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Anxiolytic/Sedative Effect of Monoterpene (–)-Borneol in Mice and In Silico Molecular Interaction with $GABA_A$ Receptor

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Abstract: Anxiety is a normal behavioral component. When it is too frequent or appears in inappropriate contexts, it can be considered pathological. Benzodiazepines (BDZs) are drugs with clinical success in anxiety treatment. BDZs act as allosteric modulators of the γ- aminobutyric acid A receptor ($GABA_AR$). However, these drugs cause adverse effects. Despite the therapeutic advances obtained with BDZs, the search for anxiolytics with fewer adverse effects is ongoing. Studies with monoterpene (–)-borneol [(–)-BOR] demonstrated pharmacological properties such as a partial agonist effect of $GABA_AR$ and an anticonvulsive effect. On the other hand, no work has been developed evaluating the anxiolytic/sedative potential. The objective of this study was to investigate the anxiolytic/sedative effects of (–)-BOR in animal models at doses of 25, 50, and 100 mg/kg (i.p.) and whether there was a molecular interaction with $GABA_AR$. The anxiolytic effect of monoterpene (–)-BOR was tested on Swiss mice (25–30 g) in three anxiety models: the elevated plus maze test, the open field test, and the light-dark box test. The thiopental-induced sleep time model was a drug screen for the sedative and hypnotic activity related to $GABA_ARs$. In the molecular docking, the interaction between the $GABA_AR$ molecule and (–)-BOR was performed using the AutoDock 4.2.6 program. The results demonstrated that (–)-BOR has sedative and anxiolytic activity. The molecular docking study revealed that (–)-BOR can interact with $GABA_ARs$ through hydrogen bonds.

Keywords: anxiety; sedation; $GABA_AR$; monoterpene; (–)-borneol; molecular docking

Citation: Amaral, M.P.d.M.d.; Silva Junior, M.P.d.; Lima, F.d.C.A.; Gutierrez, S.J.C.; Arcanjo, D.D.R.; Oliveira, R.d.C.M. Anxiolytic/Sedative Effect of Monoterpene (–)-Borneol in Mice and In Silico Molecular Interaction with $GABA_A$ Receptor. *Future Pharmacol.* **2023**, *3*, 132–141. https://doi.org/10.3390/futurepharmacol3010009

Academic Editor: Fabrizio Schifano

Received: 12 December 2022
Revised: 7 January 2023
Accepted: 11 January 2023
Published: 13 January 2023

1. Introduction

Anxiety is defined as a vague and unpleasant feeling of fear and apprehension characterized by tension or discomfort derived from the anticipation of danger, the unknown, or something strange [1]. Anxiety is a normal behavioral component and is important as a defense mechanism in relation to new and unexpected situations. However, when it is too frequent or appears in inappropriate contexts, it may interfere with the normal functioning of the organism and can be considered as pathological [2].

In the 1960s, with the introduction of Diazepam (DZP) (Valium®), a revolution began in the pharmacological treatment of anxiety. DZP innovated by dissociating the anxiolytic effect from the sedative effect [3]. Similar to DZP, the other benzodiazepines (BDZs) act as allosteric modulators of the γ- aminobutyric acid A receptor ($GABA_AR$), promoting an increase in the conductance of chloride ions inside of the cell and hyperpolarizing the

neuronal membrane. In addition to the anxiolytic effect, BDZs act as muscle relaxants and sleep inducers, and they produce anticonvulsant effects. However, these drugs cause adverse effects such as loss of motor coordination, tolerance, and dependence [4].

Despite the therapeutic advances obtained with BDZs, the search for anxiolytics with fewer adverse effects is still a goal in the pharmacological treatment of anxiety disorders. Molecules obtained from natural products are a viable source as an options with therapeutic potential. Terpenes are the most abundant and structurally more diversified group of secondary plant metabolites [5]. In addition, terpenes have a wide variety of biological functions, and most have medicinal properties [6]. They are classified according to the number of carbons used in their biosynthesis, which are monoterpenes, sesquiterpenes, diterpenes, triterpenes, and carotenoids, among others. The most frequent terpene compounds are monoterpenes (90%) and sesquiterpenes [6]. Monoterpenes such as carvacrol, menthol, limonene, and thymol are a group of natural compounds derived from two isoprene units. All these examples of monoterpenes somehow modulate $GABA_AR$, exhibiting anticonvulsive and/or anxiolytic properties [7].

