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RESEARCH ARTICLE

Robinin Isolated From Solanum Asperum Exhibits Pharmacological Actions in the Central Nervous System of Adult Zebrafish (*Danio rerio*)

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Keywords: anxiety | flavonoid | learning | neuroprotective | seizure

ABSTRACT

This study investigated the anxiolytic, anticonvulsant and memory preservation effects of the flavonoid robinin. The compound, administered at doses of 4, 20 and 40 mg/kg, did not show toxicity after 96 h of monitoring. In behavioural experiments with zebrafish, robinin did not cause significant changes in motor functions, but it impairs locomotor activity and demonstrates anxiolytic properties, evidenced by the increase in the time spent in the clean zone of the protector. A minimum effective dose (4 mg/kg) was blocked by flumazenil (FMZ), providing interaction with GABAA receptors and decreasing an anxiolytic profile similar to that of diazepam, without causing sedation. In addition, a dose of 40 mg/kg was able to reverse seizures, increasing the latency to enter the seizure stages, an effect that was also blocked by FMZ. Robinin (40 mg/kg) also prevented memory variation in an inhibitory avoidance test. In silico absorption, distribution, metabolism and excretion tests indicated that robinine presents gradual intestinal absorption and low distribution in the central nervous system. In molecular docking, the compound was exposed in the layer with CAII and GABAA receptors, corroborating the anxiolytic and anticonvulsant effects. The results suggest that robinine has therapeutic potential in the treatment of anxiety and seizures, in addition to offering memory protection, representing an advantageous alternative to benzodiazepines, with a promising neuroprotective potential for the pharmaceutical industry.

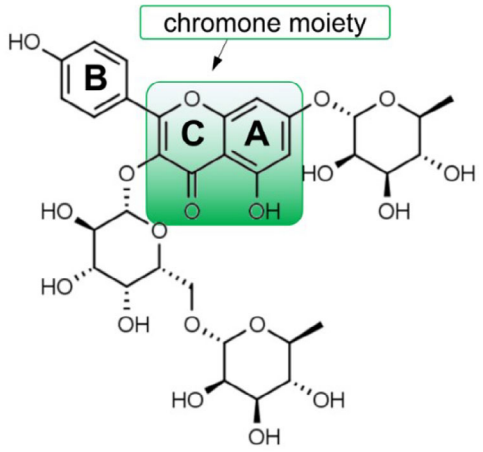


FIGURE 1 | Two-dimensional chemical structure of Robinin.

1 | Introduction

Mental disorders are becoming increasingly important, especially after the intensification of these diseases following the coronavirus disease 2019 pandemic, especially generalized anxiety disorder (GAD), which has symptoms such as trembling, sweating, blurred vision and mental confusion [1]. GAD is also common in people with epilepsy; about 15%–25% of people with epilepsy have some degree of anxiety disorder [2]. The extreme symptoms of epilepsy can result from the chemical imbalance in the central nervous system (CNS), which is characterized by a loss of control over its actions, resulting in convulsive movements of varying intensity that affect both the body and the face [3].

The class of benzodiazepines (BZDs) is used to treat and alleviate the symptoms of these conditions, but most derivatives have debilitating adverse effects, as these drugs induce sedation, promote a state of lethargy and impair patients' mental performance, including memory, causing recent memory loss and, in the long term, severe memory damage [4]. Because of the harmful consequences associated with BZDs, medical science is increasingly looking for compounds that don't pose as many risks to patients [5].

Natural products have been extensively studied since the dawn of ancient civilizations to find new biologically active compounds for the treatment of diseases [6], and research has demonstrated their properties, making them potential drugs due to their great abundance and accessibility [7]. Flavonoids are compounds isolated from plants, which stand out as natural products due to their diverse therapeutic properties, helping in the treatment and prevention of cardiac, vascular, cancer, atherosclerosis, diabetes, hypertension and gastrointestinal diseases [8]. Robinin (Figure 1) is a flavonoid found in the *Solanum asperum* plant, known as 'russara' or 'coça-coça', which is distributed in tropical regions and is widespread in South America [9]. With therapeutic properties attributed to its polyphenol structure, robinin has become the target of pharmacological studies and can be used in treatments with anti-tumour, anti-inflammatory and anti-ulcerogenic functions [10].

In order to screen new compounds at low doses, without causing sedation and control for anxiolytic and anticonvulsant effects, experiments are carried out in animal models [5]. The zebrafish (*Danio rerio*) has $GABA_A$ receptor genes similar to those of mammals, making it possible to study anxiolytic and anticonvulsant effects [11]. In addition to its genetic homology, the zebrafish is easy to handle, inexpensive and, above all, quick to respond to experiments [12]. The zebrafish's promising results in various studies allow it to be used in many areas, such as embryonic development, toxicology and drug testing, human diseases and tissue regeneration [13].

In addition to in vivo experiments, in silico studies aim to establish molecular interactions with the active sites of neuroreceptors and also to elucidate the pharmacokinetics of new drugs, which helps to understand the absorption of the substance, its chemical species with greater bioavailability and its possible properties [14].

However, this study investigated the anxiolytic and anticonvulsant effects of the flavonoid Robinin isolated from *Solanum asperum* together with its mechanism of action and its effect on memory in adult zebrafish and its effect on memory in adult zebrafish.

TABLE 1 | Evaluation of Robinin toxicity in blue zebrafish during the 96 h analysis.

Sample	Mortality				96h LD50 (mg/kg)/IV
	C	D1	D2	D3	
Robinin	0	0	0	0	> 40
C - Control (DMSO 3%)					
D1 - Dose 1 (4 mg/kg)					
D2 - Dose 2 (20 mg/kg)					
D3 - Dose 3 (40 mg/kg)					

2 | Results and Discussion

2.1 | Acute Toxicity 96 h

Robinin administered to groups of adult zebrafish at doses of 4, 20 and 40 mg/kg (20 μL; *i.p.*) showed no toxicity to the animals (Table 1), as no fish died during the 96 h observation period. This study confirms the preclinical safety of Robinin and allows further research.

