ORIGINAL ARTICLE

Modulator effect of the hydroalcoholic extract from the Amazonian plant Calycophyllum spruceanum barks on mice anxiety and oxidative stress

Efeito modulador do extrato hidroalcoólico da casca da planta amazônica Calycophyllum spruceanum sobre ansiedade e estresse oxidativo em camundongos

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Abstract

Objective: this study aimed to determine the effect of the hydroalcoholic extract from Calycophyllum spruceanum barks (HECSb) on behavioral models and neurochemical markers. **Methods**: swiss mice (N=72) received HECSb orally (100 mg kg–1) for evaluation in the tests (open field, hole board, plus maze, and tail suspension) and quantification for malondialdehyde-MDA and reduced glutathione-GSH) in brain areas (prefrontal cortex, hippocampus, and corpus striatum). **Results**: HECSb reduced the crossing number (45.25 ± 38.63 vs. saline: 83.87 ± 38.63); increased the frequency of head dips (32.57 ± 13.14 vs. saline: 19.42 ± 13.14), exploration time (19.12 ± 10.84 vs. saline: 8.28 ± 10.84 s) and permanence time in open-arms (98.28 ± 65.71 vs. saline: 32.57 ± 65.71 s) and reduced closed-arm entries (8.35 ± 7.8 vs. saline: 16.25 ± 7.8), whereas unaltered the immobilization time (63.71±12.69s vs. saline: 50.28±5.13s). Besides, HECSb increased GSH in corpus striatum (683.3 ± 118.1 vs. saline: 565.2 ± 118.1 µg/g) and MDA in prefrontal cortex (345.1 ± 191.9 vs. saline:153.2 ± 191.9 nmol/g). **Conclusion**: HECSb presents anxiolytic effects in mice via modulation of the brain oxidative stress.

Keywords: anxiolytic effect; depression; experimental models; oxidative balance; amazonian plant.

Resumo

Objetivo: avaliar o efeito do extrato hidroalcoólico das cascas de Calycophyllum spruceanum (HECSb) sobre parâmetros comportamentais e marcadores neuroquímicos. **Métodos**: camundongos Swiss (N = 72) receberam HECSb (100 mg kg–1) por via oral para avaliação nos testes (campo aberto, placa perfurada, labirinto e suspensão da cauda) e quantificação de malondialdeído-MDA e glutationa reduzida-GSH) em áreas cerebrais (córtex pré-frontal, hipocampo e corpo estriado). **Resultados**: HECSb reduziu o número de cruzamentos (45,25 ± 38,63 vs. salina: 83,87 ± 38,63); aumentou a frequência de mergulhos de cabeça (32,57 ± 13,14 vs. salina: 19,42 ± 13,14), tempo de exploração (19,12 ± 10,84 vs. salina: 8,28 ± 10,84 s) e tempo de permanência em braços abertos (98,28 ± 65,71 vs. salina: 32,57 ± 65,71 s) e reduziu entradas em braços fechados (8,35 ± 7,8 vs. salina: 16,25 ± 7,8), enquanto não alterou o tempo de imobilização (63,71 ± 12,69 vs. salina: 50,28 ± 5,13 s). Além disso, o HECSb aumentou o MDA no córtex pré-frontal (345,1 ± 191,9 vs. salina: 153,2 ± 191,9 nmol/g) e o GSH no corpo estriado (683,3 ± 118,1 vs. salina: 565,2 ± 118,1 µg/g). **Conclusão**: HECSb apresenta efeito ansiolítico em camundongos por meio da inibição do estresse oxidativo cerebral.

Palavras chave: efeito ansiolítico; depressão; modelos experimentais; balanço oxidativo; planta amazônica.

INTRODUCTION

A Neurobehavioral disorders such as anxiety and depression affect 63% and 59%, respectively, of the Brazilian population[1], being considered a public health problem. Both disorders have multifactorial origins, involve genetic, environmental, and psychosocial components, and are characterized by neurological manifestations, such as negative feelings, exacerbated fear, irritability, and lack of concentration, often related to neuroinflammation and impaired cellular redox homeostasis[1,2].

Anxiolytic and antidepressant drugs cause cognition-enhancing activity and may lead to adverse effects such as headache, nausea, decreased appetite and sexual desire, restlessness, insomnia, nervousness and tremors, memory loss, fatigue, sedation, drowsiness, decreased concentration and motor coordination, attention, and reflexes[3].