(–)-Borneol [(–)-BOR] (Figure 1), is an example of bicyclic monoterpene widely used in food, pharmaceutical, and cosmetic industries [8]. Studies with monoterpene (–)-BOR demonstrated pharmacological properties such as the partial agonist effect of $GABA_AR$ [9]; an anticonvulsive effect [10]; a vasorelaxant effect attributed to the blockade of voltage-gated calcium ion type L (Ca_VL) [11]; and the inhibition of transient receptor potential cation channel type I (TRPA1) [12]. However, despite (–)-BOR being a partial agonist for $GABA_AR$, thus far, no work has been developed evaluating the anxiolytic/sedative potential. Furthermore, (–)-BOR's ability to block calcium channels increases its potential as an anxiolytic candidate. Calcium channel blockers such as nifedipine and nimodipine have demonstrated anxiolytic effects in preclinical models [13,14]. Thus, the objective of this work was to investigate the synthetic (–)-BOR anxiolytic activity in experimental anxiety models with Swiss mice, as well as, through molecular docking, evaluate whether the more stable conformation of the molecule has some interaction with $GABA_AR$, the main inhibitory neurotransmitter of the CNS [15].

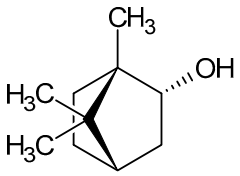

Figure 1. (–)-Borneol molecule.

2. Materials and Methods

2.1. Drugs

(–)-BOR and thiopental sodium were purchased from Sigma-Aldrich (St. Louis, MO, USA), and DZP was obtained from Cristália (São Paulo, SP, Brazil). (–)-BOR was previously solubilized in dimethylsulfoxide (1% DMSO) and then diluted in saline (0.9% NaCl). The other drugs were dissolved in 0.9% NaCl.

2.2. Animals

Swiss males (25–30 g), two months old, were housed at the Agrarian Sciences Center of the Federal University of Piauí (CCA/UFPI) and were acclimated at a temperature of 24 ± 2 °C under a light-dark cycle of 12–12 h with free access to food and water. All experiments followed protocols approved by the Animal Experimentation Ethics Committee of the Federal University of Piauí (n. 83/14).

2.3. Elevated Plus Maze Test

The elevated plus maze test [16] consists of two perpendicular open arms (30 × 5 cm) and two closed arms (30 × 5 × 25 cm), also standing perpendicular. The open and closed arms are connected by a central platform (5 × 5 cm). Male Swiss mice (25–30 g), 6 per group, were treated with vehicle, (–)-BOR (25, 50, and 100 mg/kg, i.p.), or DZP (2 mg/kg, i.p.). Thirty minutes after treatment, the animals were placed one at a time in the center of the labyrinth facing one of the closed arms, and their behavior was observed for 5 min. The parameter evaluated was time spent in the open arms in seconds. After each session, the apparatus was cleaned with 70% alcohol.

2.4. Open Field Test

The equipment used (30 × 30 × 15 cm) was a box with its floor divided into nine equal quadrants based on the model described by Archer [17]. Male Swiss mice (25–30 g), 6 per group, were treated with vehicle, (–)-BOR (25, 50, and 100 mg/kg, i.p.), or DZP (2 mg/kg, i.p.). After 30 min of drug administration, the animals were placed one at a time in the equipment, and the number of crosses with four legs (spontaneous locomotor activity) was observed for 5 min. After each session, the apparatus was cleaned with 70% alcohol.

2.5. Light-Dark Box Test

Male Swiss mice (25–30 g), 6 per group, were treated with vehicle, (–)-BOR (25, 50, and 100 mg/kg, i.p.), or DZP (2 mg/kg, i.p.). After 30 min, the animals were placed, one at a time, in the equipment composed of two compartments, one light and one dark, linked by a small door [18]. The dark (27 × 18 × 29 cm) compartment was dimly lit, and the light compartment (27 × 18 × 29 cm) was illuminated by ambient light. The total time spent in the light compartment was registered for 5 min, and between each test, the equipment was cleaned with 70% alcohol.

