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Protective effect of Amazonian *Himatanthus sucuuba* extracts in *Drosophila melanogaster* exposed to Paraquat

Efeito protetor do extrato amazônico de *Himatanthus sucuuba* em *Drosophila melanogaster* expostas ao Paraquat

Efecto protector del extracto amazónico de *Himatanthus sucuuba* sobre *Drosophila melanogaster* expuesta a Paraquat

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Abstract
The Amazon rainforest is an essential source of scientific knowledge, so several research groups seek to understand the role of Amazonian compounds in diseases. Among the vast flora, Himatanthus sucuuba has a variety of therapeutic purposes and is used for the first time in the neuroprotection induced by Paraquat (PQ) in *Drosophila melanogaster*. In our study, we carried out phytochemical assays with the hydroalcoholic extract of *H. sucuuba*, revealing qualitatively classes of secondary metabolites and quantitatively total phenols (43.33 mg GAE/g extract-1), total flavonoids (44.09 mg GAE/G Extract- 1) and antioxidant activity via DPPH and ABTS. Furthermore, exposure of adult *D. melanogaster* (wild strain, *Canton Special*) to PQ for 15 days caused increased oxidative stress, as evidenced by elevated levels of protein carbonyls, lactate, and acetylcholinesterase and citrate synthase activities. However, the diet supplemented with *H. sucuuba* (0.1 mg/mL) for 15 days prevented damage from oxidative stress triggered by PQ. Our study aims to demonstrate the protective effect of *H. sucuuba* extract on *D. melanogaster* exposed to PQ. Based on our results, we suggest that extracts from the bark of H. sucuuba can prevent or minimize human diseases caused by oxidative stress. Therefore, further studies on the mechanisms involved in such activities will be necessary.
Keywords: *Drosophila melanogaster*; Neuroprotection; Antioxidants; Amazonian plants.

Resumo

A floresta amazônica é uma importante fonte de conhecimento científico, por isso, diversos grupos de pesquisa buscam compreender a atuação de compostos amazônicos nas doenças. Dentre a vasta flora, *Himatanthus sucuuba* apresenta uma variedade de fins terapêuticos, sendo aqui, utilizada pela primeira vez na neuroproteção induzida pelo Paraquat (PQ) em *Drosophila melanogaster*. Em nosso estudo, realizamos ensaios fitoquímicos com o extrato hidro alcoólico de *H. sucuuba* revelando qualitativamente classes de metabólitos secundários e quantitativamente fenóis totais (43,33 mg EAG/g extrato^{-1}), flavonoides totais (44,09 mg EAG/G Extrato^{-1}) e atividade antioxidante via DPPH e ABTS. Ainda, a exposição *D. melanogaster* adultas (linhagem selvagem, *Canton Special*) ao PQ por 15 dias causou elevado estresse oxidativo, como evidenciado pelos elevados níveis de proteínas carboniladas, lactato e atividades de acetilcolinesterase e citrato sintase. Entretanto, a dieta suplementada com *H. sucuuba* (0.1 mg/mL) por 15 dias impediu os danos do estresse oxidativo desencadeados pelo PQ. O nosso estudo visa demonstrar o efeito protetor do extrato de *H. sucuuba* em *D. melanogaster* exposta ao PQ. Baseados em nossos resultados, sugerimos que os extratos provenientes das cascas de *H. sucuuba* podem prevenir ou minimizar doenças humanas causadas pelo estresse oxidativo, para tanto, demais estudos relacionados aos mecanismos envolvidos em tal ação serão necessários.

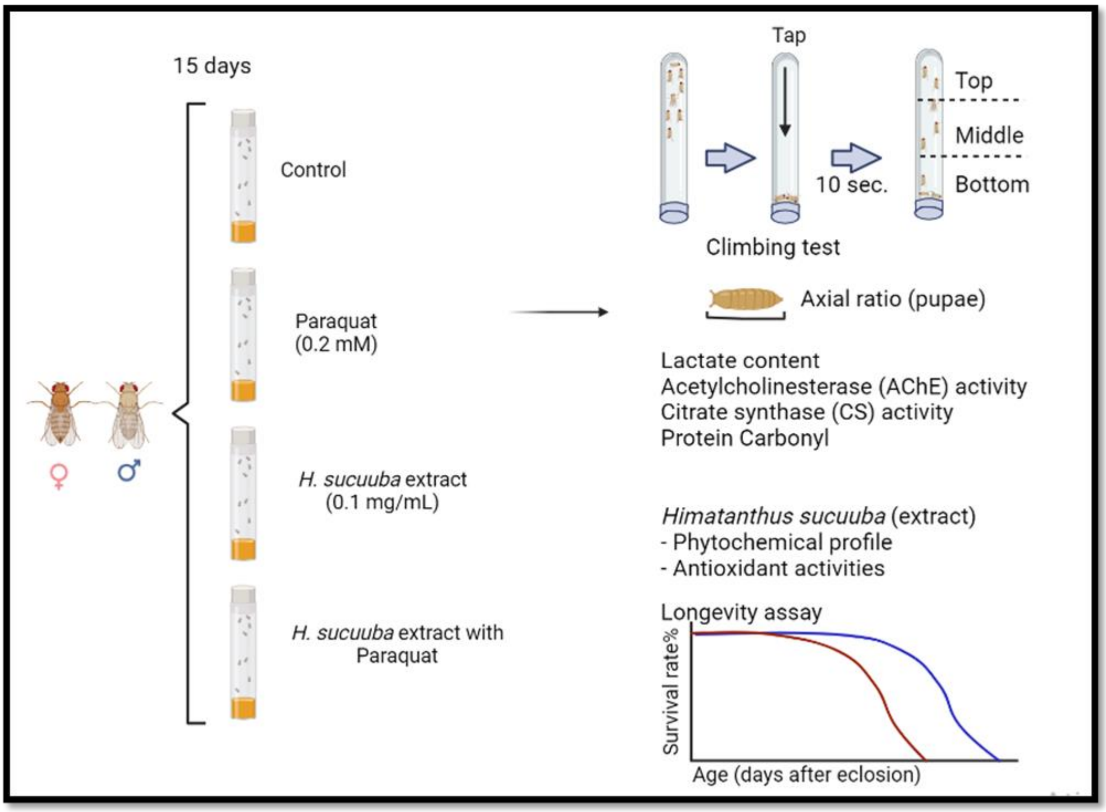
Palavras-chave: *Drosophila melanogaster*; Neuroproteção; Antioxidantes; Plantas amazônicas.