Calycophyllum spruceanum Benth. K. Schum. 1879 (Rubiaceae) is popularly known as “mulateiro” or “pau-mulato” in the Amazon region and used in folk medicine in the form of poultice, infusion, or decoction as antimicrobial, healing,

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antioxidant, analgesic, and anti-inflammatory[4]. Experimental studies demonstrated that hydroalcoholic extract from the barks of C. spruceanum (HECSb) has antinociceptive and anti-inflammatory actions via inhibition of the carragenan-induced paw edema and inflammatory nociception in mice[5], in addition to the activity of peroxidase and myeloperoxidase, key enzymes involved in inflammatory and oxidative disease[6].

The chemical analysis of HECSb revealed the presence of caffeoylquinic derivatives, small organic acids, and phenolic compounds5. Caffeoylquinic derivatives, the major constituent of HECSb, were shown to protect neuronal cell damage induced by glutamate via inhibition of reactive oxygen species (ROS)[7], along with prevention of cognitive decline, Aβ deposition in the brain[8], and antidepressant and anxiolytic effects in mice [9,10].

Therefore, the aim of this study was to evaluate the effect of the hydroalcoholic extract of *Calycophyllum spruceanum* barks (HECSb) on experimental models of anxiety and depression, along with brain oxidative stress markers.

MATERIALS AND METHODS

Preparation of the hydroalcoholic extract from Calycophyllum spruceanum

Barks of C. spruceanum were collected in Bujari (Acre, Brazil), BR-364, km 52, (-9.984004759277505, -67.33434478593384), and the exsiccate was deposited at UFAC Herbarium (No. 20307). Barks were washed, ground into fine particles, and weighed. The hydroalcoholic extract of C. spruceanum barks (HECSb) was prepared by percolation in 70% ethanol, concentrated at 45°C, lyophilized, and dissolved in distilled water before experimental protocols. HECSb chemical analysis by ultra-high pressure liquid chromatography tandem mass spectrometry (UHPLC-MS) revealed the presence of caffeoylquinic derivatives, small organic acids, and phenolic compounds[5].

Animals

Swiss male mice (25-30 g) (N=72), provided from the animal house of Centro Universitário Christus, were placed in polypropylene boxes at 26 +/- 2 ◦C, and maintained under light/dark cycles of 12/12 hours, receiving standard food and water “ad libitum”.

Ethical aspects

The experimental protocols followed the Guide for the Care and Use of Laboratory Animals, being approved by the Ethics Committee for the Use of Animals (protocol No. 022/2017-Unichristus).

Treatment

Animals received per oral (p.o.) HECSb (1, 10, and 100 mg kg–1) or sterile saline (0.9% NaCl) as control. Diazepam (1 or 2 mg kg–1) or imipramine (10 mg kg–1) were solubilized in saline and injected by intraperitoneal route (i.p.) as reference drugs. Thirty minutes after treatment, animals were submitted to behavioral tests (Open Field, Plus Maze, Hole Board, Tail Suspension) for evaluation of locomotor and exploratory activities, anxiety, and depression. Immediately after the behavioral tests, animals were euthanized, and the brain areas (prefrontal cortex, hippocampus, and striatum) were removed for quantification of the oxidative stress markers malondialdehyde (MDA) and reduced-glutathione (GSH).

Evaluation of locomotor and exploratory activities (Open Field test)

Animals were individually placed on the open-field apparatus (acrylic box: 30 x 30 x 15 cm, floor divided into 9 squares), and the locomotor and exploratory activities were evaluated during five minutes by the following behaviors: n° of crossings, rearing, and grooming[11].

Evaluation of Anxiety (Hole board test and Elevated Plus Maze)

To perform the Hole Board test, mice were placed individually on the hole board apparatus (transparent square box: 50 x 50 cm; 10 cm in height, 16 holes of 2 cm in diameter, equidistant from each other and from the edges) and evaluated during 5 minutes for the behavior of head dipping (No. and permanence time)[12].

For the Elevated Plus Maze test, animals were placed in the center of the elevated plus maze apparatus (two opposing open: 30 × 5 × 25 cm and closed arms: 30 × 5 × 25 cm), and the anxiety evaluated during 5 minutes by the parameters: time spent in the open and closed arms; number of entrances in the open and closed arms[13].

