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An Evaluation of the Anxiolytic Potential of Amentoflavone in Adult Zebrafish Undergoing Alcohol Withdrawal: In Vivo and In Silico Studies

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Abstract: The constant use of alcoholic beverages can deregulate serotonin levels, affecting neurotransmitters and triggering symptoms of anxiety. In this context, the objective of this work was to evaluate the anxiolytic potential and possible action mechanisms of the natural compound amentoflavone against the deleterious effects caused by alcohol withdrawal on the behavior of adult zebrafish (aZF). The experiments showed that amentoflavone did not change locomotion and did not cause toxicity in aZF during up to 96 h of analysis, with a median lethal concentration (LC_{50}) greater than 1.0 mg/mL. The reversal of anxiety by pretreatment with granisetron suggested that the anxiolytic effect of amentoflavone is dependent on serotonergic 5-$HT_{3A/3B}$ receptors. Furthermore, amentoflavone reversed anxiety due to flumazenil pretreatment, suggesting a dependence on the $GABA_A$ receptor. The three concentrations of amentoflavone tested were effective in treating anxiety resulting from alcohol withdrawal. In silico analysis validated the in vivo results, supporting the idea that the interaction of amentoflavone with the protein occurs in a more stable manner than reference compounds. Amid growing interest in natural alternatives to treat anxiety disorders, amentoflavone is a potential candidate for a new anxiolytic compound that acts specifically on the $5HT_{3A/3B}$ and GABAergic serotonergic pathways.

Keywords: amentoflavone; anxiolytic; alcohol abstinence; 5-$HT_{3A/3B}$; $GABA_A$; molecular modeling

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1. Introduction

Alcohol (EtOH) is a central nervous system (CNS) depressant, which means that its consumption can initially cause a feeling of relaxation and relief from anxiety. According to the Brazilian Surveillance of Risk and Protective Factors for Chronic Diseases by Telephone Survey (VIGITEL) in 2021, a total of 18.4% of the respondents were classified as abusive drinkers. Among men, this percentage was 25.6%, down from 27.0% in 2010. Alcohol intake among women, however, increased during this period, from 10.5% to 12.7% [1]. It is worth remembering that in 2021, the world was struck by the COVID-19 pandemic. Queiroga et al. [2] reported that the increase in alcohol consumption, especially among

young people, at the beginning of the pandemic was associated with factors such as fear of the disease, panic, insecurity and stress resulting from isolation measures. The researchers emphasized the importance of early intervention in cases of alcohol dependence to prevent the development of chronic alcoholism.

According to the World Health Organization (WHO), alcohol consumption can cause or contribute to more than 200 diseases and injuries. It is associated with a higher risk of developing health problems such as mental and behavioral disorders, including alcohol dependence, serious illnesses such as liver cirrhosis, some types of cancer and cardiovascular disease, as well as injuries resulting from violence and traffic accidents. Worldwide, 3 million deaths a year result from the harmful use of alcohol, accounting for 5.3% of all deaths [3].

The chronic and abusive use of alcohol can lead to a vicious cycle: as the body develops tolerance to the anxiolytic effect of alcohol, the organism requires ever-increasing doses to obtain the same relief. Furthermore, excessive alcohol consumption can lead to physical and mental health problems, further aggravating anxiety [4]. There are different scales and assessment instruments used to measure anxiety levels in patients experiencing alcohol withdrawal. In general, anxiety symptoms can be mild, moderate or severe and may include feelings of nervousness, restlessness, excessive worry, difficulty concentrating, muscle tension and irritability, among others [5]. In some more serious cases, alcohol withdrawal can lead to more intense anxiety and can even evolve into generalized anxiety disorder [6].

Anxiety generated by alcohol withdrawal is related to the interaction with GABAergic channels in the central nervous system. During chronic alcohol consumption, $GABA_A$ receptors become less sensitive, resulting in a reduction in the inhibitory activity of the neurotransmitter GABA (aminobutyric acid). When ceasing consumption, there is a lack of alcohol's sedative effect on $GABA_A$ receptors, leading to neuronal hyperexcitability and withdrawal symptoms, such as anxiety, irritability and insomnia [7,8]. The serotonergic pathway is associated with alcohol dependence, playing a key role in EtOH consumption, a vicious cycle, and recidivism [9]. Acutely, EtOH increases the release of serotonin (5-HT) in the CNS [10], with reports of a correlation between 5-HT release in the brain and specific behaviors (for example, fear, anxiety and aggression) [11].

Anxiolytic activity is characterized by the ability to reduce anxiety and the symptoms associated with it, such as tension, fear and excessive worry. In the context of alcoholism, anxiety can be a triggering or aggravating factor. Many people turn to alcohol as a way to cope with feelings of anxiety, seeking temporary relief from symptoms [12].

In this context, alcoholism treatment may include anxiolytic approaches as part of a broader rehabilitation strategy. Drugs such as disulfiram (DSF), naltrexone and acamprosate (calcium acetyl homotaurinate) can play a crucial role in reducing alcohol cravings and consumption and maintaining abstinence by acting as adjuvants for addicted people, but all of these drugs have adverse side effects [13].

In bioactivity tests by our research groups, we used adult zebrafish as a model organism to replace the use of rodents to screen new bioactive compounds with anxiolytic action, including extracts from medicinal plants, as well as synthetic substances originating from natural products. In such studies, we also investigated the mechanism of anxiolytic action via the GABAergic system, using flumazenil as a $GABA_A$ antagonist, as well as the medications granisetron (Gstn; 5-HT3A/2C antagonist), pizotifen (Piz; $5\text{-}HT_1$ and $5\text{-}HT_{2A/2C}$ antagonist) and cyproheptadine (Cypro; 5-HT2A antagonist) as antagonists of the serotonergic system [14].

Amentoflavone (AMT) is a biflavonoid obtained by the oxidative coupling of two molecules of apigenin, resulting in a bond between the C-8″ positions of the chromene ring (benzene ring fused to a pyran ring) of one molecule and the C-3′ of the hydroxyphenyl ring of the other molecule, 3′,8″-biapigenin [15] (Figure 1).

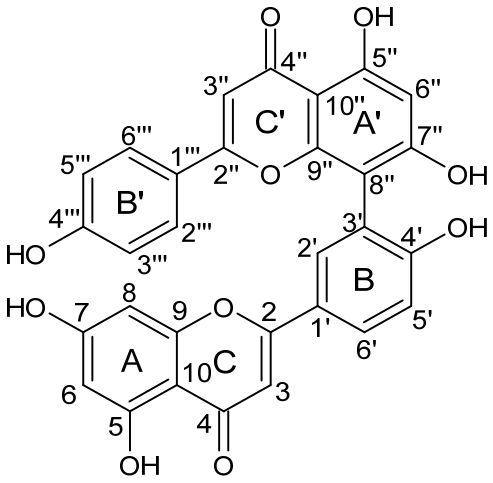


Figure 1. The chemical representation of the structure of the biflavonoid amentoflavone (AMT).

Amentoflavone has a wide range of biological activities, including anti-inflammatory, antioxidant and antimicrobial properties and the regulation of metabolism, neuroprotection, radioprotection, musculoskeletal protection, antidepressant activity and the promotion of resistance to various types of cancer. This investigation covers the bioavailability and drug delivery of amentoflavone, the molecular mechanisms underlying its activities, the simulation of molecular coupling through the in silico approach and the demonstration of the anti-SARS-CoV-2 effect of amentoflavone [16].

In previous studies, amentoflavone improved anxiety symptoms through the modulation of mTOR signaling, alleviating $A\beta_{25\text{-}35}$-induced anxiety symptoms of animals in a rat model of Alzheimer's disease [17]. These aspects highlight the importance of this molecule in exploring new approaches, particularly as an anxiolytic in zebrafish. This model has been increasingly used instead of mice, rats or other non-human mammals, due to the global strategy known as NC3Rs (Replacement, Reduction and Refinement, originating in the United Kingdom), which consists of reducing the use of animals in experimentation through replacement by other models and the refinement of research [18].

Some studies have suggested that biflavonoids may also have positive effects on the immune system, helping to strengthen the body's response to viral and bacterial infections [19,20]. Additionally, they can help regulate blood sugar levels, lower cholesterol and prevent blood clots, which can help prevent cardiovascular disease [21].