Resumen

La selva amazónica es un importante manantial de conocimientos científicos, por eso diversos grupos de investigación buscan comprender la acción de los compuestos amazónicos sobre las enfermedades. Entre la extensa flora, el *Himatanthus sucuuba* tiene una variedad de efectos terapéuticos y, en este caso, se utilizó por primera vez en la neuroprotección inducida por el Paraquat (PQ) en *Drosophila melanogaster*. En este estudio, se realizaron ensayos fitoquímicos con el extracto hidroalcohólico de *H. sucuuba* mostrando cualitativamente las clases de metabolitos secundarios y cuantitativamente los fenoles totales (43,33 mg EAG/g Extracto-1), los flavonoides totales (44,09 mg EAG/G Extracto-1) y la actividad antioxidante por DPPH y ABTS. Por otra parte, la exposición de adultos de *D. melanogaster* (cepa salvaje, *Canton Special*) frente al PQ por 15 días, causó un alto estrés oxidativo, como muestran los niveles elevados de proteínas carboniladas, el lactato y actividades de acetilcolinesterasa y por el citrato sintasa. Además, una dieta suplementada con el extracto de *H. sucuuba* (0,1 mg/mL) por 15 días evitó los daños por el estrés oxidativo provocado por el PQ. Nuestro estudio es demostrar el efecto protector del extracto de *H. sucuuba* en *D. melanogaster* sometida al PQ. De acuerdo nuestros resultados, sugerimos que los extractos de la cáscara de *H. sucuuba* podrán prevenir o minimizar las enfermedades del ser humano causadas por el estrés oxidativo.

Palabras clave: *Drosophila melanogaster*; Neuroprotección; Antioxidantes; Plantas de la Amazonia.

Graphical abstract



Source: Authors.

1. Introduction

The incidence of neurodegenerative diseases increases in the world population, and the prevalence of neurodegenerative diseases such as Alzheimer's and Parkinson's (PD) disease suggests improving the coming years. Alzheimer's disease (AD) is the most common form of dementia, accounting for approximately 50-60% of all cases and representing a primary public health concern with significant social and economic impact (Wimo et al., 2009).

The life expectancy in recent decades has caused an increase in the prevalence of degenerative diseases, which are more common in older adults. Dementia is one of the most common conditions. Despite its massive impact on individuals' health, it has been neglected in Brazil and other Latin American countries, not only by policymakers but also by the general public. Studies indicate that the prevalence of dementia will increase fourfold between 2015 and 2050 in Latin America (Prince et al., 2015). Brazil's population is aging rapidly, making dementia a growing public health priority (Nitrini & Ferri, 2020).

Phytochemicals are used for various purposes such as migraine treatment, neuroprotection, anxiolytic effect, antianxiety, antidepressant, sedative, anticonvulsant, prevention, anti-aging, and therapy for neurodegenerative diseases (Rajapakse & Davenport, 2019; Kumar & Kumar, 2020; Aguirre-Hernández et al., 2007; Hattesohl et al., 2008; Goyal et al., 2009; Gasiorowski, 2011; Costa et al., 2016; Castillo-Bautista et al., 2019; Cui et al., 2020; Li et al., 2021;` Zhou et al., 2021). Studies show that these "anti-aging herbs," such as Panax ginseng and wolfberry (*Lycium barbarum*), are often multifunctional and may protect our bodies through different mechanisms. Growing lines of evidence have suggested that these herbs are potential candidates for preventing or treating aging-related neurological disorders (Kim et al., 2007).

Apocynaceae is one of the most prominent families in the plant kingdom. Members of this family are distributed in tropical, subtropical, and temperate zones. The plants in this family are mainly trees and shrubs (Bhadone et al., 2018). The search for plant-derived acetylcholinesterase (AChE) inhibitors has accelerated, given of the benefits of these drugs not only in treating of AD (Perry et al., 1994) but in other forms of dementia.

The Apocynaceae family is reported to have AChE inhibitory potential in this study (*Himatanthus* Wild. ex. Schult. (Apocynaceae) is a genus composed of about thirteen species of trees and shrubs, widely distributed in Central and South America, particularly in Brazil (Herrera-Calderón et al., 2021). *Himantathus sucuuba* has biological activity: antimalarial, antibacterial, antifungal, anthelmintic, antileishmanial, anti-inflammatory, analgesic, antidepressant, immunoregulatory, cytotoxic, and genotoxic. However, recent studies have been looking at the enormous potential for protection against diseases associated with leishmaniasis, bacterial or fungal infection, depression, oxidative stress, and cytotoxicity or genotoxicity (Silva et al., 2021).

