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Research Article

A Mixture of α, β-amyrin Causes Anxiolytic, Sedative, and Anticonvulsant Behavior without Impairing Memory in Adult Zebrafish

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Abstract

The isomeric mixture of α- and β-amyrin isolated from Protium heptaphyllum has considerable pharmacological effects, mainly with an effect on the central nervous system. Still, no reports in the literature demonstrate its impact on zebrafish. This study evaluated the anxiolytic, sedative, anticonvulsant and memory effects of the isomeric mixture of α- and β-amyrin (ABAM) isolated from Protium heptaphyllum. ABAM was submitted to light-dark tests, seizures induced by pentylenetetrazol and inhibitory avoidance induced by electroshock in adult zebrafish. ABAM showed sedative, anxiolytic and anticonvulsant effects,preventing memory in adult zebrafish via GABAergic neurotransmission. However, ABAM has pharmacological potential for the treatment of anxiety and seizures while preserving memory, which can be explained by an interaction with the GABAA receptor.

INTRODUCTION

Diseases such as anxiety, insomnia, depression, epilepsy, dementia and chronic pain are disorders of the central nervous system (CNS) that are increasingly frequent worldwide. They are the main neuropsychiatric disorders, which tend to coexist, making their treatment even more difficult diagnosis and treatment [1]. According to the World Health Organization (WHO), approximately 264 million people suffer from anxiety disorder and epilepsy affects about 50 million people worldwide [2].

Benzodiazepines (BZs) are still widely used to treat anxiety disorders and seizures, they act on $GABA_A$ receptor subunits (mammalian neuroinhibitory receptor) at the benzodiazepine binding site, which generate allosteric modulations that potentiate the action of the gamma-aminobutyric acid neurotransmitter (GABA) on $GABA_A$ receptors [1], contributing to an increase in the frequency of opening of chloride channels, causing CNS neuroinhibition [3].

Several mechanisms of BZs are attributed to their side effects, including sedation, myorelaxation and amnestic effects if used as anxiolytics [4]. In addition, BZs have been reported to induce temporary anterograde amnesia by affecting the first stage of the memory process (coding of new information) [5,6]. The binding region of BZs in $GABA_A$ receptors involved in memory is in the CA1 region of the hippocampus [7]. In addition, BZs

cause physical and mental dependence, so rebound effects and significant withdrawal symptoms arise if treatment interruption occurs [8].

Animal models are widely used in the development of new drugs for the treatment of neuropsychiatric disorders. For example, the zebrafish (Danio rerio) has preserved neurotransmitters and a fully sequenced genome, corresponding to more than 80% of orthologous genes related to human diseases [9], expressing all $GABA_A$ and $GABA_B$ receptor genes, as well as the genes that encode the associated proteins to the $GABA_A$ receptor [10], so it is an efficient model in studies of diseases related to GABAergic neurotransmission.

Protium heptaphyllum is widespread in Brazilian territory and popularly used to treat several diseases. The isomeric mixture of α- and β-amyrin are the pharmacologically active triterpenes isolated from this species [11]. Previous studies have evidenced the sedative, anxiolytic, antidepressant and anticonvulsant activities of the mixture of α- and β-amyrin, possibly involving both the GABAergic and noradrenergic systems in mice [11,12]. In other studies, α, β-amyrin caused a periodontal anti-inflammatory effect in a rat model of ligature-induced periodontitis [13] and exhibited long-lasting antinociceptive and anti-inflammatory properties in models of persistent nociception via activation of cannabinoid receptors and by inhibition of cytokine production and expression of NF-κB and cyclooxygenase 2 [14,15]. However, this work investigated the anxiolytic, sedative, anticonvulsant activity and effect on memory in adult Zebrafish of the isomeric mixture of α- and β-amyrin isolated from P. heptaphyllum.

MATERIAL AND METHODS

Drugs and reagents

In this study, Diazepam (DZP, Neo Química®), Flumazenil (Fmz; Sandoz®), Dimethyl sulfoxide (3% DMSO; Dynamic®) and Pentylenetetrazole (PTZ, Sigma-Aldrich) were used.