Therefore, the present study aimed to investigate the anxiolytic potential of amentoflavone in vivo and in silico, to ascertain a possible effect for the treatment of anxiety resulting from alcohol withdrawal, as well as defining the mechanisms of action involved.

2. Methodology

2.1. Obtaining Amentoflavone

First, a leaf extract of *Ouratea fieldingiana* (Gardner) Engl was prepared through maceration with ethanol–water. The botanical identification of *Ouratea fieldingiana* was performed by the botanist Luiz Wilson Lima-Verde, and a specimen was deposited in the Prisco Bezerra herbarium collection under number 62,392 on 3 April 2019.

The isolation of amentoflavone was carried out from this extract using silica gel column chromatography. The column was eluted with hexane, chloroform, ethyl acetate and methanol in mixtures having increasing polarity. The fractions eluted with ethyl acetate were combined, and the solid material obtained was crystallized to obtain amentoflavone, whose structure was characterized by NMR spectroscopy [15].

2.2. Animals

Wild adult zebrafish (*Danio rerio*), aZF, of both sexes aged 60–90 days, with lengths of 3.5 ± 0.5 cm and weight 0.4 ± 0.1 g were obtained from Agroquímica Comércio de Produtos Veterinários Ltd.a, a supplier in Fortaleza (Ceará, Brazil). Groups of 40–50 fish were acclimatized in a 9 L glass aquarium at room temperature (26 ± 2 °C) for 24 h, 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. The fish received feed ad libitum 24 h before the experiments. After the experiments, the fish were sacrificed with cold water (2–4 °C) for up to 2 min until the loss of opercular movements occurred [22]. All experimental procedures were approved by the Animal Use Ethics Committee of State University of Ceará (CEUA-UECE), under protocol no. 05299177/2021.

2.3. Drugs or Pharmacological Treatments

Granisetron hydrochloride was obtained from CorePharma, LLC (Middlesex, NJ, USA). Pizotifen maleate was procured from Central Manipulation Pharmacy (São Paulo, Brazil). Cyproheptadine was acquired from Evidence Soluções Farmacêuticas (Fortaleza, Brazil). Fluoxetine was obtained from Eli Lilly (Indianapolis, IN, USA). Flumazenil was purchased from Roche Pharmaceutical (Welwyn Garden City, UK). Diazepam was from Sigma-Aldrich Corp. All other chemicals were bought from Dinamica (São Paulo, Brazil).

2.4. General Protocol

The tests were carried out based on the methods proposed by Magalhães et al. [23] and Ekambaram et al. [24]. On the day of the experiments, aZF were randomly selected, transferred to a damp sponge and treated with the test or control samples, intraperitoneally (i.p.) or orally (p.o.). They were then placed individually in glass beakers (250 mL) containing 150 mL of resting aquarium water. For intraperitoneal (i.p.) treatments, an insulin syringe (0.5 mL; UltraFine® BD) with a 30 G needle was used. For oral (p.o.) treatments, a 20 µL variable automatic pipette was used. The behavior of the animals was recorded by calibrated and blinded analysts. The activities scheme can be found in the Summary of Protocols (Figure 2).

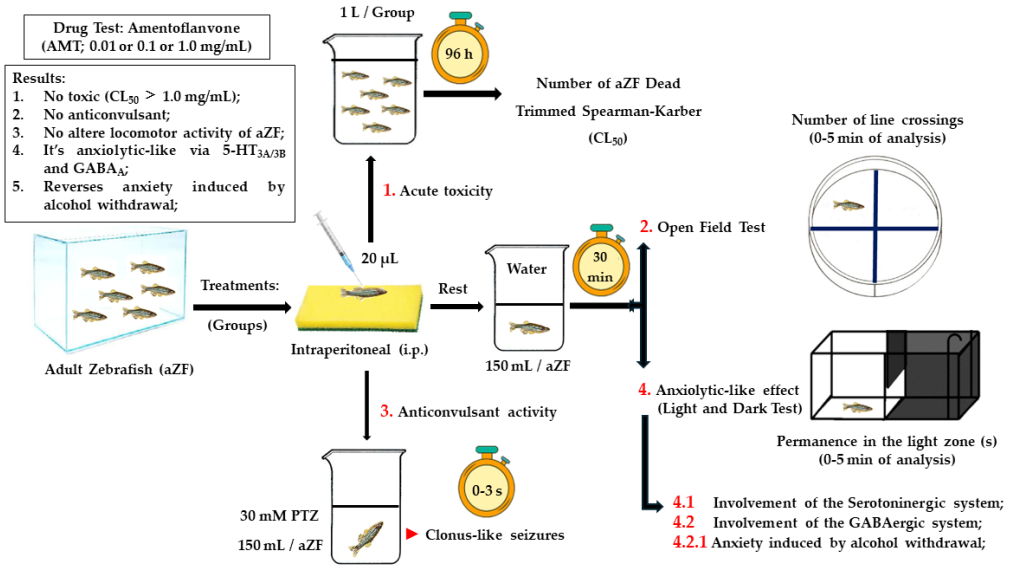


Figure 2. Protocol summary. A brief summary of the experimental protocol and behavioral analyses performed in this study.

2.5. Anxiolytic-Like Effect

The anxiolytic-like effect was explored by the Light and Dark Test, which was carried out in a glass aquarium (30 × 15 × 20 cm) with a light and a dark zone. The aquarium

was filled with 3 cm of tap water, pretreated with antichlorine and drug-free, as being shallow restricts the aquarium from living, which is a well-established anxiety behavior in a new environment [25]. The animals (n = 6/group) were treated intraperitoneally (i.p.) with 20 µL of AMT (0.01 or 0.1 or 1.0 mg/mL) or diazepam (DZP; anxiolytic control [26]; 10 mg/mL) or vehicle (3% DSMO; negative control). A group of animals without treatments was included (naive). Thirty minutes after intraperitoneal treatments, the animals were individually added to the light zone of the aquarium, and the anxiolytic-like effect was quantified as the time(s) of permanence in the light zone, during 5 min of analysis.

2.5.1. The Involvement of the Serotonergic System

The involvement of the serotonergic system in the anxiolytic effect of the test samples was explored in the Light and Dark Test, following the method of Benneh et al. [27]. Antagonist doses were standardized by Gonçalves et al. [25]. Initially, the animals (n = 6/group) were treated with 20 µL of AMT (0.01 mg/mL; i.p.), fluoxetine (Flx; 1.25×10^{-3} mg/mL; i.p.), cyproheptadine (Cypro; antagonist 5-HT_{2A} [28]; 0.8 mg/mL; p.o.), (Piz; 5-HT_1 and 5-$HT_{2A/2C}$ antagonist [29]; 0.8 mg/mL; p.o.) or granisetron (Gst; antagonist of 5-$HT_{3A/2C}$; 5-HT_{3B} [30]; 0.5 mg/mL; p.o.). Subsequently, they were pretreated with cyproheptadine (Cypro; 5-HT_{2A} antagonist; 0.8 mg/mL; p.o.), pizotifen (Piz; 5-HT_1 and 5-HT_2 antagonist; $HT_{2A/2C}$ (0.8 mg/ mL; p.o.) or granisetron (Gst; 5-$HT_{3A/3B}$ antagonist; 0.5 mg/mL; p.o.) that was administered 15 min before treatment with 20 µL of AMT (0.01 mg/mL; i.p.) or fluoxetine (Flx; 1.25×10^{-3} mg/mL; i.p.). A vehicle-treated group and an untreated group were included. After treatments, the animals were placed in the light zone of the aquarium, and the anxiolytic effect was measured by the time spent in this area for 5 min. A group of vehicle-treated (3% DSMO; 20 µL; i.p.) and untreated (naive) animals were included. After 30 min of intraperitoneal treatments and 1 h of oral treatments, the animals were individually placed in the light zone of the aquarium, and the anxiolytic-like effect was quantified as the time(s) spent in the light zone, during 5 min of analysis.