2.2 | Assessment of Locomotor Activity

One-way analysis of variance (ANOVA) showed that all doses of Robinin (4, 20 and 40 mg/kg) altered the animals' swimming (***$p < 0.001$ and *$p < 0.05$ vs. vehicle) and reduced locomotor activity (Figure 2), but there was no severe motor impairment characteristic of the sedated state as observed in the diazepam (DZP)-treated group (##$p < 0.001$ and ##$p < 0.01$ vs. DZP). It is possible to observe patterns in the behaviour of zebrafish as they show changes in their swimming behaviour according to the stimuli they are exposed to in a controlled experimental environment [15]. Sedatives such as DZP cause a drastic reduction in locomotion in adult zebrafish [16, 17]. In the study by

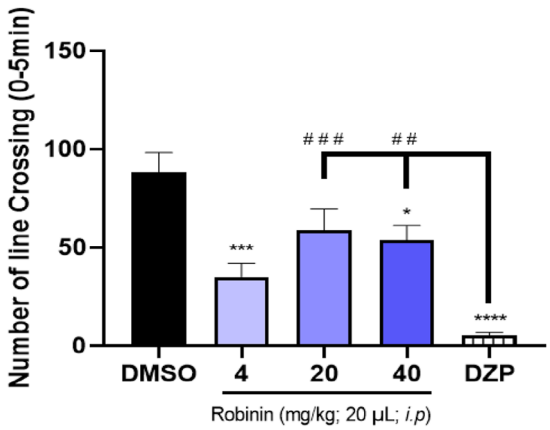


FIGURE 2 | Effect of Robinine on Adult Zebra Fish Locomotion. Values represent the mean ± standard error of the mean for six animals/group; analysis of variance (ANOVA) followed by Tukey's test (****$p < 0.0001$, ***$p < 0.001$ and *$p < 0.05$ vs. Vehicle-DMSO (3% dimethyl sulfoxide); $^{###}p < 0.001$ and $^{##}p < 0.01$ vs. Diazepam-DZP).

Thayumanavan et al. [18], flavonoids have effects on the CNS of zebrafish, showing a certain neuroprotective potential that leads to better cognitive performance, which in a rudimentary way explains the change in swimming of the animal, without seriously affecting its ability to move. As observed in our study, Robinin reduces the fish's locomotion without causing symptoms of sedation, indicating its effect on the CNS of adult zebrafish.

2.3 | Anxiolytic Activity

As shown in Figure 3A, all doses of Robinin significantly increased (*$p < 0.05$ and **$p < 0.01$ vs. vehicle) the time zebrafish spent in the clear zone of the aquarium, an anxiolytic behaviour similar to that observed in the DZP-treated group. Studies by Silva et al. [19], and Nachammai et al. [20], demonstrate the anxiolytic activity of flavonoids, the same class of compounds as robinin, in zebrafish. The anxiolytic effect of flavonoids is linked to the antioxidant property of these phenolic compounds present in natural products, where studies have linked the increased production of free radicals in the brain of animal models with a depressive state or with anxiety disorder, leading to an imbalance of redox regulators and an alteration of oxidative homeostasis in brain cells [19]. For this reason, antioxidant natural products are being investigated as an alternative for new treatments of anxiety and epilepsy.

The mechanism of anxiolytic action of Robinin was investigated by pre-treatment with FMZ in adult zebrafish (Figure 3B). Pretreatment with FMZ blocked the anxiolytic effect (##$p <$ 0.01 vs. FMZ + Robinin) of the lowest dose of Robinin, as the animals stayed longer in the dark part of the aquarium, which is an anxiety behaviour. Flumazenil also blocked the anxiolytic effect of DZP (####$p < 0.0001$ vs. FMZ + DZP).

When compared to the studies by Silva et al. [19] and Liu et al. [21], which also evaluated the anxiolytic effects of plant-derived flavonoids, similar results were observed in that FMZ prevented the anxiolytic effects of these flavonoids. Flumazenil is an antagonist of the hypnotic effects of BZDs in the CNS, due to its interaction with the $GABA_A$ receptor in the same binding region as BZDs. Compounds whose anxiolytic effects are blocked by FMZ may therefore exert this effect through GABAergic neuromodulation, as was the case with Robinin.

2.4 | Docking of Anxiolytic Activity to the GABAergic System

The theoretical mechanism of the anxiolytic action of Robinin was analyzed using the binding potential of the compound against the GABAergic system (Figure 4). At the end of the cycle of 20 independent simulations, for the ligand and the DZP control, it was observed that Robinin complexes with the GABAAR receptor between the D and E chains, as well as with natural substrates such as pyranosides (Figure 4A), while the DZP control has its modulation site for this receptor between the E and D chains. Robinin binds to the GABAAR receptor with an affinity energy of approximately -8.134 kcal/mol, indicating that the ligand has optimal specificity for the receptor and forms an energetically favourable ligand-GABAAR complex compared to DZP (-7.24 kcal/mol) [22].

The simulations showed a reproducibility parameter within the threshold considered ideal for a low root mean square deviation (RMSD < 2.0 Å) [23], with RMSD values in the order of 1.53 and 1.85 Å. The analysis of ligand-protein interactions revealed that the highly polar nature of Robininpromotes the formation of hydrogen bonds with polar side-chain aliphatic residues, specifically Glu183, Asp184, Ser186, and Arg187 in chain D, and Asn100 and Thr151 in chain E. The distances between the ligand and the residues, ranging from 1.77 to 3.06 Å, indicate a particularly strong interaction with the Asp184 residue, which is located in a different chain from the DZP binding site. This finding suggests that Robinin may exert an anxiolytic effect that is enhanced by a synergistic action with other modulators of GABAA_ R receptors, such as DZP. Å for the Robinin and DZP ligands, respectively (Table S1).

2.5 | Anticonvulsant Activity

Seizures were induced with pentylenetetrazol (PTZ). One-way ANOVA showed that the highest dose of Robinin significantly delayed (**$p < 0.01$ and *$p < 0.05$ vs. vehicle) PTZ-induced seizures in all three phases (Figure 5). Seizures were also significantly delayed by DZP (****$p < 0.0001$, **$p < 0.01$ and *$p <$ 0.05 vs. vehicle) in all three phases. In the studies by Copmans et al. [24] and Alexandre et al. [25], other flavonoids promoted anticonvulsant effects.