According to Romanova and Sweedler (2018), although the most significant percentage of animal research in neuroscience is conducted using mammalian model systems, the importance of other vertebrate and invertebrate models is well recognized. *Drosophila sp*, *Caenorhabditis elegans*, *Dario rerio*, yeast models, and other organisms have been used to gain insights into how proteins implicated in these neurodegenerative disorders cause cellular and organism pathology toxicity (Dawson et al., 2018; da Silva et al., 2021; Phulara et al., 2021; Pradhan et al., 2021; Cao et al., 2018; Pérez-González & Jiménez-Arellanes, 2021; Tello et al., 2022). Herein, we aimed to describe the protective effects of *H. sucuuba* against oxidative stress-mediated by Paraquat exposure using *Drosophila melanogaster* as a model.

2. Materials and Methods

2.1 Extract formulation

The plant samples (bark) of *Himatanthus sucuuba* at the Central Market of Manaus, Amazon, Brazil, were obtained commercially. A popular place for being served almost exclusively by extractive exploration. In preparing the plant extract, we used percolation in extractor liquid (ethanol: water 90% v/v) in the proportion of 0.833 mg/mL. Then, the extract was concentrated and stored at -20°C in methylparaben (nipagin) (Sigma cat no. 47889) (3%) until the ideal time for use.

2.2 Phytochemical profile

2.2.1 Qualitative phytochemical assays

For the saponin test, 0.5 mL of the plant extract was homogenized in 5 mL of distilled water (70º C). Vigorous agitation for 2 minutes and the formation and permanence of foam after 30 minutes were analyzed (Ezeonu & Ejikeme, 2016). The neutral detergent Extran MA02 (Merck cat no. 1.07553.500) was used as a control in this experiment.

In the phenols and tannins test, 0.5 mL of the plant extract in 5 mL of distilled water and three drops of 1% iron chloride (CRQ Chemical Products cat no. C1003480500) in an alcoholic solution solubilized. In such an experiment, test positive for phenols when a blue-red coloration occurs in the solution. However, the formation of blue or green precipitates will indicate the presence of tannins (Silva et al., 2013).

Regarding anthraquinones, we homogenized 1 mL of *H. sucuuba* extract in toluene (Dinâmica Ltda cat no. 1987-1) and 10% ammonium hydroxide (Synth cat no. H2001.01.BJ) solution. The presence of pink, red, or violet coloration in the aqueous phase indicates the presence of anthraquinones (Ojha et al., 2020).

We boiled the solution in 1% HCl for phlorotannins to observe the red deposit (Okeniyi et al., 2015). Yet, for terpenoids (Salkowski test), the 1mL of solution with 0,4 mL chloroform and carefully mixed adding 0.6 mL concentrated H_2SO_4 such that a layer could be formed to observe reddish-brown coloration (Okeniyi et al., 2015), and to the alkaloid test, modified according to Matos (2009).

2.2.2 Determination of total polyphenols

The *H. sucuuba* extract was homogenized in sodium carbonate (Dinâmica Ltda cat no. 1466-7) solution and Folin-Ciocalteu reagent (Dinâmica Ltda cat no. P.01.0132.000.00.78). After 30 minutes of incubation, readings at 760 nm were taken, with gallic acid (Sigma cat no. G7384) (10 – 100 ug/mL) as the standard sample. The results expressed the polyphenol content in mg of gallic acid equivalent (GAE)/gram of extract (Wolfe et al., 2003).

2.2.3 Determination of total flavonoids

With some modifications, total flavonoid content was determined using the aluminum chloride colorimetric method (Chang, 2002). *H. sucuuba* extract was prepared at a concentration of 500 µg/mL. 500 µL of the diluted extract was added to the tube along with 500 µL of the 60% acetic acid solution, 1 mL of the 5% Aluminum Chloride solution, 2 mL of pyridine, and 6mL of MilliQ Water. The mixture was stirred and kept in the dark. After 30 minutes, the absorbance was evaluated using the spectrophotometer at a wavelength of 420 nm. Addionatily, we used rutin standards (10, 20, 40, 60, 80, and 100µg/mL) from the solution with 1000µg/mL. Total flavonoid content was expressed as mg rutin equivalent (RE)/gram of extract using the straight-line equation based on the calibration curve.

2.3 Antioxidant activities

2.3.1 DPPH radical scavenger assay

In the plant extract (0.1 mg/mL), we added 50 µM of the DPPH radical (2,2-diphenyl-1-picryl-hydrazyl, Sigma cat no. D9132) (Sharma & Bhat, 2009), after which an incubation at room temperature in the dark for 30 minutes, reading the samples at 517 ηm. Ascorbic acid (Sigma cat no. A4544) (0.5 - 10 µg/mL) was used as a positive control in this experiment. The results were obtained according to the method of Kumara et al. (2018).

2.3.2 ABTS radical scavenger assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) solution was prepared using 5 mg of ABTS and 6 mg of potassium persulfate (Sigma cat no. 906735) and diluted in 5 mL of distilled water. Samples were prepared with 1 mg of each extract in 2 mL of DMSO, totaling 8 samples for analysis. Readings were performed at 754 nm, with gallic acid (Sigma cat no. 398225) being the standard for the experiment (Torres et al., 2017; Santos et al., 2022).

2.4 *Drosophila melanogaster* stock

Flies (wild-type, *Canton Special*) were grown and maintained at 25 +- 1ºC and 12:12 h light/dark cycle and separated into four groups, which received: (1) standard diet containing cornmeal (6.5% m/v), agar (1% m/v), yeast (6.5% m/v) and Nipagin (3% v/v) (Depetris-Chauvin et al., 2017(2) standard diet supplemented with *H. sucuuba* extracts; (3) standard diet containing Paraquat at a final concentration of 0.2 mg/mL (Souza et al., 2017); (4) standard diet containing *H. sucuuba* extracts and PQ at a final concentration mentioned above.