2.5.2. The Involvement of the GABAergic System

The involvement of the GABAergic system of the lowest effective concentration of the samples was explored in the Light and Dark Test, described above, after pretreatment with flumazenil, a $GABA_A$ antagonist, according to the method proposed by Benneh et al. [27]. Initially, the animals (n = 6/group) were treated with 20 µL of AMT (0.01 mg/mL; i.p.) or diazepam (DZP; 10 mg/mL; i.p.) or flumazenil (Fmz; 0.1 mg/mL; i.p.). In another experiment, the animals (n = 6/group) were pretreated with flumazenil (Fmz; 0.1 mg/mL; 20 µL; i.p.) 15 min before intraperitoneal treatment with AMT (0.01 mg/mL; 20 µL) or diazepam (DZP; 10.0 mg/mL). Groups of vehicle-treated (3% DSMO; 20 µL; i.p.) and untreated (naive) animals were included. After 30 min of intraperitoneal treatments, the animals were individually placed in the light zone of the aquarium, and the anxiolytic-like effect was quantified as the time(s) spent in the light zone, during 5 min of analysis.

2.5.3. Anxiety Induced by Alcohol Withdrawal

To evaluate the treatment of alcohol withdrawal-induced anxiety as described by Ferreira et al. [14], yellow cane spirit (ACAA; Ypioca®) was used as a source of ethanol (EtOH 0.38, 3.8 or 38%; *v*/*v*), which is not toxic to aZF, as reported by Ferreira et al. [14]. Animals (n = 6/group) were orally (p.o.) or intraperitoneally (i.p.) treated with 20 µL as described in groups (G) below:

Group 1—1st to 11th day: Naive (without treatments);
Group 2—1st to 11th day: Vehicle—3% DMSO (i.p.);
Group 3—1st to 5th day: ACAA (p.o); 6th to 11th without ACAA treatments;
Group 4—1st to 5th day: ACAA (p.o); 6th to 10th without ACAA treatments; 11th day: DZP (10 mg/mL; i.p.);
Group 5—1st to 5th day: ACAA (p.o.); 6th to 10th without ACAA treatments; 11th day: AMT (0.01 mg/mL; i.p.);

Group 6—1st to 5th day: ACAA (p.o.); 6th to 10th without ACAA treatments; 11th day: AMT (0.1 mg/mL; i.p.);
Group 7—1st to 5th day: ACAA (p.o.); 6th to 10th without ACAA treatments; 11th day; AMT (1.0 mg/mL; i.p.).

Thirty minutes after intraperitoneal treatments, the animals were submitted to the Light and Dark Test [25], as previously described, to characterize the anxiolytic-like effect for 11 consecutive days.

2.6. Locomotor Activity (Open Field Test)

The open field test [23] was carried out to evaluate the presence or absence of a sedative effect of the tested samples. Animals (n = 6/group) were treated intraperitoneally (i.p.) with 20 µL of AMT (0.01 or 0.1 or 1.0 mg/mL) or diazepam (DZP; sedative control; 10 mg/mL) or vehicle (3% DSMO). A group of animals without treatments was included (naive). Thirty minutes after intraperitoneal treatments, the animals were placed in Petri dishes (100 × 15 mm) containing the same water as in the aquarium, marked with four quadrants, and locomotor activity (LA) was analyzed by counting the number of line crosses, for 0–5 min.

2.7. Anticonvulsant Activity

The anticonvulsant effect of the samples was tested according to the method proposed by Siebel et al. [31]. The animals (n = 6/group) were treated intraperitoneally (i.p.) with 20 µL of AMT (0.01 or 0.1 or 1.0 mg/mL) or diazepam (DZP; anticonvulsant control; 10 mg/mL; i.p.) or vehicle (3% DSMO; negative control). Thirty minutes after intraperitoneal treatments, the animals were individually immersed in a solution of pentylenetetrazole (PTZ; 30 mM). Seizure-like behavior was characterized as clonus-like seizures, followed by the loss of posture, when the animal fell to its side and remained motionless for 1–3 s. The specific doses of the antagonists were chosen as described in the literature by Gonçalves et al. [32].

2.8. Acute Toxicity (96 h) against Adult Zebrafish

The acute toxicity study was carried out against adult zebrafish according to the standard method described by the Organization for Economic Cooperation and Development [33] to determine $LC_{50–96h}$. Animals (n = 6/group) were treated intraperitoneally (i.p.) with 20 µL of AMT (0.01 or 0.1 or 1.0 mg/mL) or vehicle (3% DSMO; control). After the treatments, aZF mortality was evaluated every 24 h. After 96 h, the number of dead fish in each group was recorded, and the lethal concentration capable of killing 50% of the animals (LC_{50}) was determined using the trimmed Spearman–Karber mathematical method with a confidence interval of 95% [34].

2.9. Statistical Analysis

The results were expressed as mean values ± the standard error of the mean for each group of 6 animals. After confirming the normal distribution and homogeneity of the data, the differences between the groups were submitted to an analysis of variance (one-way ANOVA), followed by the Tukey test. All analyses were performed using GraphPad Prism v. 5.01. The level of statistical significance was set at 5% ($p < 0.05$).

2.10. Molecular Docking Simulations

Regarding the molecular docking simulations, the target molecule was retrieved from the Protein Data Bank repository. The targets, referred to as "Cryo-EM structure of the $5HT_{3A}$ receptor in the presence of granisetron" (PDB ID: 6NP0) and "Human $GABA_A$ receptor alpha1-beta2-gamma2 subtype in complex with GABA plus flumazenil" (PDBID: 6X3U), were determined by electron microscopy with resolutions of 2.92 and 3.50 Å, respectively, being classified as membrane/transport proteins. To prepare the target, native cocrystallized ligands such as oligosaccharides, 1-methyl-N-[(1R,5S)-9-

methyl-9-azabicyclo[3.3.1]nonan-3-yl]indazol-3-carboxamide (CWB), 2-acetamido-2-deoxy-beta-D-glucopyranose (NAG), 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-imidazo[1,5-ethyl a][1,4]benzodiazepine-3-carboxylate (FYP), gamma-aminobutanoic acid (ABU) and chloride ions were used. Additionally, additional hydrogen atoms with polar and Gasteiger charges were introduced using the AutoDock Tools software [35]. To define the grid box, its center was determined at x = 133.895, y = 150.79, z = 124.352 and x = 138.138, y = 148.451, z = 124.37, respectively, with dimensions of x = 80, y = 80 and z = 80. The completeness criteria were set at 64. Finally, molecular docking simulations were performed using the AutoDock Vina software, performing 50 independent simulations for each ligand on each protein [36]. To validate the docking simulations, the re-docking technique was employed using the grid box parameters for the control binders.

The selection of the best poses was based on two criteria. The first criterion involved the root-mean-square deviation (RMSD), which serves as a validation metric for simulations performed under ideal parameters up to 2.0 Å [37]. The second criterion used the binding energy value (ΔGbinding), which is considered ideal when it presents values equal to or lower than −6.0 kcal/mol. In addition, the parameters proposed by Imberty et al. [36] were employed to evaluate the strength of hydrogen bonds considering the distances between the donor and target atoms. Hydrogen bonding distances ranging from 2.5 Å to 3.1 Å, 3.1 Å to 3.55 Å and greater than 3.55 Å were classified as strong, medium and weak, respectively.

Preparation of Binders

In the process of preparing the ligands for this study, the PubChem repository (https://pubchem.ncbi.nlm.nih.gov/ (accessed on 27 July 2023) was used to acquire the three-dimensional structures of the molecules amentoflavone (AMT), diazepam (DZP), flumazenil (antagonist) (Fmz), fluoxetine (Flx) and granisetron (antagonist) (Gstn). To optimize their conformations, the MMFF94 force field and a steeper descent algorithm were used, implementing cycles of interactions through MarvinSketch™ (https://chemaxon.com/products/marvin (accessed on 27 July 2023) [38] and Avogadro ™ (http://avogadro.cc/ (accessed on 27 July 2023) [39].