The dual pharmacological effect (anxiolytic and anticonvulsant) of Robinin is important for the combined management and treatment of anxiety and epilepsy, which often occur as comorbidities and require the use of several combined medications. This dual pharmacological effect is neither surprising nor exclusive to Robinin; BZDs themselves have the same dual effect, depending on the dose, because the neurochemical and neurobiological pathways of anxiety and epilepsy partially overlap [26]. What distinguishes robinin from BZDs is that it does not have a

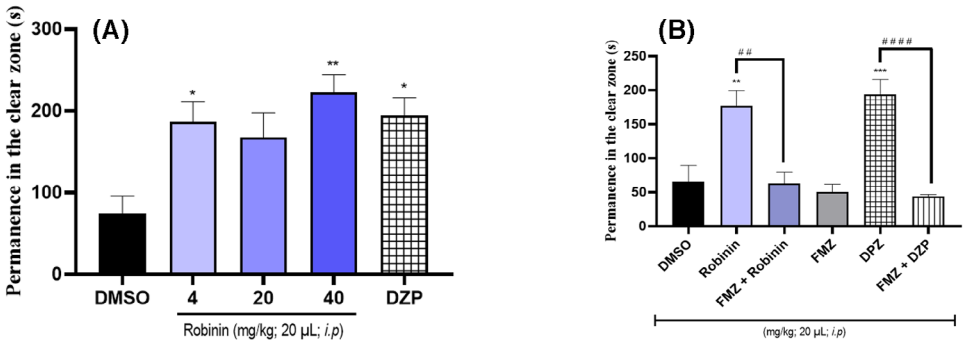


FIGURE 3 | Anxiolytic effect (A) and anxiety mechanism (B) of Robinin in the light/dark test (0–5 min). Values represent the mean ± standard error of the mean for six animals/group; (one-way ANOVA), and (two-way analysis of variance (ANOVA) in experiments with antagonists) followed by Tukey's test (***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. Vehicle–DMSO [3% dimethyl sulfoxide]; $^{\#\#\#\#}p < 0.0001$ and $^{\#\#}p < 0.01$ vs. Robinin or DZP-Diazepam). FMZ- Flumazenil.

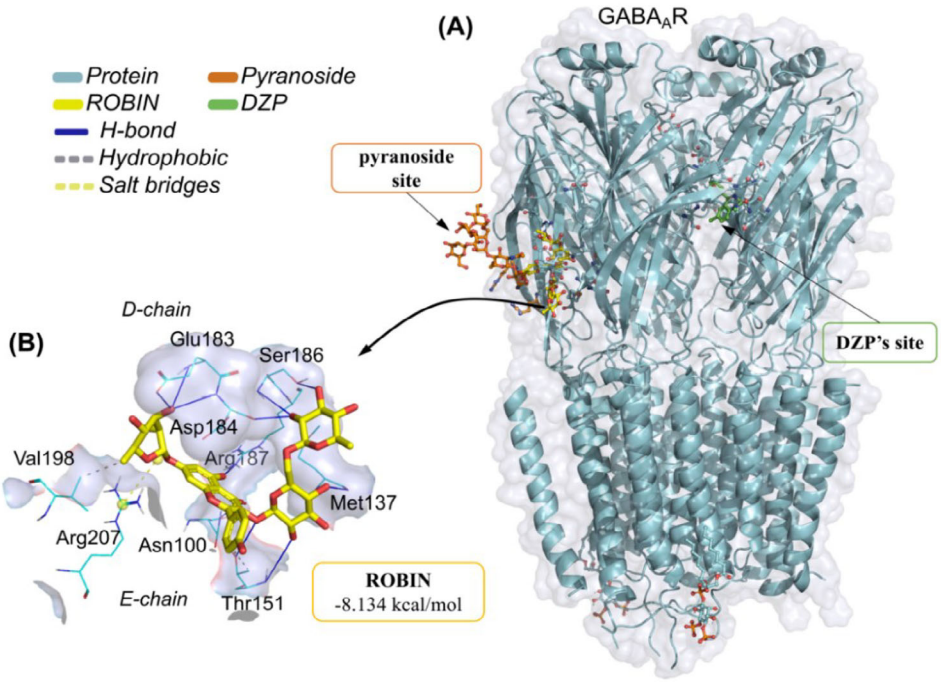


FIGURE 4 | (A) Three-dimensional visualization of the coupling of the ligand ROBIN (yellow colour), NAG (orange) and Diazepam (DZP) (green colour) in the GABAAR receptor (cyan colour) and (B) three-dimensional visualization of the ligand-protein interactions between the ligands ROBIN (yellow colour) the amino acid residues of the GABAAR binding site (cyan colour).

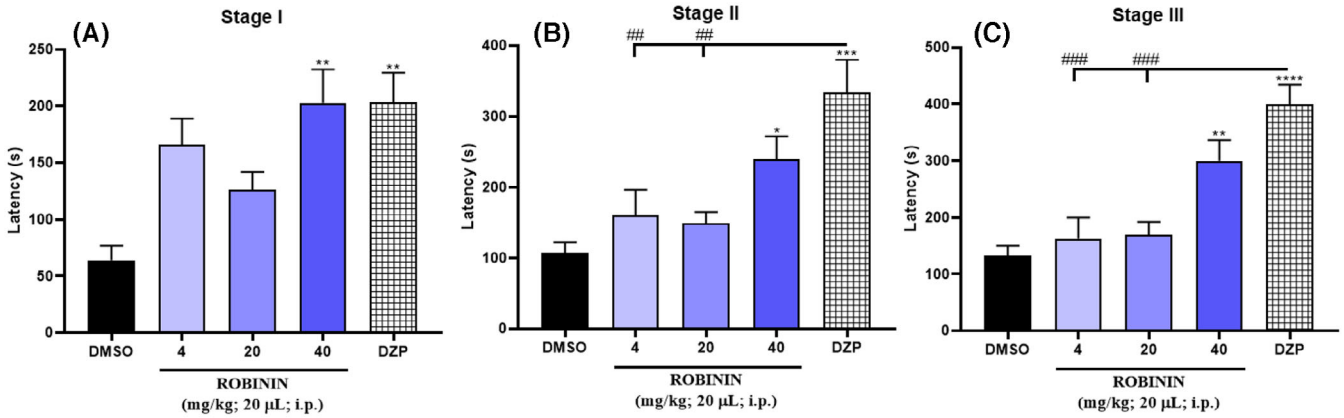


FIGURE 5 | Effect of Robinin on PTZ-pentylenetetrazol (3-stage)-induced seizure in adult zebrafish. Values represent the mean ± standard error of the mean for six animals/group; ANOVA followed by Tukey's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ vs. vehicle- DMSO (3% dimethyl sulfoxide); $^{\#\#\#\#}p < 0.0001$ and $^{\#\#}p < 0.01$ vs. DZP-Diazepam). DMSO (3% dimethyl sulfoxide).