Evaluation of depressive behavior (Tail Suspension test)

Mice were individually suspended 50 cm from the floor by a tape fixed 1 cm from the tip of the tail for evaluation of depressive behavior during 5 min by the immobility time[14].

Quantification of oxidative stress markers (MDA and GSH)

Homogenates of prefrontal cortex (PFC), hippocampus (HC), and corpus striatum (CS) were prepared in sodium phosphate buffer (pH = 7.4) to quantify the content of MDA by the method of thiobarbituric acid - TBARS at A535 nm[15], and GSH at A412nm[16].

Statistical analysis

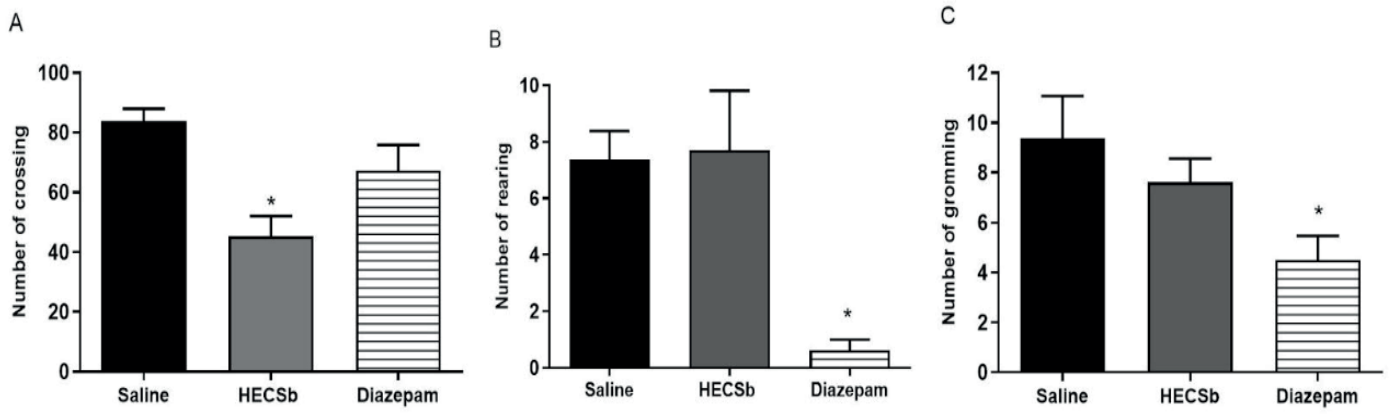
Results were expressed as mean ± standard error of the mean (SEM) using the GraphPad Prism 5.0 program (GraphPad Software, Inc., San Diego, CA, USA). Student's t-test (paired or unpaired) was used to compare two groups, and one-way Analysis of Variance (ANOVA) followed by Tukey's test. Differences were considered significant for p-values less than 0.05.

RESULTS

Effect of HECSb on locomotor and exploratory activity: Open field test

HECSb given to mice at 100 mg kg–1 (45.25 ± 38.63), but not at 1mg kg–1 or 10 mg kg–1, reduced by 33.5% the number of crossings compared to saline (83.87 ± 38.63) (Figure 1A). HECSb (100 mg kg–1) did not alter the n° of rearing (HECSb: 6.8 ± 0.5 vs. saline: 7.3 ± 0.5) (Figure 1B) or grooming (HECSb: 7.1± 2.2 vs. saline: 9.3 ± 2.2) (Figure 1C). The reference drug diazepam reduced both the number of rearing (0.625 ± 0.370) and grooming (4.5 ± 0.965).

Figure. 1. HECSb reduces exploratory activity in the open field test. Animals received p.o. HECSb (100 mg kg–1), 0.9% saline or i.p. diazepam (2 mg kg–1) 30 min before being evaluated for 5 min. (A) crossing, (B) rearing, (C) grooming. Mean ± SEM (n = 8). One-way ANOVA and Tukey test. *p<0.05 vs. saline.



