use generates symptoms of autonomic hyperactivity and epileptic seizures when alcohol is withdrawn, resulting in alcohol withdrawal syndrome [27].

For this reason, the daily alcohol content (20 µL) was equivalent to the proportion of 38% ethanol for five consecutive days, aiming at causing dependence on the fish, which increases the stress and anxiety with alcohol withdrawal [29]. As expected, PAAPFBA and diazepam were effective in decreasing (****$p < 0.0001$ *vs* Vehicle) anxiety in adult zebrafish induced by alcohol withdrawal.

5. Conclusion

The present study confirmed that N-{4′-[(E) -3- (4-Fluorophenyl) -1- (phenyl) prop-2-en-1-one]} acetamide (PAAPFBA) was nontoxic and showed anxiolytic-like effects on adult zebrafish mediated *via* the GABAergic, as well as the pharmacological potential for the treatment of alcohol withdrawal-induced anxiety.

Declaration of conflicts of interest

The authors declare no conflicts of interest.

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