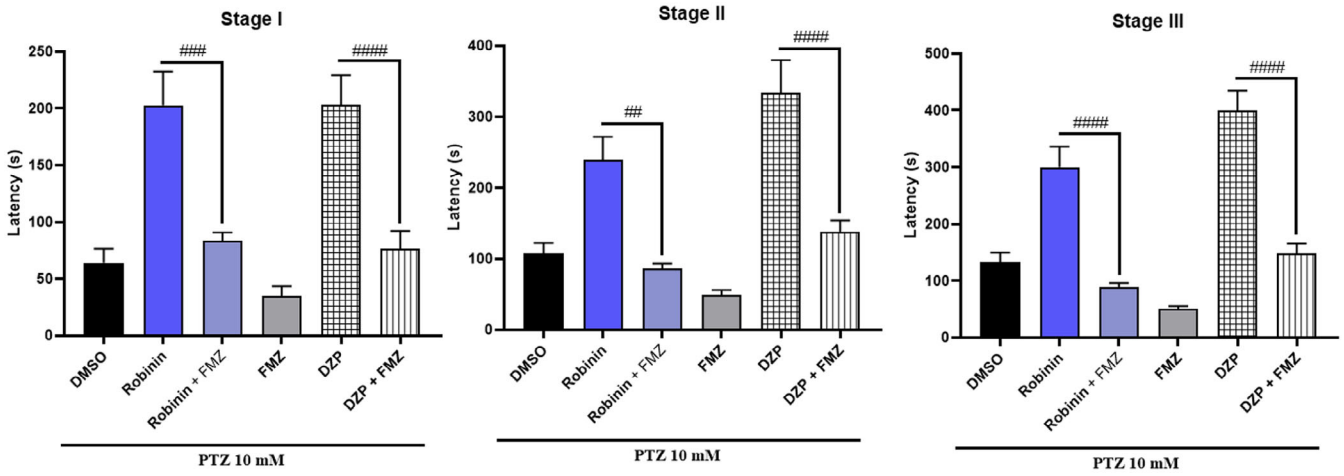


FIGURE 6 | Effect of flumazenil (FMZ) on the anticonvulsant effect of Robinin (40 mg/kg) in the PTZ- pentylenetetrazol-induced seizure test in adult zebrafish. Values represent the mean ± standard error of the mean for six animals/group; ANOVA followed by Tukey's test ($^{\#\#\#}p < 0.0001$ and $^{\#\#}p < 0.01$ vs. Robinin or DZP-Diazepam). DMSO (3% dimethyl sulfoxide).

sedative-hypnotic effect, making it a promising flavonoid for the treatment of these conditions.

To evaluate the mechanism of action of Robinin anticonvulsant via GABAergic neurotransmission, groups of zebrafish were pre-treated with FMZ (4 mg/kg, 20 µL; i.p.). Flumazenil significantly blocked ($###p < 0.001$, $##p < 0.01$ and $####p < 0.0001$ vs. Robinin or DZP) the anticonvulsant effect of Robinin and Diazepam in the three stages analyzed (Figure 6). These results suggest that the anxiolytic and anticonvulsant effect of Robinin is due to its interaction with the $GABA_A$ receptor, by interacting in a region of the receptor common to other pyranosides, as indicated by the molecular docking study.

Perhaps this binding to the $GABA_A$ receptor at a different site from BZDs explains the lack of sedation of Robinin.

Furthermore, studies by Liu et al. [21] show the association of the biological activities of the pyranosides in question, directed towards the CNS, with the hydroxyls present in the molecule, related to the potential for cell protection. These structures are also present in robinin and may be responsible for its biological activities.

2.6 | Docking of Anticonvulsant Activity

To analyze the anticonvulsant effect, the binding potential of Robinin with the enzyme Carbonic Anhydrase II was studied (Figure 7). At the end of the cycle of 20 independent simulations, an RMSD of about 1.92 Å was observed associated with the docking of robinin in the catalytic site of the enzyme, a value that expresses a pattern of reproducibility of simulations with a low root mean square deviation (RMSD < 2.0 Å) [23]. It was observed that robinin interacts with amino acid residues in common with the co-crystallized inhibitor TE1 by binding to the active site of CAII (Figure 7A), forming a ligand-CAII complex with an affinity energy of −7. 229 kcal/mol, which is within the ideal specificity spectrum for the enzyme binding site (affinity energy < −6.0 kcal/mol) [22], in addition to showing a better affinity spectrum for the enzyme with respect to TE1, with a calculated value of −5.71 kcal/mol (Table S1).

When analyzing the ligand-protein interactions, it was observed that robinin interacts with the active site residues of CAII mainly through H-bond interactions with the polar side chains of residues Asn62 (2.90 Å), Asn67 (2.14 Å) and Glu69 (2.25 Å), where the calculated distances (in Å) express H-bond interactions of moderate strength (Figure 7B) [27]. Here it is worth highlighting the π-stacking interaction formed by robinin with the aromatic side chain of residue Phe131, with perpendicular orientation (distance = 5.48 Å), with a strong contribution from its hydroxylated aromatic ring of the chromone b substructure (Figure 7B), similar to what occurs with the aromatic centre of the co-crystallized inhibitor TE1 (4.76 Å), indicating that this is a fundamental pharmacophore in the design of new CAII inhibitors [28].

2.7 | Inhibitory Avoidance Test

The effects of robinin and DZP on the inhibitory avoidance test are expressed as mean ± SEM (Figure 8A). The results show that the latency in the training session was not significant between groups.

The group of zebrafish that received the highest dose of robinin (40 mg/kg) and the control group (3% DMSO—drug diluent) had a significantly increased latency time in the test session compared to training ($^{**}p < 0.01$ and $^{***}p < 0.001$ vs. training). Fish receiving the lowest doses of robinin (4 and 20 mg/kg) and the DZP-treated group had reduced latency times in the test session. In addition, there was an increase in memory retention in the control and robinin (40 mg/kg) groups (Figure 6B). These results confirm the neuropreserver effect of Robinin, in contrast to DZP, which causes memory impairment due to sedation [29].

In this analysis, it was observed that the aromatic centres of the chemical structure of Robinin have an essentially hydrophobic molecular surface (green to blue spectra), indicating that these substructures have an affinity for non-polar/organic environments (Figure 9A) [32]. On the other hand, the presence of