Effect of HECSb on anxiety and depressive behavior

In the Hole Board test, HECSb (100 mg kg–1) increased by 1.7-fold the n° of head dips (HECSb: 32.57 ± 13.14 vs. saline: 19.42 ± 13.14) (Figure 2A) and by 2.3-fold the permanence time (HECSb: 19.12 ± 10.84 vs. saline: 8.28 ± 10.84 s) (Figure 2B). The reference drug diazepam did not alter any of these parameters (Figure 2A, B). In the Plus Maze test, HECSb (100 mg kg–1) increased 3.0-fold the time spent in the open arms (HECSb: 98.28 ± 65.71 vs. saline: 32.57 ± 65.71) (Figure 3A) and reduced by 32.3% the n° of entrances in the closed arms (HECSb: 8.35 ± 7.8 vs. saline: 16.25 ± 7.8) (Figure 3D). However, HECSb did not alter the time spent in the closed arms (Figure 3C) or the n° of entrances in the open arms (Figure 3B). Diazepam (1 mg kg–1) increased time spent in the open arms and n° of entrances in the open arms (Figure 3A, B), but reduced time spent in the closed arms and n° of entrances in the closed arms (Figure 3C, D). In the Tail Suspension test, HECSb (100 mg kg–1) did not alter the mice immobilization time (HECSb: 63.71 ± 12.69 vs. saline: 50.28 ± 5.13 s), while the reference drug, imipramine (10 mg kg–1), reduced this parameter (16.6 ± 6.04) (Figure 2C).

Figure 2. HECSb increases the mice's behavior of head dips in the hole board test and does not alter the immobility time in the tail suspension test. Animals received p.o. HECSb (100 mg kg–1), 0.9% saline or i.p. diazepam (1 mg kg–1) or imipramine (10 mg kg–1) 30 min before being evaluated for 5 min. Hole board test: (A) n° of head dips, (B) permanence time of head dips, Tail suspension test, (C) immobility time. Mean ± SEM (n = 8). One-way ANOVA and Tukey test *p<0.05 vs. saline.

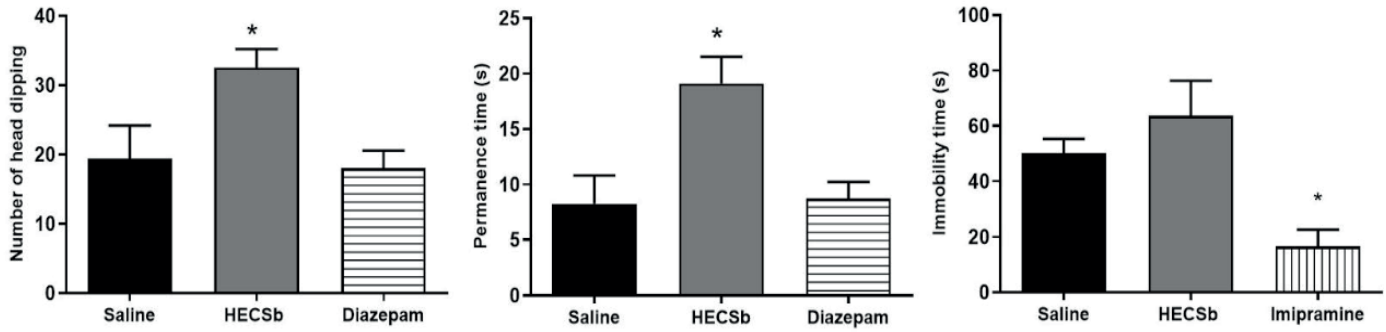
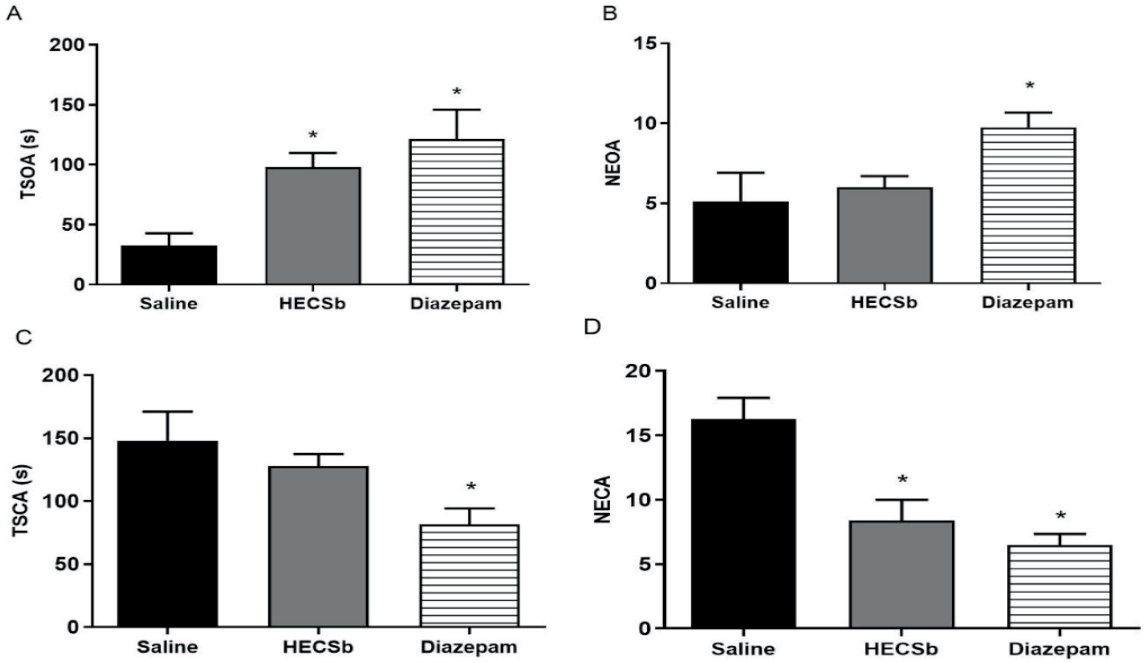