3. Results

3.1. Anxiolytic-like Effect

AMT (0.01 or 0.1 or 1.0 mg/mL; 20 µL; i.p.) significantly increased (q = 10.00, $p < 0.0001$; q = 9.051, $p < 0.0001$; q = 9.020, $p < 0.0001$ vs. naive and q = 9.518, $p < 0.0001$; q = 8.568, $p < 0.0001$; q = 8.537, $p < 0.0001$ vs. vehicle) the permanence of aZF in the light zone in the Light and Dark Test. There was no significant difference (q = 0.9502, $p > 0.05$; q = 0.9814, $p > 0.05$; q = 0.03116, $p > 0.05$) between the groups treated with AMT only, and there was no significant difference (q = 1.184, $p > 0.05$; q = 2.134, $p > 0.05$; q = 2.165, $p > 0.05$) between the groups treated with AMT and DZP (10 mg/mL; 20 µL; i.p.; q = 11.18, $p < 0.0001$ vs. naive and q = 10.70, $p < 0.0001$ vs. vehicle), Figure 3 ($F_{5, 30}$ = 25.08).

3.1.1. The Involvement of the Serotoninergic System (5-HT_{2A})

Cypro (0.8 mg/mL; 20 µL; p.o.) did not significantly prevent (q = 1.806, $p > 0.05$ vs. Cypro + AMT) the permanence in the light zone of aZF treated with AMT (0.01 mg /mL; 20 µL; i.p.) in the Light and Dark Test. However, Cypro (0.8 mg/mL; 20 µL; p.o.) significantly prevented (q = 18.11, $p < 0.0001$ vs. Cypro + Flx) the permanence in the light zone of aZF treated with Flx (1.25×10^{-3} mg/mL; 20 µL; i.p.), Figure 4 ($F_{6, 35}$ = 90.62).

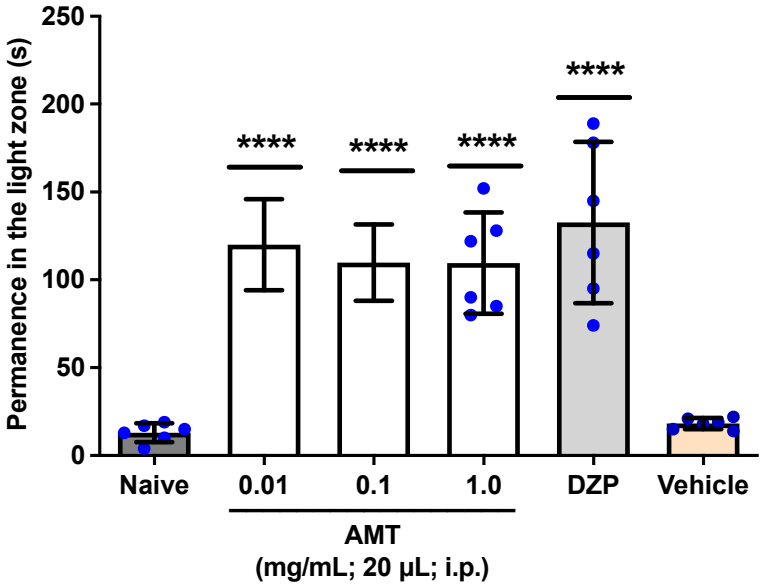


Figure 3. Anxiolytic-like effect of biflavonoid AMT in adult zebrafish (*Danio rerio*) in Light and Dark Test (0–5min). Naive—untreated animals. Vehicle—3% DMSO (20 µL; i.p.). DZP—diazepam (10 mg/mL; 20 µL; i.p.). Values represent mean ± standard error of mean (S.E.M.) for 6 animals/group. ANOVA followed by Tukey test (**** $p < 0.001$ vs. naive or vehicle).

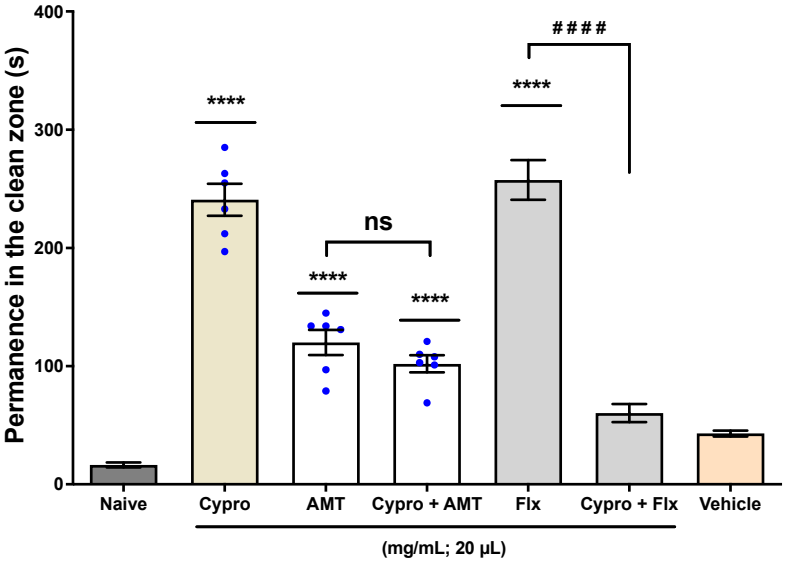


Figure 4. The effect of cyproheptadine (Cypro; 0.8 mg/mL; p.o.) on the anxiolytic-like effect of the biflavonoid AMT (0.01 mg/mL; i.p.) in adult zebrafish (*Danio rerio*) in the Light and Dark Test (0—5 min). Naive—untreated animals. Flx—fluoxetine (1.25×10^{-3} mg/mL; i.p.). Vehicle—3% DMSO (20 µL; i.p.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (**** $p < 0.001$ vs. naive or vehicle; #### $p < 0.001$ vs. Flx; ^{ns} $p > 0.05$ vs. AMT).

3.1.2. The Involvement of the Serotoninergic System (5-HT_1 and 5-$HT_{2A/2C}$)

Pizotifen (Piz; 0.8 mg/mL; 20 µL; p.o.) did not significantly prevent ($q = 1.834$, $p > 0.05$ vs. Piz + AMT) the permanence in the light zone of aZF treated with AMT (0.01 mg /mL; 20 µL; i.p.) in the Light and Dark Test. However, Piz (0.8 mg/mL; 20 µL; p.o.) significantly prevented ($q = 20.64$, $p < 0.0001$ vs. Piz + Flx) the permanence in the light zone of aZF treated with Flx (1.25×10^{-3} mg/mL; 20 µL; i.p.) in the Light and Dark Test, Figure 5 ($F_{6, 35} = 57.99$).

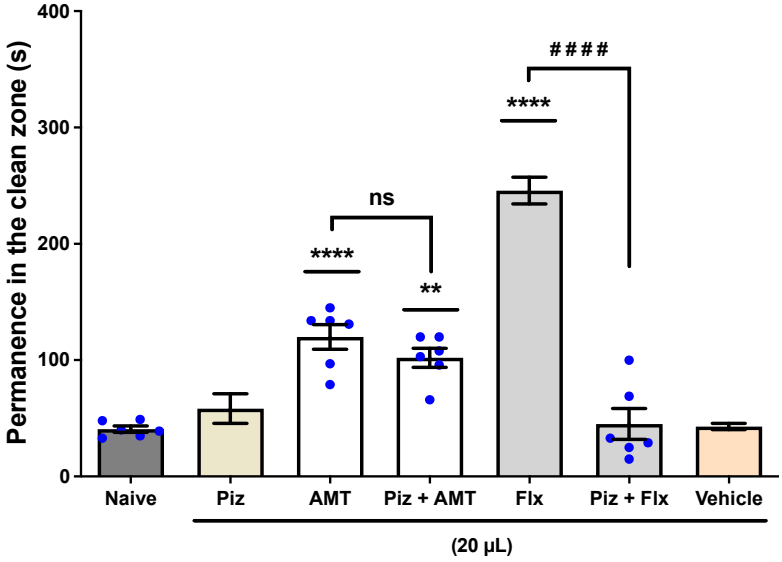


Figure 5. The effect of pizotifen (Piz; 0.8 mg/mL; p.o.) on the anxiolytic-like effect of the biflavonoid AMT (0.01 mg/mL; p.o.) in adult zebrafish (*Danio rerio*) in the Light and Dark Test (0–5 min). Naive—untreated animals. Flx—fluoxetine (1.25×10^{-3} mg/mL; i.p.). Vehicle—3% DMSO (20 µL; i.p.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (** $p < 0.01$; **** $p < 0.0001$ vs. naive or vehicle; #### $p < 0.0001$ vs. Flx; ns $p > 0.05$ vs. AMT).