2.6. Thiopental Sodium Induced Sleeping Time Test

Male Swiss mice (25–30 g), 6 per group, were treated with: vehicle, (–)-BOR (25, 50, and 100 mg/kg, i.p.), or DZP (2 mg/kg, i.p.). Immediately after substance administration, sodium thiopental (50 mg/kg, i.p.) was administered. After the animals fell asleep, they were placed in dorsal decubitus. The time was registered between the loss and recovery of righting reflex (sleep time) and was considered as the inability of the animal to return its normal position. The return of the animal to the normal position for three consecutive times was considered as criterion for the recovery of the straightening reflex [19].

2.7. Statistical Analysis

All results were presented as mean ± standard error of mean (S.E.M). Data were evaluated by analysis of variance (ANOVA) followed by the Student-Neuman-Keuls test as the post hoc test. The results were considered significant (* $p < 0.05$) when compared to the vehicle group. All analyses of the in vivo experiments were performed using GraphPad Prism software, version 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

2.8. Molecular Docking

The (–)-BOR molecule was obtained from the ZINC database (ID: 967533). After obtaining it, the geometry of the molecule was optimized in the program Gaussian 09 [20]. The density-functional theory (DFT) method [21] was used with hybrid functional B3LYP combined with base set 6–31 ++ G *. Frequency calculations were performed to check whether the molecule was at a minimum energy. The $GABA_AR$ molecule was obtained from the Protein Data Bank database (ID: 4COF) [22] and by the AutoDock Tools (ADT) program [23]. A search was made for the existence of water molecules and structures of repeated protein. Molecular docking was performed using the AutoDock 4.2.6 program, which made use of the Lamarckian genetic algorithm, in combination with global search

algorithms and local search algorithms. With the use of ADT, an affinity mesh was created encompassing the extracellular region of $GABA_AR$; the affinity maps between the ligand and macromolecule atoms were generated through the AutoGrid 4.2.6 module. In the remaining parameters, the default values of the program were used.

3. Results

3.1. Elevated Plus Maze Test

In this model, (–)-BOR (100 mg kg, i.p.) significantly increased (* p <0.05) the time of mice in the open arms of the labyrinth by 57% (49 ± 11 s) when compared to the vehicle group (21 ± 6 s) (Figure 2). DZP (2 mg/kg, i.p.) increased the time of the animals in the open arms by 69% (68 ± 14 s) when compared to the vehicle group (Figure 2).

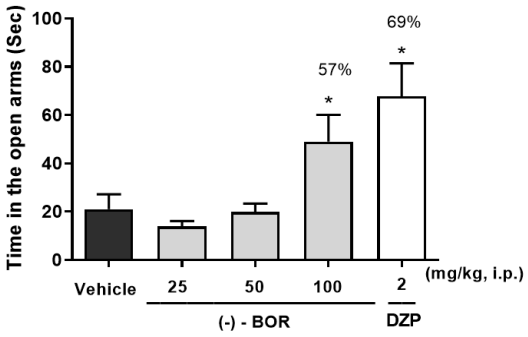

Figure 2. The effect of (–)-BOR (25, 50, and 100 mg/kg, i.p.) and DZP (2 mg/kg, i.p.) on the elevated plus maze test with mice (n = 6). Each bar represents the mean ± SEM of time in seconds (sec) in which the animals remained in the open arms. * p < 0.05 is significant compared to the vehicle group (ANOVA and t-Student-Newman-Keuls test as post hoc test).

3.2. Open Field Testing

In the evaluation of locomotor activity, (–)-BOR (50, 100 mg/kg, i.p.) had a significant effect (* p < 0.05), reducing the number of entries in quadrants by 32% (66 ± 2) and 73% (26 ± 3), respectively, when compared to the vehicle group (97 ± 8) (Figure 3). DZP (2 mg/kg, i.p.) significantly reduced (* p < 0.05) the number of entries in quadrants by 73% (25 ± 3) compared to the vehicle group (Figure 3).