2.5 Determination of the pupal volume of *D. melanogaster*

Flies adults were maintained under 25 ± 1ºC and 12:12 h light/dark cycle. After 72 hours, the larvae begin the metamorphosis into adult animals. To evaluate the effect of the *H. sucuuba* (0.05 – 0.25 mg/mL) extract against the volume of *D. melanogaster* pupae, we calculated the volume or axial ratio (length/width) of 40 pupae (Guan et al., 2006) under the conditions proposed in the diet. Each sample was run in quintuplicate.

2.6 *Himatanthus sucuuba* extract toxicity test

Drosophila melanogaster adults were maintained under 25 ± 1ºC and 12:12 h light/dark cycle for 30 days. The cultures were separated into two groups: (1) *D. melanogaster* that received a standard diet (Depetris-Chauvin et al., 2017); (2) *D. melanogaster* fed with a standard diet supplemented with *H. sucuuba* extracts in different concentrations (0.05 – 0.25 mg/mL). Each sample was run in quintuplicate.

2.7 Lifespan and Climbing assay

For lifespan, *D. melanogaster* (100/vial) was counted daily for 30 days. The climbing activity was measured using a counter-current apparatus (Simon et al., 2012), posteriorly conducted for 15 days. Results were obtained by phototaxis index $\sum(i*Ni)/N$ with N representing the total number of *D. melanogaster* assayed, i the scale number, and Ni number of flies in the tube at the end of the experiment (Ziegler et al., 2015). Each sample was run in quintuplicate.

2.8 Citrate synthase (CS) activity

Fifteen thoraces (dissected and stored at -20°C in protease inhibitor) were submitted to homogenization in Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (vol/vol) (Spinazzi et al., 2012). Then, the homogenates were centrifugated at 9000xg for 30 min at 4ºC, the supernatant was collected, and protein concentration was determined. For the CS activity, we added 0.01 mg protein to ~170 μL Tris buffer containing 10 mM Acetyl-CoA (Sigma cat no. A2181), 1 mM 5’5’-Dithiobis-2-nitrobenzoic acid (DTNB) (Sigma cat no. D8130), and 10 mM oxaloacetate (Sigma cat no. O4126) (Spinazzi et al., 2012). The reduced CoA (CoA-SH) formed by CS activity converts the DTNB into 2-nitro-5-benzoic acid (TNB). CS activities were evaluated by the rate of TNB formation, measured spectrophotometrically at 412 ηm according to Srere (1969) using a Model Cary 50MPR Varian Spectrophotometer (Varian Ltd., Melbourne, Australia). Each sample was run in quintuplicate.

2.9 Lactate content

Fifteen thoraces (dissected and stored at -20°C in protease inhibitor) were macerated and homogenized in 0.1 M sodium phosphate buffer pH 7.4. The homogenate was centrifuged at 10000xg for 10 min at 4 °C, and the supernatant was collected. Lactate content was measured by the lactate oxidase method. The reaction (5 min) was started by adding 10 µL sample to 0.2 mL of assay buffer, according to manufacturer instructions (Labtest, Brazil, cat. no. #138-1/50). Absorbance was monitored spectrophotometrically at 550 ηm using a Model Cary 50MPR Varian Spectrophotometer (Varian Ltd., Melbourne, Australia). Each sample was run in quintuplicate and the absorbance values were expressed mg/dL of lactate per total amount of protein in the sample.

2.10 Acetylcholinesterase (AChE) activity

Thoraces of *D. melanogaster* were homogenized in 100 mM sodium phosphate buffer (containing inhibitor protease), pH 7.4, to disrupt the cells. Homogenates were centrifuged at 9000x*g* for 30 min at 4ºC (Souza et al., 2017). The supernatant (0.01 mg protein) was incubated with 100 mM sodium phosphate buffer, pH 7.4, containing 150 mM acetylthiocholine (Sigma cat no. A5751) and 1 mM DTNB. AChE activity was determined spectrophotometrically using a Model Cary 50MPR Varian Spectrophotometer (Varian Ltd., Melbourne, Australia), according to Ellman et al. (1961). The results were expressed as nmol conjugated formed/min/mg protein. Each sample was run in quintuplicate.

2.11 Protein carbonyl

Thoraces of *D. melanogaster* were homogenized in ice-cold 100 mM Tris buffer, pH 7.4, and centrifuged at 1500x*g* for 10 min at 4ºC. Supernatants were separated into two fractions, incubated with 10% trichloroacetic acid (TCA) (Sigma cat no. T4885), and centrifuged at 5000x*g* for 10 min at 4ºC. One pellet was resuspended in 10 mM 2,4-dinitrophenylhydrazine (DNPH) (Sigma cat no. D199303) in 2.5 M HCl, while the other were resuspended only in 1 mL 2.5 M HCl (Reznick & Packer, 1994). After incubation (1 h, 37°C, in the dark), samples were transferred to the ice for 10 min, 10% TCA added, and centrifuged at 5000x*g* for 5 min at 4ºC. The pellets were washed three times with 1 mL ethanol/ethyl acetate (1:1) and centrifuged at 5000x*g* for 5 min at 4ºC. The pellets were dissolved in 6 M guanidine (Sigma cat no. 50950) and maintained stirring for 40 min. Protein carbonyl content was assessed spectrophotometrically at 340 ηm using a Model Cary 50MPR Varian Spectrophotometer (Varian Ltd., Melbourne, Australia).

2.12 Protein assay

The protein concentration was determined by Bradford assay using BSA (Sigma cat no. A6003) as the standard (Bradford, 1976).

2.13 Statistical analyzes

The data are presented as mean ± S.E.M. N means the number of flies per group used in each experiment. Statistical analysis was performed using one-way ANOVA for multiple comparisons using the GraphPad ©Prism version 8.0 software (San Diego, CA, USA). Results were considered significant at $P < 0.05$.