3.1.3. The Involvement of the Serotoninergic System (5-$HT_{3A/3B}$)

Granisetron (Gstn; 0.5 mg/mL; 20 µL; p.o.) significantly prevented ($q = 8.344$, $p < 0.0001$ vs. Gstn + AMT) the permanence in the light zone of aZF treated with AMT (0.01 mg /mL; 20 µL; i.p.) and significantly prevented ($q = 18.72$, $p < 0.0001$ vs. Gstn + Flx) the permanence in the light zone of aZF treated with Flx (1.25×10^{-3} mg/mL; 20 µL; i.p.) in the Light and Dark Test, Figure 6 ($F_{6, 35} = 58.12$).

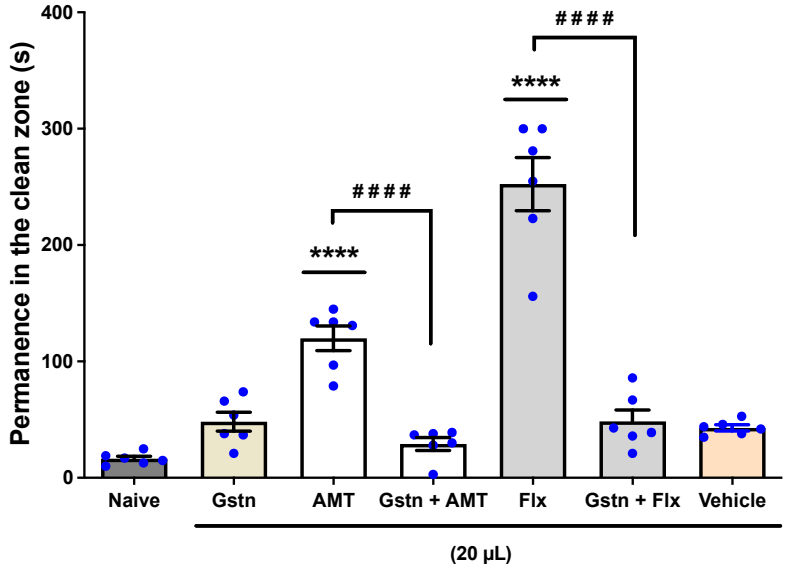


Figure 6. The effect of granisetron (Gstn; 0.5 mg/mL; p.o.) on the anxiolytic-like effect of the biflavonoid AMT (0.01 mg/mL; i.p.) in adult zebrafish (*Danio rerio*) in the Light and Dark Test (0–5 min). Naive—untreated animals. Flx—fluoxetine (1.25×10^{-3} mg/mL; i.p.). Vehicle—3% DMSO (20 µL; i.p.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (**** $p < 0.0001$ vs. naive or vehicle; #### $p < 0.0001$ vs. AMT or Flx).

3.1.4. The Involvement of the GABAergic System

Flumazenil (Fmz; 0.1 mg/mL; 20 µL; i.p.) significantly prevented ($q = 17.84$, $p < 0.0001$ vs. Fmz + AMT) the permanence in the light zone of aZF treated with AMT (0.01 mg /mL; 20 µL; i.p.) and significantly prevented ($q = 30.28$, $p < 0.0001$ vs. Fmz + DZP) the permanence in the light zone of aZF treated with diazepam (DZP; 10.0 mg/mL; 20 µL; i.p.) in the Light and Dark Test, Figure 7 ($F_{6, 35} = 169.7$).

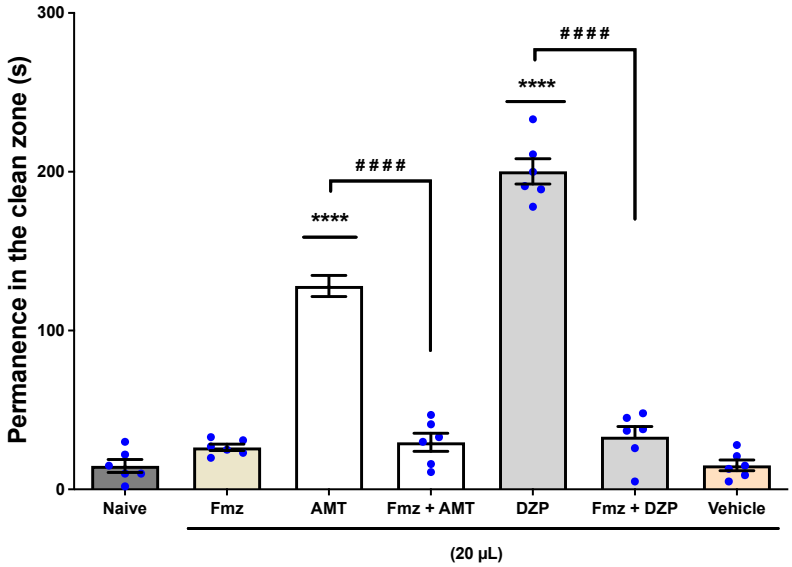
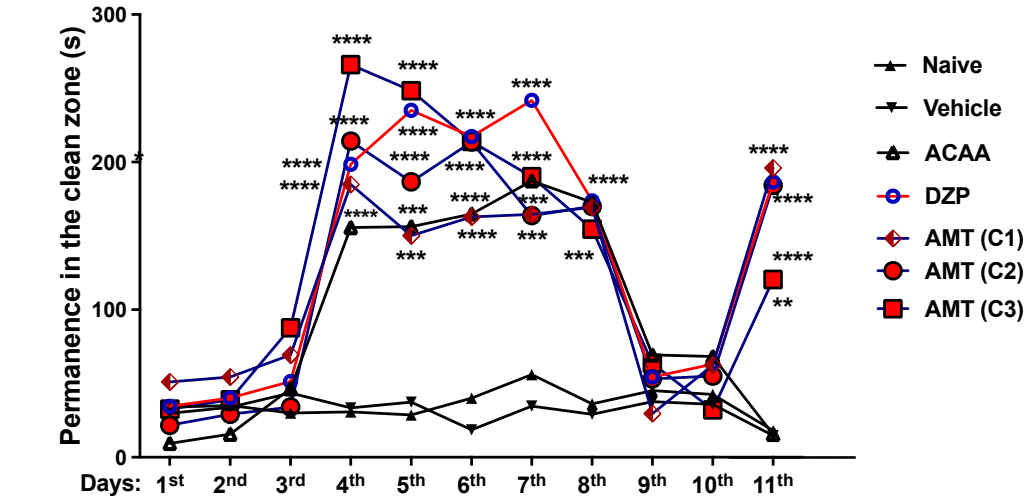


Figure 7. The effect of flumazenil (Fmz; 0.1 mg/mL; i.p.) on the anxiolytic-like effect of AMT biflavonoids (0.01 mg/mL; i.p.) in adult zebrafish (*Danio rerio*) in the Light and Dark Test (0–5 min). Naive—untreated animals. DZP—diazepam (10 mg/mL; p.o.). Vehicle—3% DMSO (20 µL; p.o.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (**** $p < 0.0001$ vs. naive or vehicle; #### $p < 0.0001$ vs. AMT or DZP).