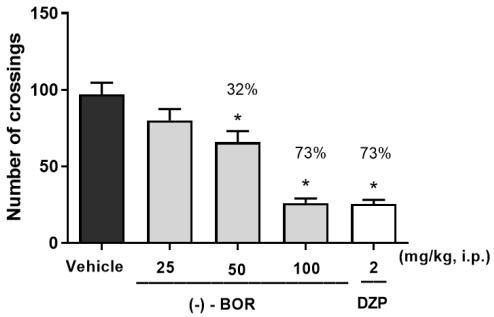

Figure 3. The effect of (–)-BOR (25, 50, and 100 mg/kg, i.p.) and DZP (2 mg/kg, i.p.) on the open field test with mice (n = 6). Each bar represents the mean ± S.E.M. of the number of quadrants crossed by the animals. * p < 0.05 is significant compared to the vehicle group (ANOVA and Student-Newman-Keuls test as the post hoc test).

3.3. Light-Dark Box Test

In the light-dark box test, (–)-BOR (25, 50, and 100 mg/kg, i.p.) showed a significant effect (* $p < 0.05$), increasing by 47% (143 ± 6 s), 65% (161 ± 3 s), and 78% (174 ± 8 s), respectively, the residence time of the mice in the clear compartment when compared to the vehicle group (98 ± 11 s) (Figure 4). In comparison to the vehicle group, DZP (2 mg/kg, i.p.) increased the time by 63% (160 ± 12.2 s) (Figure 4).

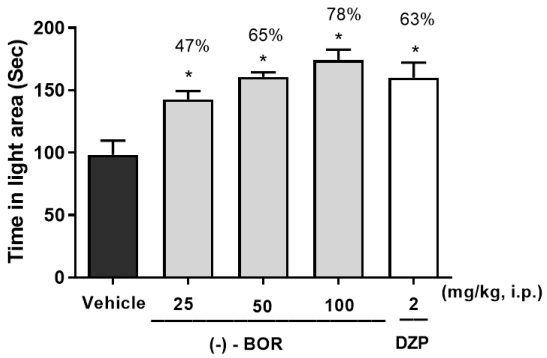

Figure 4. The effect of (–)-BOR (25, 50, and 100 mg/kg, i.p.) and DZP (2 mg/kg, i.p.) on the light-dark box test with mice ($n = 6$). Each bar represents the mean ± S.E.M of the time in seconds (sec) of the animals in the clear compartment. * $p < 0.05$ is significant compared to the vehicle group (ANOVA and the Student-Newman-Keuls test as the post hoc test).

3.4. Thiopental Sodium Induced Sleeping Time Test

In the thiopental sodium-induced sleeping time test, (–)-BOR (100 mg/kg, i.p.) had a significant effect (* $p < 0.05$) when 57% (13 ± 1 min) reduced the onset of sleep and increased the total sleep time in 57% (103 ± 9 min) in relation to the vehicle group (31 ± 2; 66 ± 3 min, respectively) (Figure 5A,B, respectively). Similarly, DZP (2 mg/kg, i.p.) significantly decreased (* $p < 0.05$) the time required for the onset of sedation and sleep duration induced by thiopental in 74% (8 ± 1 min) and 65% (108 ± 2 min), respectively, when compared to the vehicle group (Figure 5A,B, respectively).

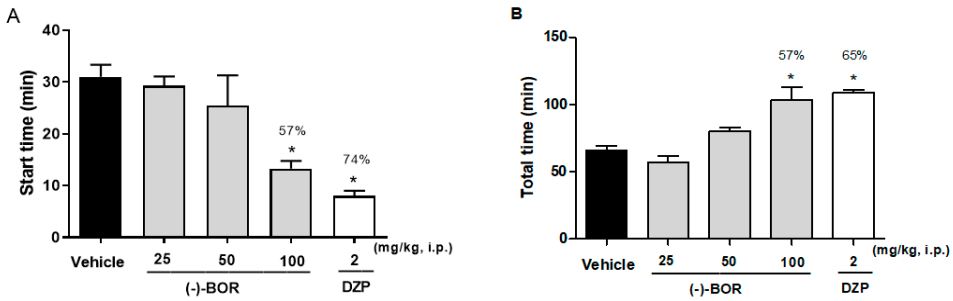

Figure 5. The effect of (–)-BOR (25, 50, and 100 mg/kg, i.p.) and DZP (2 mg/kg, i.p.) on the thiopental sodium-induced sleeping time test in Swiss mice ($n = 6$). Each bar represents the mean ± S.E.M of sleep onset time (**A**) or total sleep duration time (**B**) in minutes (min). * $p < 0.05$ was significant compared to the vehicle group (ANOVA and Student-Newman-Keuls test as the post hoc test).