3. Results

3.1 Phytochemical profile of *H. sucuuba*

According to the results obtained by phytochemical screening of the hydroalcoholic extract of the bark of the *Himatanthus sucuuba* plant revealed the presence of phenols, quinones, terpenoids, and gallic tannins; saponins, tannins, and anthraquinones were not detected (Table 1).

Table 1 - Analysis of metabolites from *H. sucuuba* hydroalcoholic extract.

Phytochemical compounds	**Observation**
Saponins	-
Phenols	+
Tannins	-
Quinones	+
Alkaloids	+
Anthraquinones	-
Floblatanins	-
Terpenoids	+
Gallic tannins	+

Legend: (-) not detected; (+) detected. Source: Authors.

The results for total phenolic and total flavonoid contents in the hydroalcoholic extract of *H. sucuuba* the contents were found as their mean values, respectively (mg GAE/100 g of extract) (Table 2).

Table 2 - Total Phenolic and total flavonoid contents of *H. sucuuba* extract.

Test	**Values**
Total phenolic compounds	43.33 mg GAE/g
Total flavonoid compounds	44.09 mg GAE/g

Legend: mg GAE/g: miligrams of galic acid equivalent to grams of vegetal extract. Source: Authors.

Antioxidant activity

The antioxidant activity of the *H. sucuuba* were determined in vitro using DPPH (31,98 µg/mL) and ABTS (49,51 µg/mL) radical scavenging activities (Table 3).

Table 3 - Antioxidant potential of the bark from the *H. sucuuba* extract.

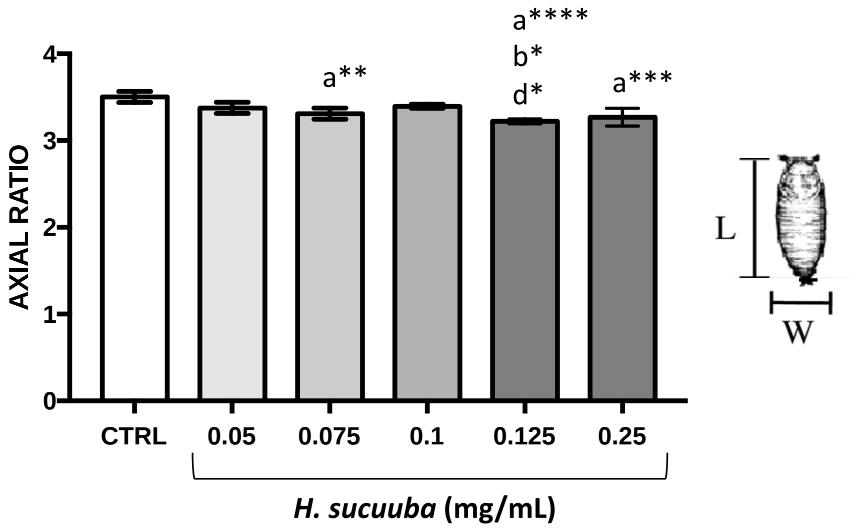
Test	**Average of Inhibition (%)**
DPPH	31,98[1] µg/mL
ABTS	49,51[2] µg/mL

[1]Expressed in % of inhibition of DPPH radical
[2]Expressed in % of inhibition of ABTS radical
Source: Authors.

3.2 Toxicity of the *H. sucuuba* extract on the developing *Drosophila melanogaster*

During the pupal stage, the body shape of a fly can be conveniently described by its cuticle's axial ratio (AR, length/width). Our results demonstrate ratios referring to concentrations of the *H. sucuuba* extract feeding *D. melanogaster* larvae for five days (Figure 1).

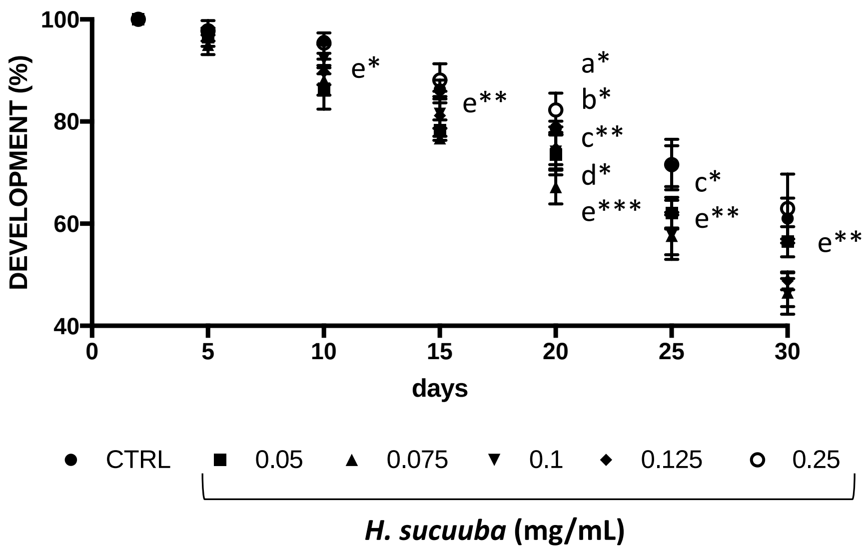
Figure 1 - Axial ratio on *D. melanogaster* pupae.



The body shape of a pupa can be described by the length (L) / width (W). The values represent the mean ± S.E.M of five experiments. The results were considered statistically significant when *p<0.05, **p<0.01, ****p<0.0001: [a] *vs* CTRL, [b] *vs* 0.05 mg/mL, [c] *vs* 0.075 mg/mL, [d] *vs* 0.1 mg/mL. Source: Authors.