Anxiety Induced by Alcohol Withdrawal

As indicated in Figure 8, ($F_{6, 385} = 67.58$), continuous exposure to ACAA (EtOH 38%; v/v; 20 µL; p.o.) until the 5th day of treatment in adult zebrafish (Groups 3–7) produced an anxiolytic-like effect on the 4th ($q = 6.986$, $p < 0.0001$ vs. naive and $q = 6.827$, $p < 0.0001$ vs. vehicle) and 5th ($q = 7.135$, $p < 0.0001$ vs. naive and $q = 6.650$, $p < 0.001$ vs. vehicle) days. On the 6th ($q = 6.976$, $p < 0.0001$ vs. naive and $q = 8.178$, $p < 0.0001$ vs. vehicle), 7th ($q = 7.340$, $p < 0.0001$ vs. naive and $q = 8.532$, $p < 0.0001$ vs. vehicle) and 8th ($q = 7.619$, $p < 0.0001$ vs. naive and $q = 8.020$, $p < 0.0001$ vs. vehicle) days of abstinence from ACAA (38% EtOH; v/v), an anxiolytic-like effect was also detected in aZF. Alcohol withdrawal (EtOH 38%; v/v; 20 µL; p.o.) induced anxiety in aZF from the 9th ($q = 1.351$, $p > 0.05$ vs. naive and $q = 1.770$, $p > 0.05$ vs. vehicle) to 10th days ($q = 1.425$, $p > 0.05$ vs. naive and $q = 1.807$, $p > 0.05$ vs. vehicle). The treatment of anxiety induced by alcohol withdrawal in aZF was performed with DZP (Group 4) and AMT (Groups 5–7), on the 11th day. As a result, AMT (0.01 or 0.1 or 1.0 mg/mL; 20 µL; i.p.) significantly increased ($q = 9.948$, $p < 0.0001$; $q = 9.269$, $p < 0.0001$; $q = 5.728$, $p < 0.01$ vs. naive or $q = 10.13$, $p < 0.0001$; $q = 9.482$, $p < 0.0001$; $q = 5.915$, $p < 0.01$ vs. vehicle) the permanence in the light zone of aZF in the Light and Dark Test. On the 11th day, there was no statistically significant difference between the anxiolytic-like effect of AMT at any dose and DZP group (10 mg/mL; 20 µL; i.p.; $q = 10.29$, $p < 0.0001$ vs. naive or $q = 10.49$, $p < 0.0001$ vs. vehicle).



Groups	With treatment	Without treatment	With treatment
G1-Naive	No treatment	No treatment	No treatment
G2-Vehicle	3% DMSO	3% DMSO	3% DMSO
G3-ACAA	ACAA	No treatment	No treatment
G4-DZP	ACAA	No treatment	10 mg/mL
G5-AMT (C1)	ACAA	No treatment	0.01 mg/mL
G6-AMT (C2)	ACAA	No treatment	0.1 mg/mL
G7-AMT (C3)	ACAA	No treatment	1.0 mg/mL

Figure 8. The effect of AMT (C1—0.01 or C2—0.1 or C3—1.0 mg/mL; 20 µL; i.p.) on the treatment of anxiety in adult zebrafish (11th day), induced by abstinence from alcohol (EtOH 38%) in the Light and Dark Test (0–5 min). G—group. C—concentration. Naive—untreated animals (control). ACAA—yellow cane spirit (20 µL; p.o.). Vehicle—3% DMSO (20 µL; i.p.). DZP—diazepam (10 mg/mL; 20 µL; i.p.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. naive, vehicle or ACAA).

3.2. Assessment of Locomotor Activity (Open Field Test)

AMT (0.01 or 0.1 or 1.0 mg/mL) did not change the animals' locomotor activity (Figure 9). However, diazepam (DZP; 10 mg/mL; 20 µL; i.p.) decreased the animals' locomotor activity ($q = 0.3163$, $p > 0.05$; $q = 1.965$, $p > 0.05$; $q = 2.530$, $p > 0.05$ vs. naive or $q = 0.09036$, $p > 0.05$; $q = 2.372$, $p > 0.05$; $q = 2.937$, $p > 0.05$ vs. vehicle), Figure 9 ($F_{5, 30} = 111.4$).

3.3. Anticonvulsant Activity

AMT (0.01 or 0.1 or 1.0 mg/mL, 20 µL; i.p.) did not delay the onset of clonus-like seizures, followed by the loss of posturing in aZF (Figure 10). However, diazepam (DZP; 10 mg/mL; 20 µL; i.p.) significantly reversed ($q = 20.58$, $p < 0.0001$ vs. control) the onset of clonus-like seizures, followed by the loss of posture in aZF, Figure 10 ($F_{4, 25} = 74.31$).

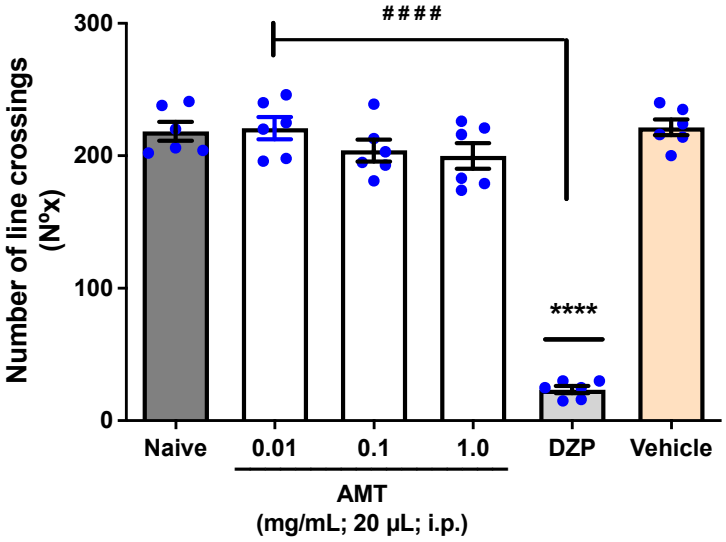


Figure 9. The effect of the biflavonoid AMT on the locomotor activity of adult zebrafish (*Danio rerio*) in the open field test (0–5 min). Naive—untreated animals. DZP—diazepam (10 mg/mL; 20 µL; i.p.). Vehicle—3% DMSO (20 µL; i.p.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (**** $p < 0.0001$ vs. naive or vehicle; #### $p < 0.0001$ vs. DZP).

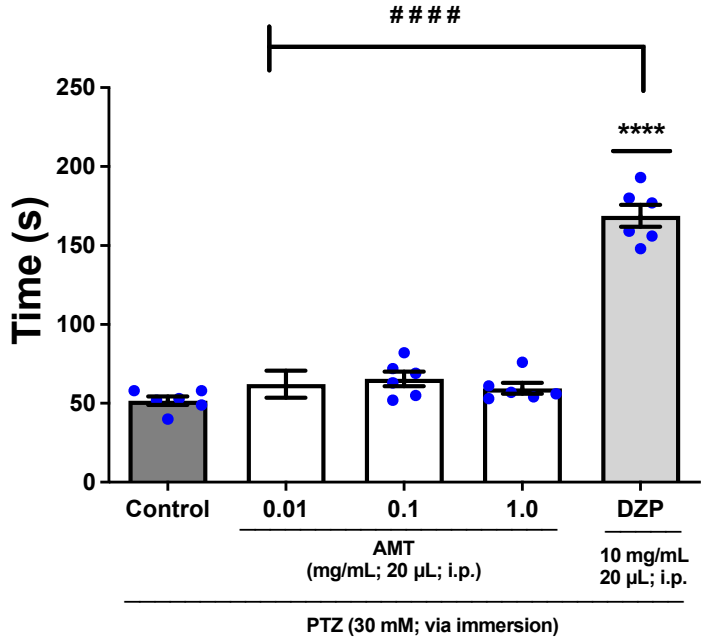


Figure 10. The effect of the biflavonoid AMT on convulsive activity (clonus and loss of posture) in adult zebrafish (*Danio rerio*) induced by pentylenetetrazole (PTZ). Vehicle—3% DMSO (20 µL; i.p.); DZP—diazepam (10 mg/mL; 20 µL; i.p.). Values represent the mean ± the standard error of the mean (S.E.M.) for 6 animals/group. ANOVA followed by the Tukey test (**** $p < 0.0001$ vs. control; #### $p < 0.0001$ vs. DZP).

3.4. Acute Toxicity against Adult Zebrafish

For all doses of AMT tested (0.01 or 0.1 or 1.0 m/mL; 20 µL), no toxicity against aZF was observed over the 24 h observation intervals up to 96 h, as determined by the trimmed Spearman–Karber method. This suggests that the LC_{50} for AMT is greater than 1.0 mg/mL (Table 1).

Table 1. Results of AMT acute toxicity tests against adult zebrafish.

Sample	Adult Zebrafish Mortality				96 h of Analysis LC_{50} (mg/mL)/IV
	Vehicle	0.01 mg/mL	0.1 mg/mL	1.0 mg/mL	
AMT	0	0	0	0	>1.0

AMT—amentoflavone. Vehicle—DMSO 3% (control; 20 µL; p.o.). LC_{50}—lethal concentration to kill 50% of adult zebrafish; IV—confidence interval.