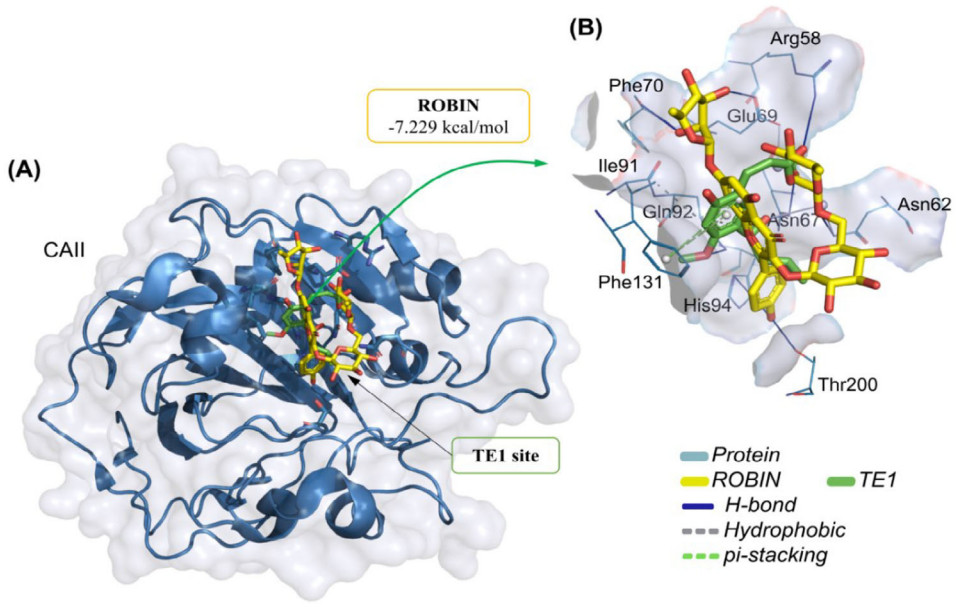


FIGURE 7 | (A) Three-dimensional visualization of the coupling of the ligand ROBIN (yellow colour) and the inhibitor TE1 (green colour) in the CAII protein (blue colour) and (B) three-dimensional visualization of the ligand-protein interactions between the ligands ROBIN (yellow colour) and TE1 (green colour) and the amino acid residues of the CAII active site (blue colour).

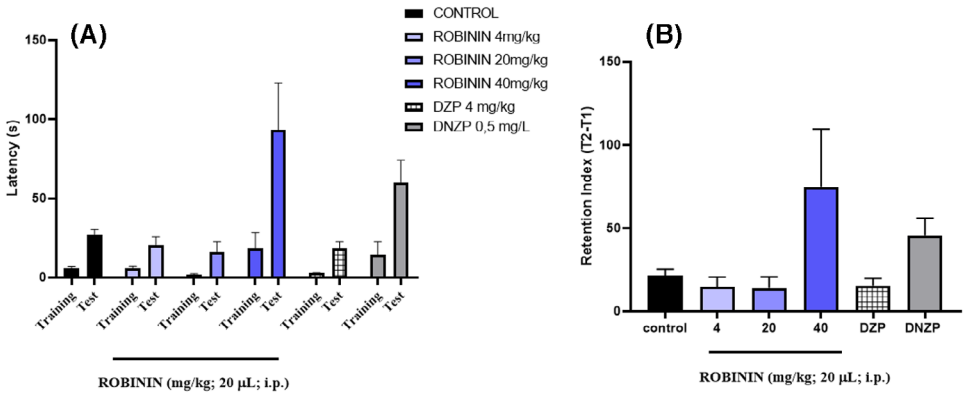


FIGURE 8 | Assessment of Robinin on aversive memory in zebrafish studied in the inhibitory avoidance task. (A) Latency to enter the dark area in training and test sessions. (B) Memory retention indices. Values represent the mean ± standard error of the mean for 6 animals/group; ANOVA followed by Tukey's test (**$p < 0.01$ and ***$p < 0.001$ vs. training). Control- DMSO (3% dimethyl sulfoxide). DNZP- Donazepil. DZP-Diazepam.

glycosylated structures contributes to the formation of a polar surface, with a strong contribution from H-bond donor hydroxyl groups (yellow to red spectra), resulting in a total polar surface area of 304.21 Å^2 (Fig. A). Thus, the log *P* index of the order of -1.79 indicates that the substance has a strong affinity for hydrophilic environments and a gradual absorption in lipophilic environments (Table 2).

3 | Multi-Parameter Optimization-Based ADME Study

3.1 | Molecular Lipophilicity Potential and Topological Analysis

Molecular lipophilicity potential (MLP) analysis can provide a topological view that relates physicochemical properties such as molecular volume, lipophilicity and polarity to pharmacokinetic attributes such as permeability across the cellular lipid bilayer and transport through physiological environments [30, 31]. These physicochemical properties are closely related to drug absorption and distribution.

3.2 | Predicted Pharmacokinetic Descriptors

A multi-parameter optimization (MPO) system developed by Wager et al. [33] and applied to a set of experimental data for drugs in clinical trials and drug candidates suggests that weakly basic compounds (pKa < 8.0) that are slightly lipophilic (log *P* < 3) and more polar than CNS agents, whose sum of the polar surface areas of the H-bond donor and acceptor groups results in a TPSA > 75 Å^2, present an alignment between safety and in vitro ADME profile. These attributes include high passive cell permeability (Papp > 10 × 10^{-6}cm/s) and metabolic stability resulting from