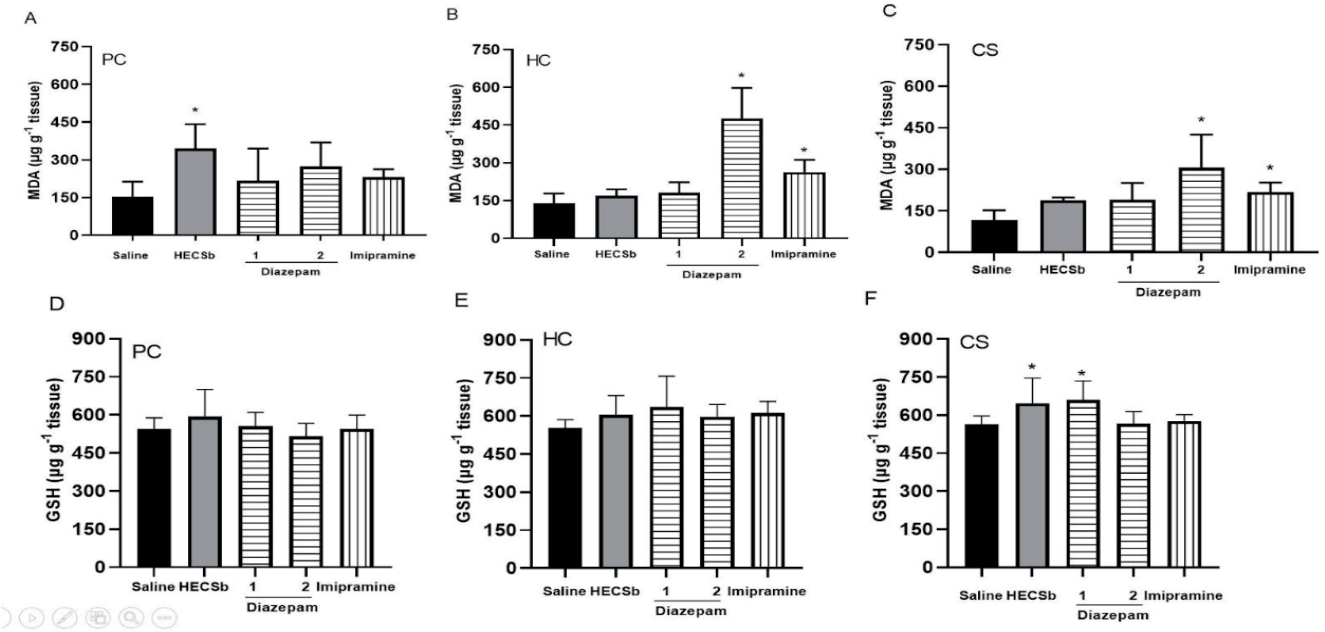
Figure 3. HECSb increases the mice's permanence time in the open arms and reduces the number of entries in the closed arms in the elevated plus maze test. Animals received p.o. HECSb (100 mg kg–1) or 0.9% saline, or i.p. diazepam (1 mg kg–1) 30 min before being evaluated for 5 min. Mean ± SEM (n = 8). One-way ANOVA and Tukey test *p<0.05 vs. saline. (A) Time spent in the open arms (TSOA); (B) Number of entrances in the open arms (NEOA); (C) Time spent in the closed arms (TSCA); (D) Number of entrances in the closed arms (NECA).