3.5. Molecular Docking

In the docking study, the most stable conformation (Figure 6) presented -4.96 kcal.mol^{-1} and 231.31 Ki (μM) as binding free energy and constant inhibition, respectively (Table 1). (–)-BOR interacted with the following amino acid residues of $GABA_AR$: lysine 274 (Lys274), valine 50 (Val50), glutamine 185 (Gln185), phenylalanine 186 (Phe186), and methionine 49 (Met 49) (Table 1). The three-dimensional docking design between $GABA_AR$ and (–)-BOR indicated that the interaction occurred near the cell membrane in an extracellular region (Figure 7).

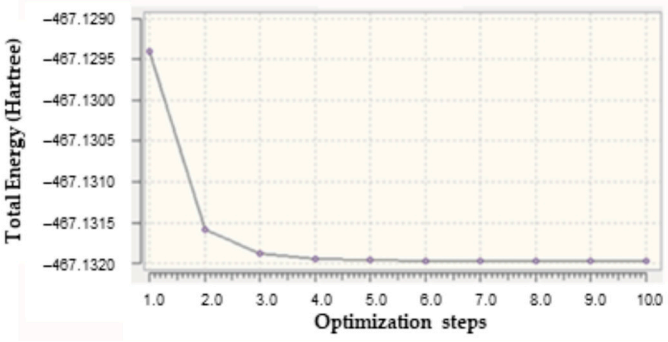

Figure 6. Energy diagram correlated with the conformation obtained at the end of functional density calculation. The conformation chosen was one that presented the lowest energetic state.

Table 1. Comparative data of the three best conformations between (–)-BOR and $GABA_AR$.

Conformation	Binding Free Energy (Kcal/mol)	Inhibitory Constant Ki (μM)	$GABA_AR$ Amino Acid Residues	Binding between (–)-BOR and Val50	Energy (Kcal/mol)	Length (Å)
1	−4.96	231.31	Lys274, Val50, Gln185, Phe186, Met49	Hydrogen bridge	1.41	2.045
2	−4.96	230.41	Lys274, Val50, Gln185, Phe186, Met49	Hydrogen bridge	−1.43	2.056
3	−4.89	259.46	Val50, Met 49, Pro184, Gln185, Lys274	Hydrogen bridge	−1.72	1.915

Legend: Lys274 (lysine 274), Val50 (valine 50), Gln185 (glycine 185), Phe186 (phenylalanine 186), Met 49 (methionine 49), Pro184 (proline 184), and Gln185 (glutamine 185).

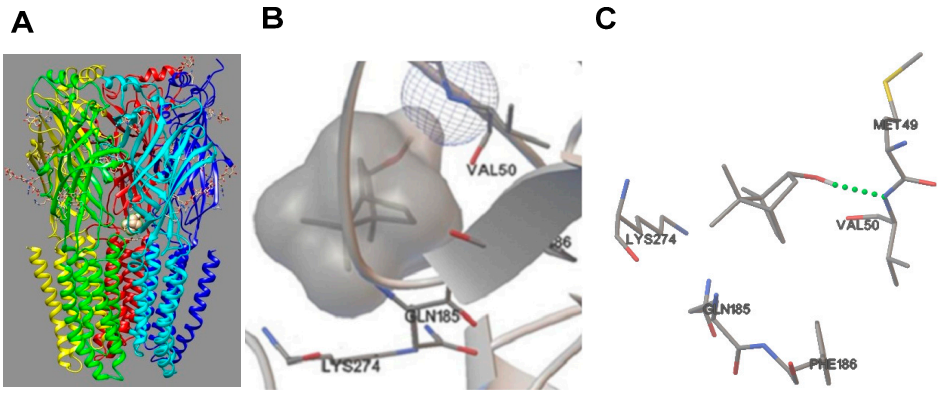

Figure 7. Molecular mapping between (–)-BOR and $GABA_AR$: (**A**) representative frontal image of the interaction between the monoterpene (–)-BOR and $GABA_AR$; (**B**) mesh of energy between molecules; and (**C**) the hydrogen bond between the amino acid Val50 and the molecule of (—)-BOR.