Obtaining a mixture of α- and β-amyrin

The isomeric mixture of α, β-amyrin [Figure 1] was isolated from P. heptaphyllum resin. The resin fractionation (20 g) was carried out by chromatography on a silica gel column with hexane, chloroform, ethyl acetate and methanol. Fractions extracted with chloroform (5.2 g) were repeatedly chromatographed on silica gel and eluted with increasing amounts of hexane-ethyl acetate. Fractions obtained from hexane: acetate [1:1] were analyzed by TLC and contained 450 mg of alpha and beta-amyrin [16].

Animals and Maintenance

Wild adult zebrafish (Danio rerio) (age 90 to 120 days; 0.4 ± 0.1 g, 3.5 ± 0.5 cm) of both sexes (approximately 50:50 male to female ratio) were purchased at a local store (Fortaleza, CE). Before the experiments, the fish were kept for at least two weeks in a glass aquarium (30 × 15 × 20 cm) of 10 L (n = 3/L), at a temperature of 25 ± 2°C, in light-dark cycles of 24 hours with chlorinated water (ProtecPlus®) and air pump with submerged filters, the temperature of 25°C and pH 7.0, circadian cycle of 10–14 h (light/dark), fed twice a day with commercial feed in flakes (Alcon BASIC™, Alcon, Brazil) up to 12 hours before the experiments. For anesthesia, the animals were anesthetized in ice water before drug applications, orally or intraperitoneally. After the experiments, the animals were euthanized by immersion in ice water (0 and 3°C) for 1 min until the loss of opercular movements. All protocols used in this work were approved by the Ethics Committee on Animal Use of the State University of Ceará (CEUA-UECE; No. 04983945/2021), following the Ethical Principles of Animal Experimentation.

General protocol: Adult zebrafish were randomly selected in the experiments, transferred to a damp sponge, and treated with 20 µL of the test sample (mixture of α, β-amyrin - ABAM at doses of 4 mg/kg, 20 mg/kg and 40 mg/kg) or controls (3% DMSO) orally (p.o.) using an automatic pipette. Then, the animals were placed individually in a container (500 mL) containing 350 mL of aquarium water and kept at rest for 1 h until the experiments.

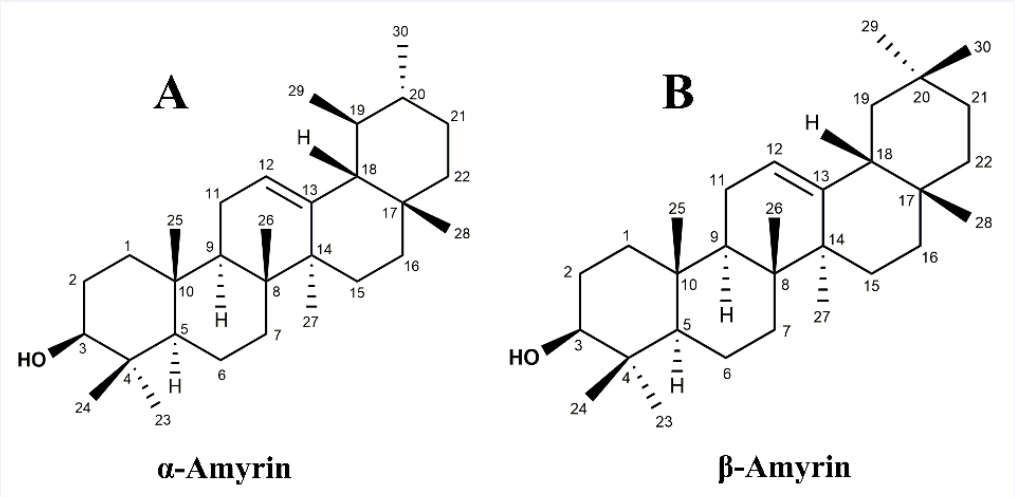

Figure 1 Molecular structures of α, β-amyrin compounds: (A) α-amyrin (3α-hydroxyurs-12-ene) and (B) β-amyrin (3β-hydroxyolean-12-ene).