Effect of HECSb on MDA and GSH levels in brain areas

HECSb (100 mg kg–1) increased MDA in the prefrontal cortex (HECSb: 345.1 ± 191.9 vs. saline: 153.2 ± 191.9) (Figure 4A), but not at hippocampus (Figure 4B) or corpus striatum (Figure 4C). HECSb (100 mg kg–1) increased GSH only in corpus striatum (HECSb: 683.3 ± 118.1 vs. saline: 565.2 ± 118.1) (Figure 4F), but not in prefrontal cortex (Figure 4D) and hippocampus (Figure 4E). Diazepam and imipramine also increased MDA in the hippocampus (Figure 4B) or corpus striatum (Figure 4C) and GSH in the corpus striatum (Figure 4F).

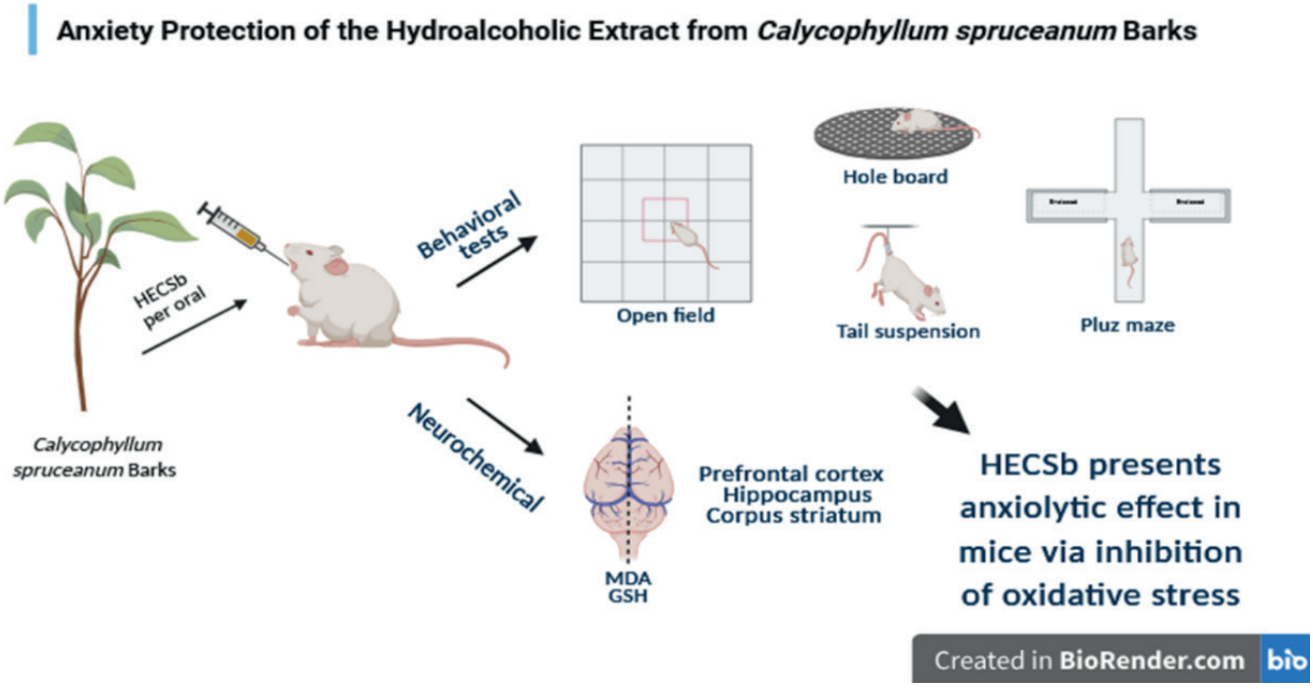
Figure 4. HECSb increases MDA in the prefrontal cortex and GSH in the corpus striatum. Mice received p.o. HECSb (100 mg kg–1) or 0.9% saline, and i.p. diazepam (1 or 2 mg kg–1) or imipramine (10 mg kg–1) 30 min before behavior tests. (A-C) MDA; (D-F) GSH. Mean ± SEM (n = 8). One-way ANOVA and Tukey test *p<0.05 vs. saline. PC: prefrontal cortex; HC: hippocampus; CS: corpus striatum.