3.5. Molecular Modeling

In molecular modeling assays, further analysis revealed that amentoflavone had a direct and significant interaction with the $5HT_{3A}$ active site. This interaction was particularly notable when compared with reference substances such as fluoxetine (used as a positive control) and granisetron (an antagonist). These interactions are clearly visualized in Figure 11, indicating that amentoflavone has the ability to compete effectively with these compounds, suggesting prominent anxiolytic potential.

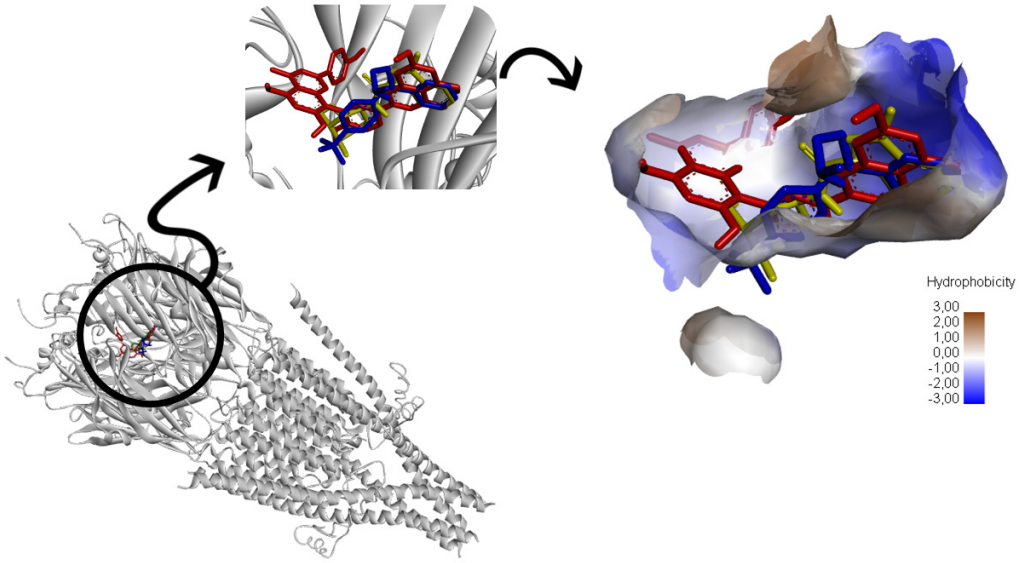


Figure 11. Representation of AMT (red), Flx (blue), Gstn (green) on target $5HT_{3A}$.

The detailed analysis of Table 2 reveals crucial information about amentoflavone and its interaction with the protein's active site compared to the positive control and antagonist.

Table 2. The binding energy and RMSD values between the $5HT_{3A}$ target and the molecules analyzed.

Ligands	ΔG_{bind} (kcal/mol)	RMSD (Å)
AMT	−9.8	1.207
Flx	−7.7	1.226
Gstn	−8.3	1.304

AMT—amentoflavone; Flx—fluoxetine; Gstn—granisetron; RMSD—root-mean-square deviation.

The visualization in Figure 12 of these interactions, where amentoflavone fits into the active site of the GABA protein, reinforces the possibility of targeted and specific action. Furthermore, the analysis of the values presented in Table 3 is remarkable. The ΔG of −9.8 kcal/mol and the RMSD of 1.177 demonstrate that amentoflavone maintains an extremely stable interaction with the active site of the $GABA_A$ protein. This stability may indicate amentoflavone's ability to effectively influence the activity of the GABA protein, which plays a crucial role in modulating anxiety.

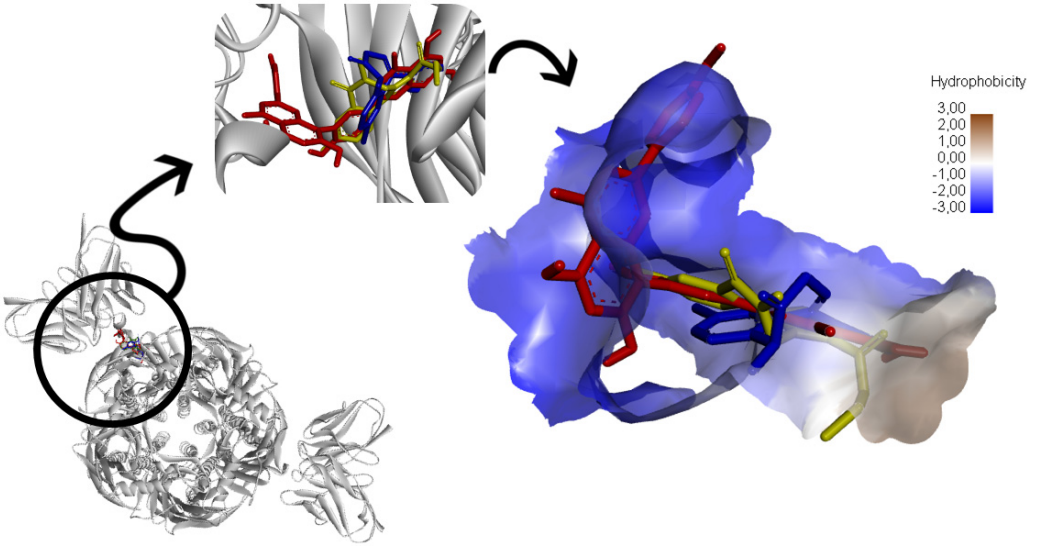


Figure 12. Representation of AMT (red), DZP (blue), Fmz (green) on $GABA_A$ target.

Table 3. The binding energy and RMSD values between the $GABA_A$ target and the molecules analyzed.

Ligands	ΔG_{bind} (kcal/mol)	RMSD (Å)
AMT	−9.8	1.177
DZP	−6.3	1.250
Fmz	−6.4	1.994

AMT—amentoflavone; DZP—diazepam; Fmz—flumazenil; RMSD—root-mean-square deviation.

4. Discussion

The open field test is commonly used to evaluate sedative effects and drug-related behavior in murine models [40], and it has been adapted to aZF [41]. According to Benneh et al. [27] and Gupta et al. [42], diazepam (sedative control), a benzodiazepine, attenuates the locomotor activity (mobility) of aZF in the open field. In this work, aZF treated with AMT showed the same effect as aZF treated with diazepam in the open field test (Figure 3), suggesting a possible sedative effect of AMT. The absence of a reduction in the locomotor activity of aZF induced by the biflavonoid AMT indicates the lack of a sedative effect of the sample. The sedative effect can be compared to the effects of benzodiazepines (anxiolytic drugs; diazepam), which decrease the locomotor activity (mobility) of adult zebrafish (*Danio rerio*) in open field tests, as highlighted by Benneh et al. [27], Gupta et al. [43] and Lin et al. [28]. In this context, we investigated the possible anxiolytic-like effect and the potential treatment of anxiety induced by the alcohol withdrawal of this dietary supplement.

Among the various tests to assess anxiety in adult zebrafish, the Light and Dark Test is one of the most commonly used tests, since it is based on the paradigm of the innate aversion of zebrafish to well-lit areas, similar to that of rodents. In such a test, the animals not treated with anxiolytic drugs present the same behavior presented in mice, having an aversion to light zones, as indicated by Gebauer et al. [25] and Maximino et al. [44]. In this context, we also used the same animal model to investigate the anxiolytic-like effect of the natural product AMT. In this study, all concentrations of AMT increased the time the animals remained in the illuminated area of the aquarium, indicating an anxiolytic-type effect (Figure 4). The use of this model corroborates the same results reported by other researchers [27].