Flies fed different concentrations of the *Himatanthus sucuuba* extract exhibited a significant decrease in survival until day 30 of the experiment (Figure 2). Locomotor ability of *D. melanogaster* diets supplemented with plant extracts was performed for 30 days, but data with antioxidant effects (0.1 mg/mL) were significant at 15 days (Figure 3).

Figure 2 - The concentration of the *H. sucuuba* extracts on *D. melanogaster* development.

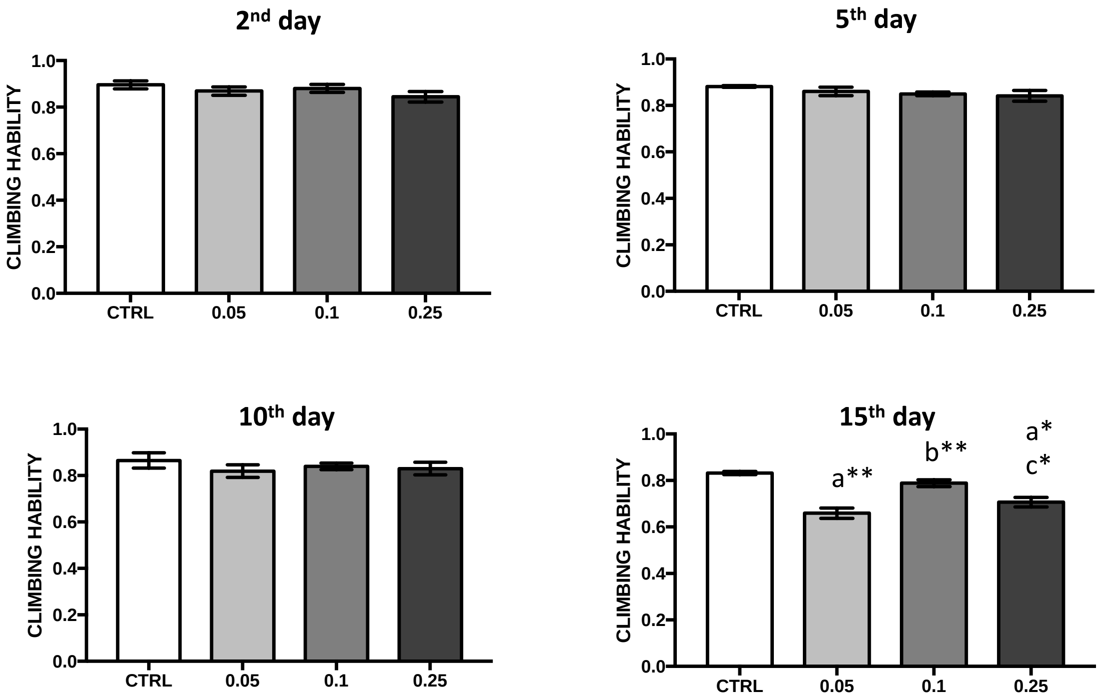


The values represent the mean ± S.E.M of five experiments. The results were considered statistically significant when *p<0.05, **p<0.01, ***p<0.0001: [a] *vs* CTRL, [b] *vs* 0.05 mg/mL, [c] *vs* 0.075 mg/mL, [d] *vs* 0.1 mg/mL, [e] *vs* 0.125 mg/mL. Source: Authors.

3.3 Locomotor activity (Climbing test)

The climbing test on day 15 differed from the control showing significant differences concerning the concentrations of *H. sucuuba* extract versus the control ($p<0.05$, $**p<0.01$).

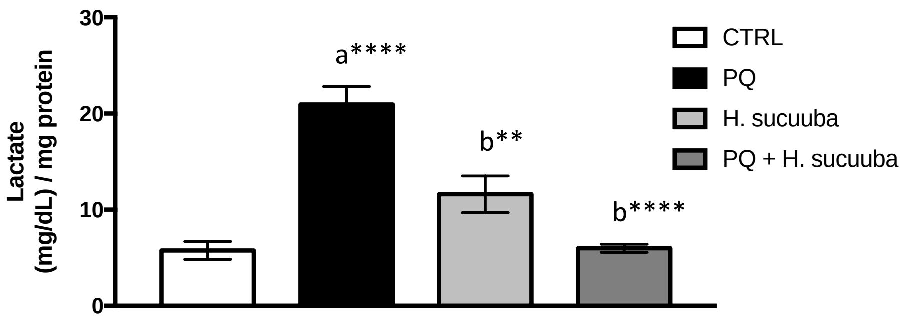
Figure 3 - Climbing activity of *D. melanogaster* fed a diet supplemented with *H. sucuuba*.



The flies were fed with standard diet or standard diet supplemented with *H. sucuuba* extracts in different concentration (0.05 – 0.25 mg/mL). The values represent the mean ± S.E.M of five experiments. The results were considered statistically significant when $*p<0.05$, $**p<0.01$: [a] *vs* CTRL, [b] *vs* 0.05 mg/mL, [c] *vs* 0.1 mg/mL. Source: Authors.

PQ supplementation induced enzymatic lactate activity. Enzyme lactate is a key enzyme of energy metabolism in the brain, catalyzing the reduction of pyruvate to lactate. Its potential was investigated because of the relevance of this enzyme and the ability to highlight neuronal oxidative metabolism that depends on lactate derived from astrocytes where they are most abundant in the brain. Exposure to PQ increased the enzyme lactate activity and treatment with the *H. sucuuba* plant extract prevented the change (Figure 4).