Light-dark test: Anxiety behavior in zebrafish was observed using a light/dark test according to the protocol developed by Gebauer et al. [17], with adaptation. The experiment was carried out in a glass aquarium (30 cm × 15 cm × 20 cm) divided into white areas (covered with white matte adhesive paper) and another black area (covered with black adhesive paper) without the entry of light. The aquarium was filled with tap water without chlorine, with a water level of 3 cm, which simulated a new shallow environment different from the conventional aquarium and capable of inducing anxiety behaviors. Adult fish (n = 6/group) were administered orally with ABAM at 4; 20 or 40 mg/kg doses. Negative and positive control groups consisted of 3% DMSO and 1 mg/kg DZP solution, respectively. After 1 h, each fish was placed in the light zone of the aquarium for 60 s to provide acclimatization. After that, the barrier that divided the two sides were opened, allowing free circulation in the tank for another 300 s, and data were collected by trained evaluators at mowing. The following parameters evaluated the anxiolytic effect: a) the time spent by each animal on the light side suggests less anxiety; b) the latency time to pass from the light side to the dark side, indicating a sedative effect; c) the number of crossings between both sides, investigating locomotor impairment. Each group was tested, followed by the previous one.

Assessment of GABAergic neuromodulation: The mechanism of action involved in the anxiolytic effect of ABAM was identified through pre-treatment with flumazenil (a GABAA receptor modulator that acts in the same region as Diazepam) [18]. Adult zebrafish (n = 6/group) were pretreated with flumazenil (4 mg/kg; 20 µL; intraperitoneally - i.p.), and after 15 min, the lowest effective dose of ABAM (4 mg/kg; 20 µL; p.o.) found in the pilot test (see previous section); One group was treated with 3% DMSO (vehicle; 20 µL; p.o.) and used as a negative control, and DZP (1 mg/kg, 20 µL; p.o.) and was used as a $GABA_A$ receptor agonist. After 1 h of treatment, the animals were submitted to the light/dark test as described in the previous section, and the time spent in the light region of the aquarium (anxiolytic behavior), latency time and crossing from the light side to the dark side during 300 s was observed.

PTZ-induced convulsive behavior at 10 mM: The reversal of PTZ-induced seizures was investigated in this study. Animals (n = 6/group) were orally treated with ABAM at doses of 4; 20 or 40 mg/kg, DZP (1 mg/kg; 20 µL; p.o.) or negative control - vehicle (3% DMSO; 20 µL; p.o.). After 1 h, the animals were individually exposed to 10 mM PTZ, dissolved in water in a 250 mL beaker, and the convulsive behavior was evaluated in the three stages: stage I - increased swimming; stage II - swirling behavior; and stage III - clonus-like seizures, (loss of posture when the animal falls to the side and remains motionless for 1-3 s). At the end of the evaluation of the three test stages, the animals were euthanized on ice [19]. The mechanism of action was further evaluated.

Inhibitory avoidance task: The assessment of ABAM in inhibitory avoidance was performed as described by Bertoncello et al [20]. The apparatus consisted of a glass tank (28 cm long x 14.7 cm wide x 19 cm high) filled with 1.3 L of non-chlorinated water. The tank was divided into two equal compartments (black and white) separated by a manually operated guillotine-type partition (10 × 10 cm). The black compartment contained three pairs of metal bars (1 cm in diameter) spaced 3 cm apart and connected to an electro-stimulator. To cause the aversive stimulus, the fish received a pulsed shock of 100 Hz for 5 s. Animals (n = 6 animals per group) previously separated individually into 500 mL pots and identified were submitted to the training session, in which the fish were placed individually in the white compartment of the device. After 1 min of acclimatization, the guillotine door was lifted, and each animal's latency time to enter the black area was recorded. After the fish crossed the dark compartment, the door was lowered, and a mild electric shock (125 mA, 3 ± 0.2 V) was delivered. Then, the fish were removed from the tank and orally treated (n = 6 fish per group) with ABAM in three doses (4; 20 or 40 mg/kg, one dose for each group), another group treated with DZP (4 mg /kg, p.o.) and another with 3% DMSO (negative control; drug diluent). The test session was performed after 24 hours, similarly to the training session, but without applying an electric shock.