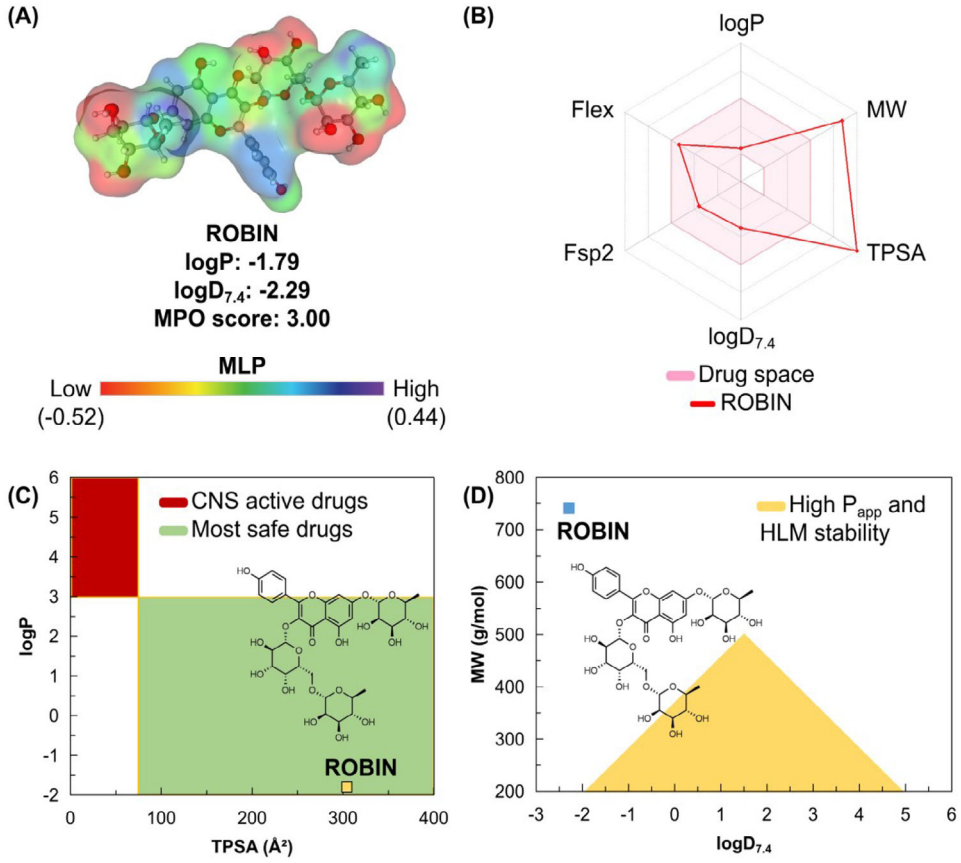


FIGURE 9 | (A) topological analysis of the molecular lipophilicity potential (MLP) map, where the colour spectrum varies from red (low MLP) to violet (high MLP), (B) oral bioavailability radar that reveals the limiting physicochemical attributes of ROBIN pharmacokinetics, (C) alignment between lipophilicity (log *P*) and topological polarity (TPSA) in estimating the access of the substance to the CNS and (D) alignment between molecular weight (MW) and lipophilicity at physiological pH (log *D*7.4) in estimation of oral absorption spectrum and hepatic clearance.

TABLE 2 | Physicochemical properties calculated and applied to the Pfizer, Inc. biopharmaceutical classification system, by the multi-parameter optimization (MPO) algorithm and Pfizer and Golden Triangle rules.

Property	Value	T0
log *P*	−1.79	1.00
log *D*	−2.29	1.00
MW	740.66 g/mol	0.00
TPSA	304.21 Å^2	0.00
HBD	11	0.00
pKa (basic)	−3.68	1.00
MPO score	3.00	
Golden Triangle rule	Rejected	
Pfizer rule	Accepted	

low clearance of the intrinsic molecular fraction in blood plasma ($CL_{int,u}$ < 8.0 mL/min/kg), properties that ensure good absorption and oral bioavailability [33, 34].

In the oral bioavailability radar of Figure 9B, it is possible to observe that the high topological polarity (TPSA > 120 Å^2) leads to an increase in the MW of Robinin at a value > 500 g/mol, indicating that these properties represent a weight factor of 0.00 (0 to 1) and strongly contribute to the MPO score = 3.0 (on a scale of 0–6), as physicochemical violations implying pharmacokinetic limitation (Table 2). This polarity range, when aligned with low lipophilicity (logP < 3), indicates that Robinin is in a physicochemical space where toxicity by CNS permeation is unlikely (Figure 9C) [6], which is corroborated by the predicted CNS access descriptor (Cbrain/Cblood) of 0.02, expressing a higher concentration of the compound in blood plasma (Table 3) [35]. This attribute may be related to the hydrophilic nature of the compound, with the estimated VD of 0.80 L/kg indicating that the absorbed molecular fraction tends to be better distributed in blood plasma than in biological tissues (Table 3), including the blood-brain barrier [32].

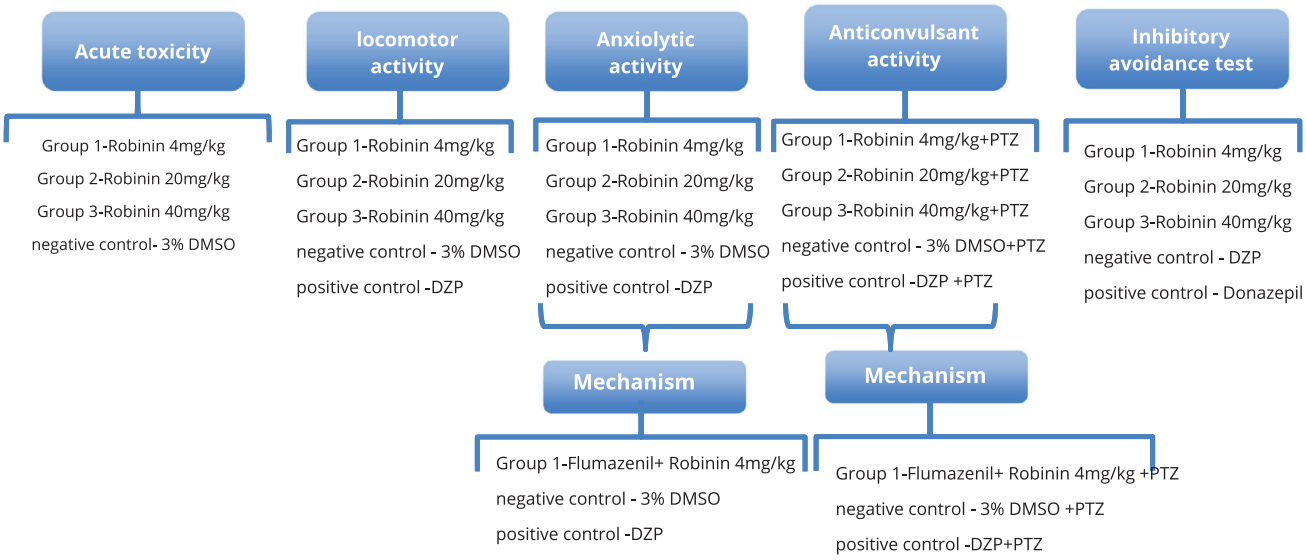
On the other hand, the calculated molecular weight (MW) of Robinin of 740.66 g/mol (>500 g/mol) places the compound outside the ideal physicochemical spectrum for good permeability in biological membranes (Figure 9D) [34]. In support of this, a Papp Madin-Darby canine kidney (MDCK) of 7.43 × 10^{-9} cm/s was predicted, which, when reconciled with the likelihood of the compound being a P-gp substrate, indicates gradual diffusion across the cellular lipid bilayer (Table 3), suggesting a bioavailability of less than 30% (F < 30%). The low first-pass clearance, with a predicted value of $CL_{int,u}$ = 0.80 mL/min/kg, suggests a low

TABLE 3 | Pharmacokinetic properties predicted by the absorption, distribution, metabolism and excretion (ADME) consensus test between the PreADMET and ADMETlab 3.0 platforms.