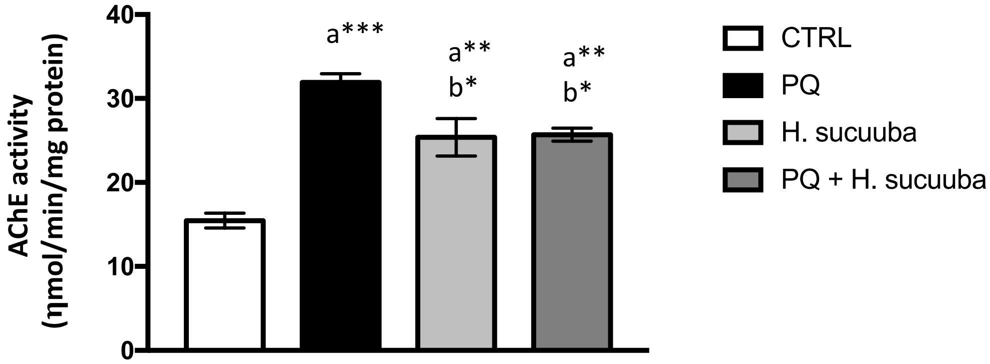
Figure 4 - Lactate concentration in thoraces of *D. melanogaster* fed on a diet supplemented with PQ and/or *H. sucuuba* extracts.



The values represent the mean ± S.E.M of five independent experiments. The results were considered statistically significant when $*p < 0.05$, $**p < 0.01$, $***p < 0.001$: [a] vs CTR, [b] vs PQ. Source: Authors.

AChE levels exposed to paraquat is shown to be high. Exposure to *H. sucuuba* extract with Paraquat restored AChE activity to the levels found in flies in the control group. The group with *H. sucuuba* extracts also obtained significant data compared to the control group (Figure 5).

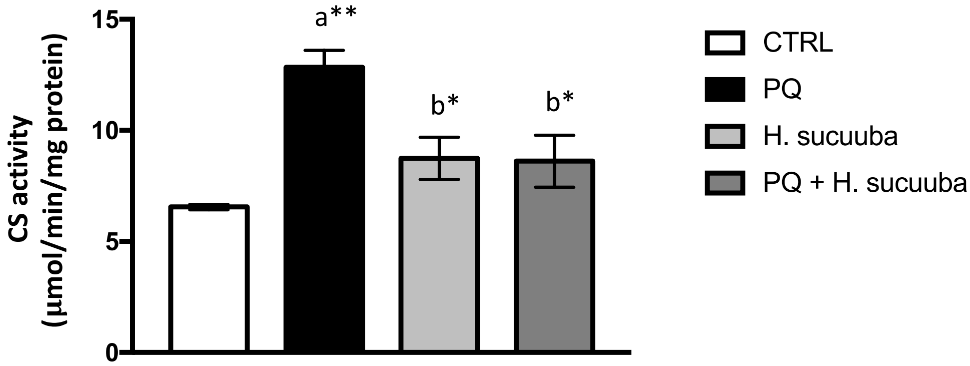
Figure 5 - AChE activity in thoraces of *D. melanogaster* fed on a diet supplemented with PQ and/or *H. sucuub*a extracts.



The values represent the mean ± S.E.M of three independent experiments. The results were considered statistically significant when *p < 0.05, **p < 0.01, ***p < 0.001: [a] vs CTR, [b] vs PQ. Source: Authors.

PQ exposition induced citrate synthase (CS) activity, an enzyme of the Krebs Cycle that can indicate mitochondrial content or improve in several mitochondrial tissues. However, flies fed with *H. sucuuba* showed the same physiological effect as control animals (Figure 6).

Figure 6 - CS activity in thoraces of *D. melanogaster* fed on a diet supplemented with PQ and/or *H. sucuub*a extracts.



The values represent the mean ± S.E.M of three independent experiments. The results were considered statistically significant when *p < 0.05, **p < 0.01: [a] vs CTR, [b] vs PQ. Source: Authors.

PQ influenced an intracellular redox cycle that improved oxidative stress in the muscle expressed by protein carbonyl. Also, the diet PQ supplemented with *H. sucuuba* was able to block the redox cycle and ROS generation by PQ (Figure 7).

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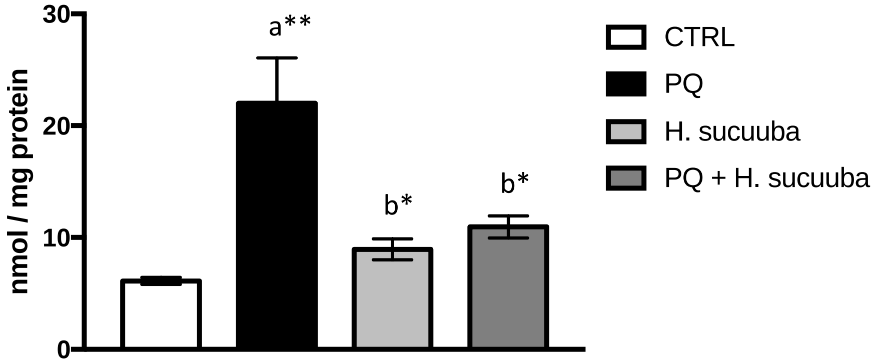
Figure 7 - Protein carbonyl content.



The values represent the mean ± S.E.M of three independent experiments. The results were considered statistically significant when *p < 0.05, **p < 0.01: [a] vs CTR, [b] vs PQ. Source: Authors.