Statistical analysis

Data were analyzed using Graphpad-Prism software version 8.0. After confirmation of normal distribution and data homogeneity, differences between groups were submitted to analysis of variance (One-Way ANOVA) and Two-Way ANOVA in experiments with antagonists, followed by Tukey's test. The level of statistical significance was set at 5% ($p < 0.05$).

RESULTS

Light-dark test

ABAM at the three doses tested and Diazepam significantly reduced anxiety (****$p<0.0001$ vs. Control) in adult zebrafish, as the time spent in the clear region of the aquarium increased significantly when compared to animals in the negative control group [Figure 2A]. In addition, the evaluated doses of ABAM did not change the latency time [Figure 2B] and the number of crossings between the light and dark sides [Figure 2C] when compared to the animals in the control group, since there was no significant difference, suggesting that there were no changes in the locomotor activity and exploratory capacity of the fish, unlike the group treated with Diazepam, which had an increase in latency (***$p<0.001$ vs. Control) and a reduction in the number of crossings, indicating the sedative effect and motor impairment of Diazepam.

Assessment of GABAergic neuromodulation

The involvement of ABAM with the $GABA_A$ receptor was assessed by pre-treatment with flumazenil (a $GABA_A$ receptor benzodiazepine antagonist). As a result, it was identified that flumazenil blocked (# # # # $p<0.0001$ vs. ABAM) the anxiolytic and sedative effect of ABAM (4 mg/kg), reducing the time spent in the clear area of the aquarium and latency, similar to what happened with the group pre-treated with flumazenil and DZP

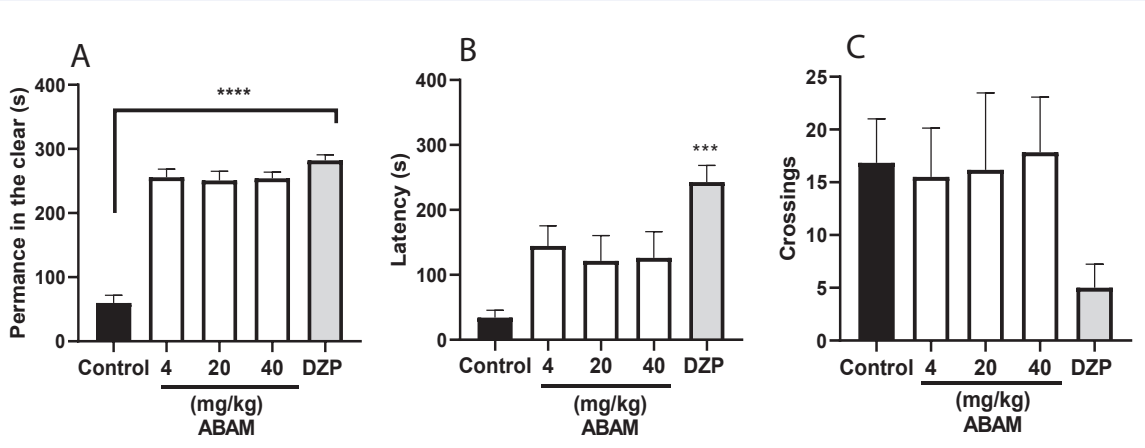

Figure 2 Effect on ABAM in the light-dark test (0 –5 min). (A) Staying in the light; (B) Latency; (C) crossing from light to dark. Control group (3% DMSO); DZP – Diazepam (1.0 mg/kg; 20 µL; p.o.). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Turkey's test (*** $p < 0.001$; **** $p < 0.0001$ vs. Control).

(1 mg/kg), which also had the time spent in the light region and latency decreased (# # # #$p<0.0001$, # # # $p<0.001$ vs. DZP) [Figure 3A and B]. Furthermore, flumazenil pre-treatment restores locomotion in the DZP-treated group ($p>0.05$) [Figure 3C].