4. Discussion

Anxiety disorders are serious psychiatric conditions that daily affect commitments and cause a high cost to public health. If we consider the costs in terms of debility, associated financial costs, risk of suicide [24], and the serious adverse effects of anxiety disorders treatment [4], the search for therapeutic alternatives should be considered. Substances obtained from natural products are a viable source for new anxiolytics. Several monoterpenes, such as carvacrol acetate and linalool oxide, have demonstrated anxiolytic properties in experimental animal models [25,26]. In this work, the pharmacological potential of (–)-BOR was evaluated in anxiety animal models.

In the investigation of the anxiolytic effect of (–)-BOR, three models were used: the elevated plus maze test, the open field test, and the light-dark box test. Three models are widely used in the evaluation of animals' exploratory behavior, mainly rodents [27,28]. The elevated plus maze test is a valuable tool in new anxiolytics research and in anxiety neurobiology studies [29]. This model is based on the conflict between the natural behavior of exploring a new environment and the tendency to avoid potential danger [30]. Rodents show a behavior pattern, avoiding open spaces and preferring enclosed spaces. This tendency is suppressed by anxiolytics [31]. In this test, animals treated with (–)-BOR remained longer in open arms, reducing the anxiogenic effect triggered by the model, similar to DZP, indicating anxiolytic property. However, it is possible that motor activity interference by drugs can give a fake positive/negative in the elevated plus maze test [32]. Therefore, for greater safety, the open field test was used.

The open field test is a classic model applied to evaluate drugs' autonomic effects and the animals' general behavior [33]. In the open field test, anxiolytic drugs reduce animal curiosity about new environments by decreasing locomotor activity [34,35]. Locomotor activity is an alertness indicator, and its decrease can be interpreted as CNS excitability reduction [36]. The treatment with (–)-BOR reduced the mice locomotor activity by reducing the quadrants crossed by the animals, thus corroborating with the data in the elevated plus maze test.

The light-dark box model was the last test for anxiety used. This model is similar to the elevated plus maze test and open field test. In the light-dark box model, a new environment is presented to the animal. The new environment triggers a stress level that results in blocking typical behaviors, such as exploratory and locomotor. Untreated animals usually move to the dark area of the box and avoid the stress of a lighter environment [30]. After anxiolytic substance administration, this behavior was altered, the shift to dark environment decreases, and the animal remained longer in the clear area [31]. The data agree with found results in previously tested models. The (–)-BOR administration resulted in an anxiolytic effect, increasing the time of mice in the clear area.

Anxiolytic and sedative drugs activate $GABA_A$Rs [3]. The thiopental-induced sleep time model is a drug screen for the sedative and hypnotic activity related to $GABA_A$Rs. Sodium thiopental binds at barbiturate sites in $GABA_A$Rs and hyperpolarizes the post-synaptic membrane [37]. BDZs, such as DZP, potentiate the sedative effect of thiopental. This increase is due to the agonist action of BDZs on $GABA_A$Rs [38,39] growing the Cl^- influx to inside of cell [40]. Any substance that decreases the time of onset and prolongs the sedative effect of thiopental presents possible activity on $GABA_A$Rs [39]. In thiopental sodium-induced sleeping time, (–)-BOR decreased the onset and prolonged sleep time, constituting a probable GABAergic effect. However, in this model, only a 100 mg/kg dose had a significant effect on the two evaluated parameters.

Despite that Quintans-Júnior et al. [10] suggest that (–)-BOR modulates $GABA_A$Rs, Granger et al. [9] already investigated the ability of (+)-BOR and (–)-BOR to modulate the recombinant human $\alpha1\beta2\gamma2L$ $GABA_A$R. In the study, the authors concluded that (–)-BOR is a partial agonist of $GABA_A$Rs. However, the activation of these receptors occurs at sites unrelated to BDZs, as their effect is not reversed by flumazenil.