DISCUSSION

In this study, the effects of the hydroalcoholic extract of C. spruceanum barks were observed in mice's neurobehavioral tests, such as open field, elevated plus maze, hole board, and tail suspension. These tests were used for screening the HECSb effects on the Central Nervous System (CNS), providing information on the locomotor, anxiolytic, and antidepressant activity.

Rodents naturally have the instinct to explore new areas, although they have a present aversion to remaining in open areas, since they feel exposed to predators[11]. In the open field test, the animals treated with HECSb showed a decrease in the number of crossings, indicating a reduction in locomotor and exploration behaviors, suggesting an anxiogenic behavior. On the other hand, they showed unaltered rearing and grooming, suggesting a lack of anxiety or stress in the open field environment. Of note, exposure to an open field represents a form of stress for a rodent[11].



To assess the anxiety behavior, the hole-board test was performed to evaluate the number of animal dives, since non-sedating doses of benzodiazepines and other anxiolytic drugs increase this behavior, while sedation is characterized by the reduction of dives[17]. The number of head dips over the edge of the open area is another marker of anxiety, with increased head dips signaling a reduced level of anxiety[18]. In our study, animals pre-treated with HECSb showed a significant increase in head dipping and in the permanence time at the hole board apparatus, indicating an anxiolytic effect.

The HECSb anxiolytic effect was confirmed in the elevated plus maze test. Animals treated with HECSb presented an increase in the permanence time in open arms, along with a reduction in the entrance number in the closed arms, suggesting an inhibitory anxiety-like behavior. A selective increase in the parameters corresponding to open arms reveals an anxiolytic effect, since anxiolytic drugs increase the time spent and number of entries in the open arms, which are typically avoided by rodents[13,18]. Conversely, anxiogenic substances reduce this open arm exploration, showing a preference for the closed arms[13].

Anxiety is also associated with depression and other alterations in cognition and fear. Drugs with an antidepressant effect can prolong the time during which the animal exhibits behavior and escape, which is inversely proportional to the time of immobility[19]. HECSb did not alter the mice's immobilization time, showing no depressant effect.

It is possible to suggest that HECSb presents anxiolytic effects, but does not induce, enhance, or suppress despair-like behavior. This effect could be assigned to caffeoylquinic derivatives, the major constituent of HECSb[5], since the literature has shown that these substances protect against neuronal damage induced by glutamate via inhibition of reactive oxygen species (ROS)[7], along with prevention of cognitive decline, Aβ deposition in the brain[8], and antidepressant and anxiolytic effects in mice[9,10]. Besides, mulateiro (C. spruceanum) is known for its antioxidant and anti-inflammatory properties[20,21]. Besides, HECSb had already been described for its anti-inflammatory effect [5].

In this line, the possible protection of HECSb on brain oxidative stress was investigated. HECSb increased both malondialdehyde (MDA) and glutathione (GSH) levels in the prefrontal cortex and corpus striatum, respectively, suggesting a complex and potentially contradictory situation related to oxidative stress, as seen with diazepam and imipramine. MDA is a biomarker of oxidative stress and the end product of lipid peroxidation, while

GSH is the most potent weapon against oxidative and nitrosative stress[22]. When the biochemical balance between oxidants and antioxidants is disturbed in favor of oxidants, "oxidative stress" conditions emerge and can promote neurological disorders, such as anxiety and depression[23]. Reduced levels of glutathione in the brain increase its vulnerability to oxidative stress and may be associated with the development and progression of several psychiatric disorders, including generalized anxiety disorder[24]. Although HECSb had increased both MDA and GSH, we believe that its protective effect could be due to glutathione, commonly referred to as the main antioxidant of the brain, playing a critical role in the maintenance of redox homeostasis24. Additionally, HECSb chemical analysis revealed the presence of caffeoylquinic derivatives and phenolic compounds[5], known to inhibit molecular signaling pathways activated by oxidative stress[7,9,25,10].

The main limitation of our research was the failure to elucidate the anxiolytic mechanism of HECSb, mainly due to the analysis of only a few oxidative stress markers, alongside the investigation of a single dose of HECSb. Despite this, our study provides an important contribution to the traditional knowledge of an Amazonian plant and its effects on the central nervous system.

In conclusion, HECSb exhibits anxiolytic effects in mice through modulation of brain oxidative stress

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