Property	Value	Source
P_{app} MDCK	7.43×10^{-9} cm/s	PreADMET
P-gp	Substrate	ADMETlab 3.0
$F_{<20\%}$	0.03	ADMETlab 3.0
$F_{<30\%}$	0.90	ADMETlab 3.0
BBB	0.02	PreADMET
VD	0.80 L/kg	ADMETlab 3.0
CYP2D6 inhibitor	Non-inhibitor	PreADMET
CYP2D6 substrate	Non-substrate	PreADMET
CYP3A4 inhibitor	Inhibitor	PreADMET
CYP3A4 inhibitor	Weakly	PreADMET
$CL_{int,u}$	0.80 mL/min/kg	ADMETlab 3.0

excretion of the intrinsic molecular fraction of the substance in the human liver microsomal (HLM) system. This low clearance is attributed to the low susceptibility of the ligand to be a substrate of CYP450 isoforms 2D6 and 3A4 (Table 3), indicating that Robinin is predominantly biotransformed in phase II metabolism for subsequent excretion [36].

4 | Experimental Section



4.1 | Drugs and Reagents

The drugs/reagents used were DZP and PTZ purchased from Sigma-Aldrich, Missouri, USA. Flumazenil was purchased from Roche Pharmaceutical (Welwyn Garden City, UK).

4.2 | Obtaining the Material

Robinin was obtained by extraction, from the plant species *Solanum asperum*, carried out by Prof. Dra. Otília Deusdênia Loiola Pessoa, Coordinator of the Laboratory of Natural and Marine Products, Department of Organic and Inorganic Chemistry, Federal University of Ceará.

4.3 | Animal Model

Zebrafish (*Danio rerio*) were purchased from a supplier in Fortaleza (Ceará, Brazil), wild and adult, with a mixture of males and females. The animals were properly acclimated for 24 h in 10 L aquariums, 25°C and pH 7.0. The fish were monitored, as were the temperature and water quality conditions. At the end of the experiments, the animals were euthanized by freezing, using loss of eye movement as a parameter. The experiments were approved by the Ethics Committee for the Use of Animals under protocol number 04983945/2023.

4.4 | Acute Toxicity 96 h

The experiment was performed using an adaptation of the method of Ekambaram et al. [37], where the fish ($n = 6$/group) were treated with 20 µL of the sample at doses of 4, 20 or 40 mg/kg via the intraperitoneal (i.p.) route. As a negative control, one group was treated with 3% DMSO (20 µL; i.p.). Animals were monitored for 96 h, with mortality checks performed every 24 h. The lethal dose was considered to be 50% (LD_{50}) according to the Organization for Economic Co-operation and Development [38].

4.5 | Assessment of Locomotor Activity (Open Field Test)

The experiment was performed according to the proposal of Ahmad and Richardson [39], with adaptations, ZFa were exposed to 20 µL doses (4, 20 or 40 mg/kg) intraperitoneally (i.p.), a group with vehicle (DMSO 3%, 20 µL; i.p.) was used as negative control and DZP (4 mg/kg; 20 µL; i.p.) was used as positive control. After 30 min, the groups were placed in Petri dishes marked in quadrants and the number of line crossings (CL) was evaluated to measure the effect of the drugs on the animal's exploratory

commitment when crossing the lines of each quadrant for 5 min. Anxiolytic behaviour is observed when there is a reduction in locomotor activity of the zebrafish, and the hypnotic sedative effect is observed when the fish remains virtually immobile during the analysis period [16].

4.6 | Anxiolytic Activity (Light/Dark Test)

According to the criteria proposed by Siebel et al. [40], anxiolytic activity was analyzed using the light/dark test, where animals ($n = 6$/group) were treated intraperitoneally with the sample (4, 20 or 40 mg/kg). A vehicle group (DMSO 3%, 20 µL; i.p.) and a positive control group: DZP (4 mg/kg; 20 µL; i.p.) were also tested. After 30 min of treatment, the animals were placed in aquariums of 30 × 15 × 20 cm, with dark and light sides evenly distributed, and with water treated with anti-chlorine at a height of 3 cm. The low water level and the new environment cause anxiety in zebrafish [41]. In the evaluation, the parameters are counted for 5 min, observing the time spent in the clear zone, an anxiolytic behaviour [16].

4.7 | Mechanism of Anxiolytic Action

To evaluate the effect of robinin via GABAergic neurotransmission, FMZ, a BZD antagonist at the $GABA_A$ receptor, was used [16]. The zebrafish groups ($n = 6$/group) received the sample application 15 min after prior treatment with FMZ (4 mg/kg; 20 µL; i.p.) and after 30 min the animals were individually analyzed in the light-dark test. The dose of Robinin used was the lowest dose that showed anxiolytic activity in the preliminary study (4 mg/kg; 20 µL; i.p.). A vehicle group (DMSO 3%, 20 µL; i.p.) was used as a negative control and a group treated with the reference drug (DZP, 4 mg/kg; 20 µL; i.p.) was also used as a positive control.

4.8 | Anticonvulsant Activity

To assess anticonvulsant activity, a seizure was induced in animals previously treated with Robininin (4, 20 or 40 mg/kg; 20 µL; i.p.) immersed in PTZ according to the methodology of Ferreira et al. [16]. After 30 min, the fish were individually placed in a 250 mL beaker and the three stages of the seizure were observed in the animal, where the first stage consisted of increased swimming, and in the second stage the fish swam in a whirlpool rising to the water's edge, and in the third stage it presents an interruption of swimming, remaining immobile and reaching the bottom of the beaker for 3 s. The parameter used in this experiment is the latency time taken by the animal to enter each stage. A vehicle group (DMSO 3%, 20 µL; i.p.) was used as a negative control and DZP (4 mg/kg; 20 µL; i.p.) as a positive control.

4.9 | Mechanism of Anticonvulsant Action

To verify its action via the GABAergic pathway, animals were pretreated with the BZD antagonist FMZ (FMZ, 4 mg/kg; 20 µL; i.p.). After 15 min, an effective anticonvulsant dose of Robinin (40 mg/kg; 20 µL; i.p.) was administered and after 30 min, each zebrafish was individually induced to seizure and the latency of each seizure stage was analysed as described in the previous section. A vehicle group (DMSO 3%, 20 µL; i.p.) was used as a negative control and a positive control group was also treated with the reference drug (DZP, 4 mg/kg; 20 µL; i.p.) [16].

4.10 | Inhibitory Avoidance Test

Based on the experiment conducted by Bertoncello et al. [42], groups of zebrafish were individually isolated and identified, and then the animals were placed in an aquarium (28 × 14.7 × 19 cm) with 1.3 L of previously treated water. The glass tank was equally divided into light and dark compartments, separated by a manual barrier (10 × 10 cm), similar to a guillotine, containing a set of three metal bars (1 cm diameter) spaced 3 cm apart, coupled to an electrostimulation device that delivered a mild 100 Hz electric shock for 5 s as an aversive stimulus.