PTZ-induced convulsive behavior at 10 mM

Higher doses of ABAM reversed PTZ-induced seizure behavior in all three stages (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. Control), an effect similar to that of DZP, which also significantly (****$p < 0.0001$ vs. Control) delayed onset of seizures in all three stages when compared to Control treatment [Figure 4].

Inhibitory avoidance task

The inhibitory avoidance caused by electroshock indicated that the doses of 4 and 20 mg/kg of ABAM retained the memory (*$p < 0.05$ vs. Training) of the Zebrafish in the test session, unlike the group treated with Diazepam that had memory retention impaired, as the latencies to enter the dark compartment between the training and test sessions did not differ [Figure 5A]. Furthermore, there were no significant differences in the retention index between the groups. However, it was observed that the group treated with Diazepam had reduced performance in the test session ($p > 0.05$) [Figure 5B].

DISCUSSION

Although studies on the isomeric mixture of α- and β-amyrin with anxiolytic, sedative and anticonvulsant effect in mice have been studied [11,12], this is the first report of its effects on anxiety, seizure and memory retention in adult zebrafish.

The light-dark paradigm is a commonly used preclinical test for anxiolytic drug screening [18-21]. Anxiolytic drugs such as benzodiazepines are reported to increase the time of adult zebrafish in the light region of the aquarium, increase the latency to enter the dark area (sedation behavior) and decrease the number of crossings from light to dark area in the test [22–25]. This study identified that ABAM causes anxiolytic and sedative behavior without impairing the locomotor activity of the animals, unlike Diazepam, which, despite causing anxiolytic and sedative effects, compromises the locomotion of the fish [22–24, 26]. Furthermore, the anxiolytic and sedative effect of ABAM has already been identified in mice through GABAergic neurotransmission through the same binding site of Benzodiazepines [12], corroborating to the results.

Sedative and anxiolytic drugs, such as Benzodiazepines, increase the action of gamma-aminobutyric acid (GABA) on the $GABA_A$ receptor. Therefore, compounds capable of combating anxiety and causing sedation can act through GABAergic neurotransmission in the same region of action of benzodiazepines on the $GABA_A$ receptor. For this investigation, it is possible to use flumazenil, a competitive antagonist of Benzodiazepines commonly used in the clinic in cases of overdose and preclinical studies of new compounds with anxiolytic and sedative effects [22,23-26]. A high-resolution cryoelectron microscopy study indicates that Diazepam and flumazenil bind to the same benzodiazepine binding pocket on $GABA_A$ receptors but use different modes. Diazepam binds to the α1β3γ2 subunit and flumazenil to α1β2γ2 [27]. Thus, compounds whose action is blocked by flumazenil can act in the same region as Diazepam. Therefore, in this study, pre-treatment with flumazenil was used and blocked the anxiolytic and sedative effect of ABAM, increasing the time of the animals in the dark area and drastically reducing the latency, the same occurring with the animals treated with Diazepam, in addition, flumazenil preserved the locomotor activity of fish treated with Diazepam, similar to what happened with Zebrafish larvae in which Diazepam-induced hypolocomotion (sedation-like state) was effectively antagonized by flumazenil .

The evaluation of the anticonvulsant effect of ABAM in the PTZ model has already been investigated after oral and intraperitoneal administration and after acute or subchronic treatments in mice