Cyproheptadine (Cypro) is an antagonist of the serotonergic system $5\text{-}HT_{2A}$. It antagonizes the effects of fluoxetine (Flx), used as a positive control [27]. In this study, the non-reversal of the anxiolytic-like effect of AMT by pretreatment with cyproheptadine suggests that the anxiolytic effects of AMT are independent of the serotonergic receptor

5-HT_{2A} (Figure 5). Pizotifen is a non-selective antagonist of serotonergic systems 5-HT_1 and 5-$HT_{2A/2C}$. It antagonizes the effects of fluoxetine (Flx) used as a positive control [27]. In this work, the non-reversal of anxiolysis by pretreatment with pizotifen suggests that the anxiolytic effect of the biflavonoid AMT is not dependent on serotonergic 5-HT_1 and 5-$HT_{2A/2C}$ receptors. (Figure 6). Granisetron (Gstn) is an antagonist of the serotonergic 5-$HT_{3A/3B}$ system. It antagonizes the effects of fluoxetine (Flx) used as a positive control [27]. In this work, the reversal of anxiolysis by pretreatment with Gstn suggests that the anxiolytic effect of the biflavonoid AMT is dependent on serotonergic receptors 5-$HT_{3A/3B}$ (Figure 7). Flumazenil is an antagonist of benzodiazepine action at $GABA_A$ receptors, thus inactivating the anxiolytic effects, sedation and hypnosis of benzodiazepines such as diazepam [28,45]. In this work, the reversal of anxiolysis by pretreatment with flumazenil suggests that the anxiolytic effect of the biflavonoid AMT is dependent on the $GABA_A$ receptor (Figure 8). This study corroborated the same results obtained from our previous studies, such as Ferreira et al. [14] who reported the anxiolytic-like effect of synthetic chalcone, as well as Silva et al. [46] who reported that *Combretum lanceolatum extract* reversed anxiety on aZF with the participation of the GABAergic system.

The GABAergic system is related to the production and action of the GABA neurotransmitter, which is one of the main inhibitory neurotransmitters in the brain. The activation of $GABA_A$ and $GABA_B$ receptors promotes the hyperpolarization of nerve cells, reducing neuronal excitability and causing a calming and anxiolytic effect [47]. On the other hand, the $5HT_{3A/3B}$ system refers to a specific class of serotonin type 3 ($5HT_3$) receptors found in the central and peripheral nervous system. Serotonin is another important neurotransmitter, which plays a crucial role in regulating mood, sleep, appetite and other brain functions. $5HT_{3A/3B}$ receptors are involved in modulating the effects of serotonin in the brain. Studies have shown that the activation of $5HT_3$ receptors may be associated with anxiety induction, while the inhibition of these receptors may have an anxiolytic effect [48]. On the other hand, the activation of the GABAergic system, enhancing GABA action, has a well-established anxiolytic effect [49].

Adult zebrafish have been used to investigate the potential of new alternative drugs for the treatment of alcohol withdrawal-induced anxiety [14,46,50]. In this context, we employed the same methods to assess the potential of AMT during alcohol withdrawal-induced anxiety in aZF. As expected, all doses of AMT were effective in treating the anxiety-like behavior in adult zebrafish induced by alcohol withdrawal on the 11th day of treatment, a significantly similar effect to that of diazepam (Figures 9 and 10). Our results corroborate the same results as from Ferreira et. al. [14], da Silva et al. [46] and Marques et al. [50].

Amentoflavone shares the same active site with these compounds, which suggests the possibility of similar or even superior action. However, the most evident aspect was the stability of this interaction. We observed that amentoflavone exhibited a more stable interaction compared to the positive control at the active site of the protein, as evidenced by the ΔG value of -9.8 kcal/mol. This value suggests a strong bond between amentoflavone and the target protein, which is a significant indication of its potential efficacy as an anxiolytic agent. Furthermore, the RMSD value of 1.207 also demonstrated that amentoflavone maintains a conformation relatively close to that of the active site, which is fundamental to ensure the effectiveness of the interaction.

For the three substances, the presence of moderate (3.1 Å to 3.55 Å) and weak (>3.55 Å) interactions stood out [27] from the type of hydrogen bonds in AMT (six) and Flx (one). Gstn, like the other substances, will have Van der Waals bonds and π-type interactions, which are weaker and more unstable than the first one. Here, we can highlight the interactions performed with the amino acid ASN101. All substances interacted with this residue, favorably ($Gstn_{hb}$ and Flx_{vw}) or unfavorably ($AMT_{d\text{-}d}$), and with interaction forces of a greater (Gstn) or smaller (Flx) domain. Further regarding the forces of interaction, we believe the phenomenon that occurred in vivo (depicted in Figure 7) reversed the effectiveness of Gstn (antagonist), Flx and AMT.

The observation of the interactions of amentoflavone with the $GABA_A$ protein in in silico tests brings intriguing revelations and suggests a remarkable potential of this substance in the context of anxiety treatment. The similarity in the binding of amentoflavone to the same active site occupied by diazepam (positive control) and flumazenil (antagonist) on the $GABA_A$ protein is particularly interesting and may provide valuable clues about its anxiolytic effects. The in silico evaluation confirmed the data found in vivo, and these results reinforce the hypothesis that amentoflavone not only has the ability to interact with the active site of the protein but also to do so in a substantially more stable way compared to the reference compounds. This greater stability may play a key role in its effectiveness as an anxiolytic agent. Therefore, evidence from molecular modeling provides a promising scientific basis for a further investigation and the potential development of amentoflavone as a treatment for anxiety.

The relationship between these systems is still not completely understood, but some evidence suggests they can interact and influence each other. For example, the activation of $5HT_{3A/3B}$ receptors can modulate GABA release in certain areas of the brain, affecting the activity of the GABAergic system [46].

However, it is important to emphasize that anxiety is a complex and multifaceted phenomenon, involving several neurotransmitter systems and neural pathways. The interaction between the GABAergic and $5HT_{3A/3B}$ systems is just one of several neurochemical pathways involved in the regulation of anxiety. Having a compound with such specificity as amentoflavone will allow for carrying out more focused studies for the development of new anxiolytic treatments. In addition, AMT has been shown to be effective in reversing anxiety induced by alcohol withdrawal, thus confirming its action on the GABAergic system. The results obtained corroborate those in the literature that have already investigated the anxiolytic activity of amentoflavone via the GABAergic pathway in mice [51] and through the modulation of mTOR signaling [20] in rats.

These findings are promising for the search for new therapeutic approaches in the treatment of anxiety and may provide important insights into the interaction between different neurotransmitter systems in the brain. Understanding the neurochemical pathways involved in anxiety is crucial for the development of more effective therapies with fewer side effects.

5. Conclusions

The data obtained in the present study revealed that the natural biflavonoid amentoflavone (AMT) isolated from the leaves of *Ouratea fieldingiana* was not toxic and did not present a sedative to aZF. AMT had specific anxiolytic effects through the serotonergic $5\text{-}HT_{3A/3B}$ system, as well as GABAergic. It is noteworthy that the anxiolytic effect of AMT was also evidenced in the in silico study, and the anxiolytic effect of AMT through the GABAergic system was confirmed through the treatment of anxiety induced by alcohol withdrawal. The absence of toxicity and such actions of these natural products in the central nervous system demonstrate their pharmacological potential and thus insights into the development of new anxiolytic drugs. Amentoflavone, as a compound with specific activity on the GABAergic system, may represent a promising therapeutic option for the treatment of anxiety and may also be an alternative for the management of alcohol withdrawal symptoms. However, more research is needed to confirm its effectiveness and safety in humans. In the context of growing interest in natural and alternative medicines for anxiety disorders, amentoflavone represents a possible source of new anxiolytic compounds. Continuing studies in this area could lead to significant advances in the treatment of anxiety disorders and contribute to a better quality of life for those who suffer from these conditions.

Author Contributions: The authors declare individual contributions to the article as follows: data collection, analysis and the preparation of the manuscript: L.S.F.: conceptualization; writing—original draft and writing—review and editing; W.M.B.d.S.: writing—original draft; D.R.A.: methodology; S.A.A.R.S.: methodology; G.A.d.N.: methodology; F.E.A.M.: conceptualization; data curation; formal analysis; supervision and writing—review and editing; A.R.C.: funding acquisition; supervision; validation and writing—review and editing; S.M.d.M.: conceptualization; funding acquisition and writing—review and editing. The completed manuscript underwent comprehensive review by all authors, resulting in approval for publication. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This work was approved by the Ethics Committee on the Use of Animals of State University of Ceará (CEUA-UECE; no. 05299177/2021) following the Ethical Principles of Animal Experimentation.

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Conflicts of Interest: The authors declare no conflicts of interest.

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