Structurally, $GABA_A$Rs are composed of one γ subunit, two α subunits, and two β subunits. BDZs, such as DZP, bind in the extracellular portion of $GABA_A$Rs in $\gamma2$ and $\alpha1$ subunits [41]. Molecular docking research between DZP and $GABA_A$Rs indicate that drugs bind to the amino acids Lys 105, Tyr 160, Tyr 210, and Val 212 in the $\alpha1$ subunits and to the Phe 77 amino acid in the $\beta2$ subunit [15,38]. In this work, we observed that (–)-BOR, in a more stable configuration, interacts with amino acids Lys 274, Val 50, Gln 185, Phe186, and Met 49, which are different amino acids found between DZP and $GABA_A$Rs (Table 2).

(–)-BOR is not the only molecule that activates $GABA_A$Rs at the binding site other than BDZs. Other studies have demonstrated anxiolytic effects of substances such as valeric acid [42] and carvacrol [43] when binding to $GABA_A$Rs at sites other than BDZs. More than 11 binding sites were proposed in $GABA_A$Rs [44] and various drugs (e.g., BDZs,

barbiturates, steroids, picrotoxin, ethanol, etc.) activate $GABA_A$Rs at different binding sites. Therefore, the molecular docking of (–)-BOR and $GABA_A$R indicates that the interaction occurs near the cell membrane, not corresponding to the site where BDZs bind (Figure 8).

Table 2. Comparison between (–)-BOR binding sites and DZP binding sites with $GABA_A$R.

Molecule	Linking Subunit	Amino Acid Residue	References
(-)-BOR	___-__	Lys274, Val50, Gln185, Phe186, Met49	This work
DZP	α1/γ2	Lys105, Tyr160, Tyr210, Val212/ Phe77	(Ci et al., 2008; Bergmann et al., 2013)

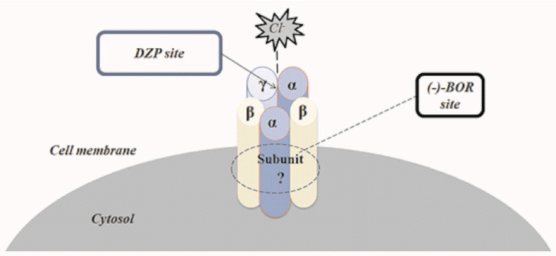

Figure 8. Illustration of the DZP binding site and the possible extracellular region in which the monoterpene (–)-BOR binds to $GABA_A$R.

5. Conclusions

The results demonstrate that (–)-BOR has sedative and anxiolytic activity. The molecular docking study revealed that (–)-BOR can interact with $GABA_A$Rs through hydrogen bonds. Theoretically, this interaction would be able to allosterically activate the receptor at a site other than the BDZs, producing the anxiolytic and sedative effects observed with the animal models. In this context, this work opens for further studies to determine in which $GABA_A$R subunit the interaction occurs and how receptor activation occurs.

Author Contributions: Conceptualization, M.P.d.M.d.A.; methodology, M.P.d.M.d.A. and M.P.d.S.J.; validation, M.P.d.M.d.A. and S.J.C.G.; formal analysis, M.P.d.M.d.A. and F.d.C.A.L.; investigation, M.P.d.M.d.A., M.P.d.S.J., F.d.C.A.L. and S.J.C.G.; resources, F.d.C.A.L., D.D.R.A. and R.d.C.M.O.; writing—original draft preparation, M.P.d.M.d.A. and M.P.d.S.J.; writing—review and editing, F.d.C.A.L., D.D.R.A. and R.d.C.M.O.; visualization, M.P.d.M.d.A. and M.P.d.S.J.; supervision, R.d.C.M.O.; project administration, M.P.d.M.d.A.; funding acquisition, M.P.d.M.d.A. and D.D.R.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Animal study protocols were approved by the Ethics Committee for the Use of Animals of UNIVERSIDADE FEDERAL DO PIAUÍ (CEUA/UFPI), No. 083/2014, approved on 24 November 2014.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be available under request.

Acknowledgments: This work was supported by UFPI (Federal University of Piauí, Brazil), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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

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