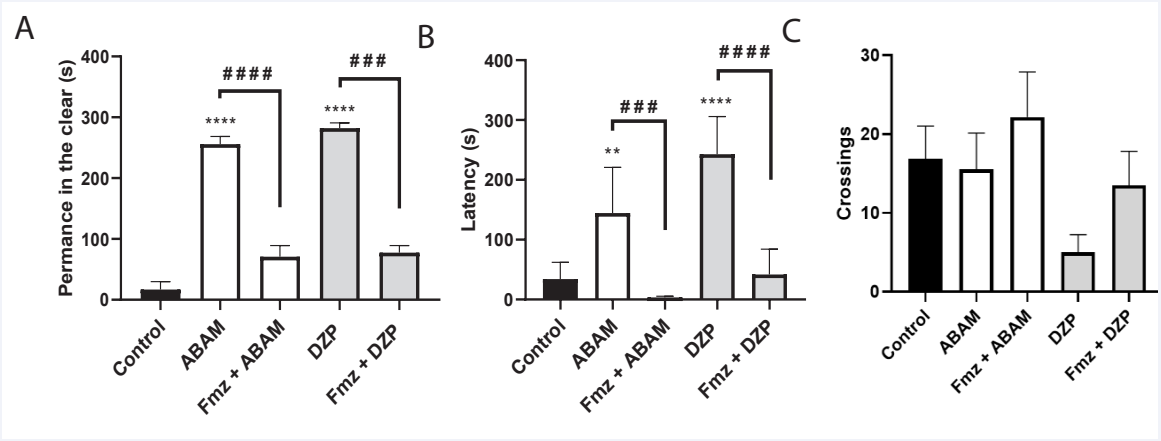

Figure 3 Effect of flumazenil on anxiolytic behavior induced by ABAM (4 mg/kg; 20 µL; p.o.) in the light-dark test (0 –5 min). (A) Staying in the light; (B) Latency; (C) crossing from light to dark. Control group (3% DMSO); DZP – Diazepam (1.0 mg/kg; 20 µL; p.o.). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Turkey's test (*** $p < 0.001$; **** $p < 0.0001$ vs. Control. # # # $p < 0.001$; # # # # $p < 0.0001$ vs. ABAM or DZP).

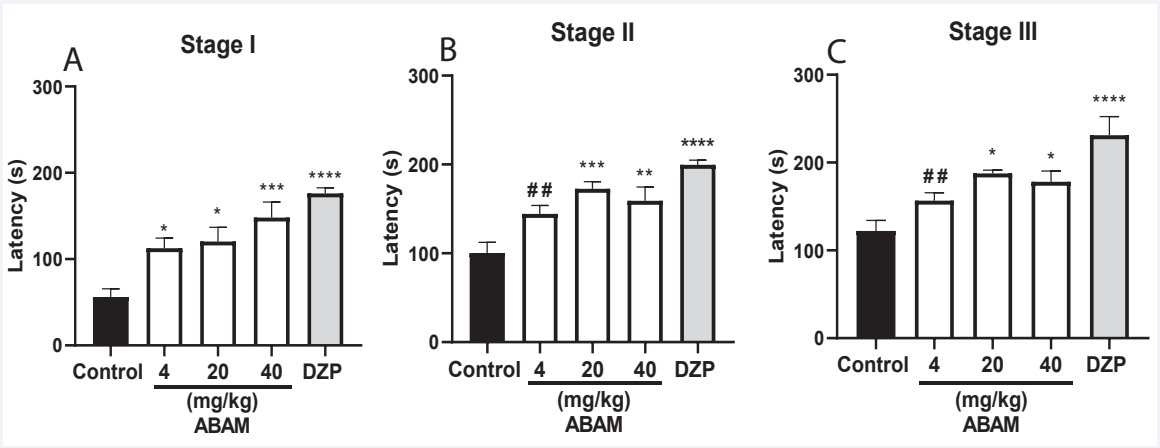

Figure 4 Effect of ABAM (p.o.) on seizure induced by pentylenetetrazole (0 mM) in adult zebrafish, Stage I (A), Stage II (B), Stage III (C). Control group (3% DMSO); DZP – Diazepam (1.0 mg/kg; 20 µL; p.o.). Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Turkey's test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. Control. # # $p < 0.01$ DZP).