4. Discussion

The secondary metabolites found in plants belonging to the families Acanthaceae, Apocynaceae, Amaryllidaceae, Angelicae, Araceae, Asclepiadaceae, Berberidaceae, Buxaceae, Combretaceae, Compositae, Coniferae, Cyperaceae, Ebenaceae, Ericaceae, Euphorbiaceae, Fumariaceae, Gentianaceae, Guttiferae, Lamiaceae, Leguminosae, Lilliaceae, Lycopodiaceae, Malvaceae, Magnoliaceae, Menispermaceae, Mollugi naceae, Moraceae, Musaceae, Nelumbonaceae, Papa veraceae, Piperaceae, Rubiaceae, Rutaceae, Sapotaceae, Solanaceae, and Tamaricaceae has alkaloids, terpenoids, glycosides, and coumarins, compounds used in neuro diseases therapeutic applications (Mukherjje, 2007).

Compounds of the Amazon plants with different proven properties, combined with in vitro and in vivo tests, contribute to developing new treatments (Yamaguchi & Souza, 2020). Herein, our results demonstrated the presence of quinones, alkaloids, terpenoids, gallic tannins, phenols. Also, we quantified the total phenolic (46.06 mg GAE/g extract) and flavonoid (44.09 mg GAE/g extract) compounds. A similar effect in Amazonian plants as *B. japurensis* (59.4 mg GAE/g extract), *C. spruceanum* (60.2 mg GAE/g extract), *M. guyanensis* (58.7 mg GAE/g extract) (Vargas et al., 2016), *B. gasipaes* (30 mg GAE/g extract) (Santos et al., 2015), and seeds of *R. gardneriana* (54.11 mg GAE/g extract) (Montero et al., 2018).

The ABTS assay is based on the generation of a blue/green ABTS+ that antioxidants can reduce, whereas the DPPH assay is formed by lowering the purple DPPH to 1,1-diphenyl-2-picrylhydrazine. Studies with DPPH and ABTS assays with Amazonian plants such as *B. japurensis* (8.4 µg/mL and 12.3 µg/mL), *C. spruceanum* (7.5 µg/mL and 5 µg/mL), *M. guyanensis* (28.4 µg/mL and 8.2 µg/mL), *P. nitida* (49.9 µg/mL and 38.5 µg/mL), and *P. olacoides* (29.7 µg/mL and 8.7 µg/mL) (Vargas et al., 2016) demonstrated different scavenging activity of the natural compounds depends of the source to secondary metabolism. Our results showed 31.98 µg/mL for DPPH and 49.51 µg/mL for ABTS assays, a significant impact for the bark from *Himatanthus sucuuba* compared to the Amazonian plants described.

The neurotransmitter acetylcholine in the motor neuron receptors contributes to the muscle cells' muscular contraction (Jones, 2009). To measure the toxic effect of *H. sucuuba* extracts, we tested different concentrations (0.05 - 0.25 mg/mL), and only 0.1 mg/mL showed a protective effect against cellular aging after 15 days. Afterward, PQ intoxication was used as a cellular damage model to analyze the neuroprotective effect of *H. sucuuba* extracts.

Natural products extracted from plants have demonstrated acetylcholinesterase inhibitory action, such as physostignin (alkaloid) extracted from the seeds of *Physostigma poisonum*, terpenoids as found in the essential oil of *Salvia lavandulaefolia*, tarazerol (pentacyclic triperpenpoide) obtained from the roots of *Clitoria ternatea*, as well as thymosaponin, a steroidal saponin from the roots of *Anemarrhena asphodeloides* (Zengin et al., 2016; Abeysinghe et al., 2020; Yagi, et al., 2020).

Additionality, our results showed PQ exposition increased the activity of acetylcholinesterase (AChE), improved the acetylcholine hydrolysis, and reduced muscular contraction in the muscle cell membranes. Diet supplemented with *H. sucuuba* demonstrated a protective effect in AChE activity against damage caused by PQ intoxication, as reported by Carmo et al. (2021).

In *Drosophila melanogaster*, perineural glial cells have enzymes responsible for metabolizing trehalose, whose metabolites, including lactate, are secreted and absorbed by neurons. Additionally, astrocytic glycolysis removes glucose from the endothelium and generates lactate, destined for the citrate cycle and the respiratory chain in neuronal mitochondria (Volkenhoff et al., 2015). However, raised lactate levels are a valuable biomarker of neuronal degeneration related to mitochondrial dysfunction detected in Alzheimer's disease (Harris et al., 2016). In this study, PQ ingestion by *D. melanogaster* significantly increased lactate concentration in thoraces, an effect prevented by *H. sucuuba* extracts ingestion in combination.

The normal function of metabolism illustrates the importance of the delicate balance between oxidant and antioxidant processes. However, natural or synthetic compounds can induce oxidative stress, a classic mechanism for cellular damage and disease dysfunction (Frohnet & Bernlohr, 2013; Scialò et al., 2020). Our results showed PQ improved a redox cycle in the thoraces of *D. melanogaster* that increased the citrate synthase activity (CS, an enzyme of Krebs Cycle indicative of mitochondrial content) as a protective effect against the redox environment cellular, reduced ATP production, and higher oxidative stress (Hosamani & Muralidhara, 2013; Bélanger et al., 2011). In a restored condition, *H. sucuuba* extracts in 0.1 mg/mL for 15 days demonstrated a significant effect on oxidative stress, as evidenced by protein carbonyl levels and CS activity.

Finally, based on this study, we proposed using *D. melanogaster* in the initial screening of prospective Amazonian extracts in neuromuscular protection, especially after the chronic exposition of PQ. Also, these results indicate more research into the possible compounds that may be related to this activity.

5. Conclusion

In this study, we demonstrated, for the first time, that a diet supplemented with *H. sucuuba* protected *D. melanogaster* from the changes in PQ-induced intoxication, preserving neuromuscular functions such as AChE activity, mitochondrial content, lactate, and protein carbonyl levels. Our group reinforces the importance of in-depth research with mutant lines of D. melanogaster that express neurodegenerative diseases to describe molecular mechanisms.

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