Animals ($n = 6$/group) were individually placed in 500 mL jars and identified during the training day. Each zebrafish was placed in the light part of the aquarium with the partition lowered. After one minute of adaptation, the partition was raised and the latency of the fish to enter the dark environment, where the electric shock (125 mA, 3 ± 0.2 V) was applied, was recorded. This latency time was used as a parameter for statistical analysis. The animals were then removed from the aquarium and treated with Robinin (4, 20 or 40 mg/kg; 20 µL; i.p.), one group per dose, and a vehicle group (DMSO 3%, 20 µL; i.p.) and a DZP-treated group (4 mg/kg; 20 µL; i.p.) were added to the test. A positive control group was inserted–Donazepil. The test was repeated after 24 hours, but the electric shock was not administered.

4.11 | Statistical Analysis

Analyses were performed using GraphPad Prism v. 8.01 software, with a statistical significance level of 95% ($p < 0.05$). Analysis of variance (one-way and two-way ANOVA) was used to compare the assessment groups for each test and their respective doses. Results are expressed as the mean ± standard error of the mean for each group ($n = 6$/group).

4.12 | Molecular Docking Simulations

The two-dimensional representation of the chemical structure of Robinin was plotted and rendered in the academic licence software MarvinSketch version 24.1.0, Chemaxon (https://chemaxon.com/marvin), where it was prepared for structural optimisation according to the formalism of the Merck Molecular Force Field method (MMFF94), performed in the Avogadro2 software (https://two.avogadro.cc/), configured to perform optimisation by the Steepest Descent algorithm according to an energy convergence criterion of the order of 10^{-6} units, yielding the structure with the lowest potential energy, which was prepared for molecular docking simulations.

The theoretical evaluation of the anticonvulsant and anxiolytic activity of Robinin followed the methodology of da Silva et al.

[43], where the protein structures were obtained from the RCSB Protein Data Bank server (https://www.rcsb.org/) and prepared for molecular docking simulations using the AutoDockToolsTM software (https://autodock.scripps.edu/). The structure of carbonic anhydrase II (CAII) is deposited in the repository under the identification code PDB 3F8E, identified as 'Coumarins are a novel class of suicide carbonic anhydrase inhibitors', classified as an enzyme of the lyase class in the organism Homo sapiens, and its structure was solved by X-ray diffraction at a resolution of 2.0 Å [28]. The structure of the GABAAR receptor is deposited in the database under PDB code 6HUP, identified as 'CryoEM structure of human full-length alpha1beta3gamma2L GABA(A)R in complex with DZP (Valium), GABA and megabody Mb38', classified as a membrane receptor and expression system in the organisms Homo sapiens and Escherichia coli, whose co-crystallised structure was solved by electron microscopy at a resolution of 3.58 Å [44].

In the AutoDockTools program, the polar hydrogens and Gasteiger charges were added to the protein structure and the grid box was adjusted to encompass the entire conformational space of the macromolecules. CAII was fitted in a space defined by the dimensions 49 × 47 × 60 Å (x, y, z) with coordinates x = −6.650, y = −0.180 and z = 16.240, while the GABAAR receptor was fitted in the dimensions 127 × 99 × 125 Å (x, y, z) with coordinates x = 125.280, y = 139.530 and z = 136.020. The AutoDockVina code was then configured to run a series of 50 independent simulations of 20 positions each for the Robinin ligand and the co-crystallised ligands TE1 (CAII inhibitor) and DZP (DZP—GABAAR modulator), using the Lamarkian Genetic Algorithm (LGA) [45]. The best-fit selection criterion includes energetic and statistical parameters within the ideality threshold defined by affinity energy < −6.0 kcal/mol and RMSD < 2.0 Å [46].

4.13 | MPO-Based ADME Study

In the MarvinSketch program (https://chemaxon.com/marvin), the MLP surface map was generated, where the lipophilicity spectrum was related to the partition coefficients (log *P*), distribution (log *D*7.4) and Topological Polar Surface Area (TPSA). The descriptors were then applied to the Multiparameter Optimization (MPO) system of Pfizer, Inc., for quantitative estimation of drug-likeness, as shown in Equation (1):

$$d = \sum_{i=1}^{N} w_k T_k \left(x_k^0\right) \quad (1)$$

where w is the weighting factor assigned to each physicochemical attribute k that is within the ideal threshold (T(x)), taking into account the limits: log $P \leq 3$, log $D7.4 \leq 2$, MW ≤ 360 g/mol, TPSA 40–90 Å^2, H-bond donors (HBD) ≤ 1 and base pKa ≤ 8.0 ($N = 6$ attributes). The sum results in a desirability score (d) ranging from 0 (poor drug-likeity) to 6 (optimal drug-likeity), which was related to the biopharmaceutical classification systems of the Pfizer rule [6] and the Golden Triangle rule [34].

Following the methodology proposed by da Rocha et al. [47], the results of the MPO analyses were related to the ADME descriptors predicted by the online servers PreADMET (https://preadmet.qsarhub.com/) and ADMETlab 3.0 (https://admetlab3.scbdd.com/), including apparent permeability (P_{app}) in the Madin-Darby Canine Kidney cell model, P-glycoprotein (P-gp) substrate, oral bioavailability (%F), the volume of distribution (VD), blood-brain barrier permeability, cytochrome P450 isoform substrates and intrinsic hepatic clearance ($CL_{int,u}$).

5 | Conclusions

Robinin was shown to cause anxiolytic behaviour in adult zebrafish without sedation, and the $GABA_A$ receptor may be involved in this effect. Robinin also delayed the onset of seizures, showing an anticonvulsant effect, which may be related to its interaction with $GABA_A$ and CAII receptors. Robinin also prevented memory impairment in adult zebrafish. Thus, robinin is a promising pyranoside as a therapeutic agent in the development of treatments for anxiety and seizures without memory impairment and without sedative effects.

Author Contributions

Cecília Guimarães Lemos: supervision and writing–review and editing. **Jéssica Bezerra Maciel**: investigation; formal analysis and writing–original draft. **Antônio Wlisses Da Silva and Maria Kuerislene Amâncio Ferreira**: formal analysis; software; validation and reviewed the manuscript. **Hélcio Silva dos Santos**: conceptualization; methodology and determination of the molecular structures. **Francisco das Chagas L. Pinto; Emmanuel Silva Marinho and Otília Deusdênia Loiola Pessoa**: writing–original draft and aided in the analysis of the spectra. **Matheus Nunes da Rocha; Márcia Machado Marinho and Jane Eire Silva Alencar de Menezes**: Project administration and writing–review and editing. **Hélcio Silva dos Santos**: methodology, supervision, project administration.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.