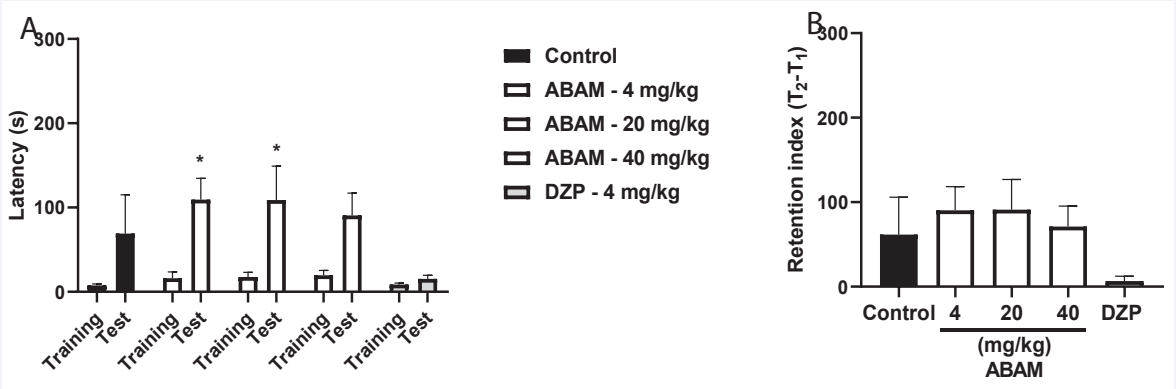

Figure 5 Effect of ABAM (p.o.) on the aversive memory of adult zebrafish tested in the inhibitory avoidance task. (A) Latency to enter the dark area in training and testing sessions. (B) Memory retention rates. Control group (3% DMSO); DZP – Diazepam (4 mg/kg; 20 µL; p.o.). Values represent the mean ± standard error of the mean for 6 animals/group; Two-Way ANOVA followed by Turkey's test (*$p < 0.05$ vs. Control).

[11]. The anticonvulsant effect of ABAM was evidenced by a dose-dependent increase in the latency to enter seizures having the maximum effect at a dose of 25 mg/kg. Furthermore, subchronic treatment increased the concentrations of taurine (116 and 76%) and tyrosine (135 and 110%) in the basal ganglia and hippocampus, respectively, and decreased by 68, 65 and 62% of glutamate, aspartate and GABA in the basal ganglia in the brain of mice, indicating that the anticonvulsant effect of ABAM occurs by balancing inhibitory and excitatory neurotransmitters. In this study, ABAM also caused a neuroprotective effect by blocking seizures caused by PTZ at 10 mM, similar to that obtained with Diazepam, having a peak of action at a dose of 20 mg/kg, delaying the latency to enter the three seizure stages, confirming that a dose of 20 mg/kg is sufficient to abolish acute seizures as previously observed [11]. Furthermore, Diazepam causes an anticonvulsant effect when interacting with the α1, α2 and α5 subunits of the $GABA_A$ receptor [29]. Therefore, the anticonvulsant effect of ABAM is possibly related to its interaction with these subunits.

$GABA_A$ receptors containing the α5 subunit have a restricted distribution in the brain, expressed mainly in the dendritic fields of the hippocampus, where they represent approximately 20% of $GABA_A$ receptors [30], This location suggests that α5 may be involved in the physiological processes underlying learning and memory [31]. This study investigated the effect of ABAM on memory retention using the electroshock-induced inhibitory avoidance test. It was observed that ABAM preserved the animals' memory, which was not identified in the group treated with Diazepam. Furthermore, neurobehavioral and in silico studies indicate that the impairment in memory and learning caused by Diazepam is related to an interaction with the amino acid His101 of the α1 receptor on the $GABA_A$ receptor and that compounds with an anxiolytic effect that does not cause these symptoms do not interact with this amino acid residue [32], indicating that ABAM causes anxiolytic, sedative and anticonvulsant effects without compromising memory and learning because it does not bind with His101 and has an affinity with the α5 subunit of the $GABA_A$ receptor.

CONCLUSION

The isomeric mixture of α- and β-amyrin has anxiolytic and sedative activity without causing motor impairment and an anticonvulsant effect that attenuates tonic-clonic seizures in adult Zebrafish. The effects of ABAM are possibly related to GABAergic mechanisms, acting in the same binding region of benzodiazepines. These findings confirm the relevance of ABAM as a potential target for developing new treatments for central nervous system disorders.

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