Paullinia cupana (Kunth) stimulates behavior patterns and regulates oxidative stress markers in lobster cockroach *Nauphoeta cinerea*

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Abstract

Paullinia cupana (Kunth), popularly known as guarana, is a plant species from the Amazon Region of Brazil that stands out for being one of the most promising herbal medicines of the Brazilian flora since it has relevant biological properties. However, studies are still needed to use this species as a direct approach to evaluate aspects related to behavior and oxidative stress in invertebrate model animals. In this context, we aimed to evaluate the stimulant and the antioxidant effects of *P. cupana* in lobster cockroach *Nauphoeta cinerea*. For that, cockroaches were exposed to a diet separately supplemented by the decoction of *P. cupana* powder decoction at increasing concentrations of 1, 25, 50 and 100 mg/g for 42 days. Behavioral and biochemical assays were performed, respectively, to assess the locomotor/exploratory performance and oxidative stress marker levels of the cockroaches. They exhibited an outstanding increase in the locomotion performance and in the cell viability content, as well as in the protein and non-protein thiol levels. Moreover, there was a decrease in lipid peroxidation levels and in free Fe^{2+} ion contents. Together, our results demonstrate the stimulant and the antioxidant capacity of *P. cupana* by acting positively in behavioral patterns and by regulating oxidative stress markers in lobster cockroach *N cinerea*. These findings encourage further laboratory analyzes in order to better enlighten the specific mechanisms of action attributed to guarana.

Keywords: Antioxidant properties, Behavioral patterns, *Paullinia cupana* (Guarana), Oxidative stress markers.

Resumo

Paullinia cupana (Kunth), popularmente conhecida como guaraná, é uma espécie de planta da Região Amazônica do Brasil que se destaca por ser um dos fitoterápicos mais promissores da flora brasileira uma vez que apresenta atividades biológicas relevantes. No entanto, ainda são necessários estudos que utilizem esta espécie como uma abordagem direta para avaliar aspectos relacionados ao comportamento e estresse oxidativo

em animais modelo invertebrados. Neste contexto, objetivamos avaliar os efeitos estimulante e antioxidante de *P. cupana* na barata-lagosta *Nauphoeta cinerea*. Para tanto, as baratas foram expostas a uma dieta suplementada separadamente pela decocção do pó de *P. cupana* em concentrações crescentes de 1, 25, 50 e 100 mg/g por 42 dias. Ensaios comportamentais e bioquímicos foram realizados, respectivamente, para avaliar o desempenho locomotor/exploratório das baratas e os níveis de marcadores de estresse oxidativo. As baratas exibiram um notável aumento no desempenho de locomoção e no conteúdo de viabilidade celular, bem como nos níveis de tiol proteico e não proteico. Além disso, houve uma diminuição nos níveis de peroxidação lipídica e nos teores de íons Fe^{2+} livres. Tomados em conjunto, nossos resultados demonstram a capacidade estimulante e antioxidante de *P. cupana* ao atuar positivamente em padrões comportamentais e ao regular os marcadores de estresse oxidativo em barata-lagosta *N cinerea*. Esses achados encorajam novas análises laboratoriais para melhor esclarecer os mecanismos específicos de ação atribuídos ao guaraná.

Palavras-chave: Propriedades antioxidantes, Padrões comportamentais, *Paullinia cupana* (Guarana), Marcadores de estresse oxidativo.

Resumen

Paullinia cupana (Kunth), conocida popularmente como guaraná, es una especie de planta de la región amazónica de Brasil que se destaca por ser una de las fitoterapéuticas más prometedoras de la flora brasileña debido a que posee actividades biológicas relevantes. Sin embargo, aún se necesitan estudios que utilicen esta especie como un enfoque directo para evaluar aspectos relacionados con el comportamiento y el estrés oxidativo en animales modelo de invertebrados. En este contexto, nuestro objetivo fue evaluar los efectos estimulantes y antioxidantes de *P. cupana* en la cucaracha-langosta *Nauphoeta cinerea*. Para ello, las cucarachas fueron expuestas a una dieta suplementada por separado con decocción de *P. cupana* en concentraciones crecientes de 1, 25, 50 y 100 mg/g durante 42 días. Se realizaron ensayos de comportamiento y bioquímicos, respectivamente, para evaluar el desempeño locomotor/exploratorio de las cucarachas y los niveles de marcadores de estrés oxidativo. Las cucarachas exhibieron un aumento notable en el rendimiento de la locomoción y el contenido de viabilidad celular, así como en los niveles de tioles proteicos y no proteicos. Además, hubo una disminución en los niveles de peroxidación lipídica y en los niveles de iones Fe^{2+} libres. En conjunto, nuestros resultados demuestran la capacidad estimulante y antioxidante de *P. cupana* para actuar positivamente en los patrones de comportamiento y regular los marcadores de estrés oxidativo en la cucaracha-langosta *N cinerea*. Estos hallazgos alientan más análisis de laboratorio para aclarar mejor los mecanismos de acción específicos del guaraná.

Palabras clave: Propiedades antioxidantes, Patrones de comportamento, *Paullinia cupana* (Guaraná), Marcadores de estrés oxidativo.

1. Introduction

Paullinia cupana (Kunth) is a plant species native to the Amazon region of Brazil that belongs to Sapindaceae family and it is popularly known as guarana (Mendes et al., 2019). Commonly, the branches of *P. cupana* are used to obtain syrup or guarana powder, which can be packaged in bottles and capsules, as well as used in the industrial production of soft drinks, shakes and functional ingredients (Ribeiro et al., 2012; Silva et al., 2019). According to Yonekura et al. (2016), the intake of any industrial and natural form of guarana is recommended in low doses, approximately 3 gram per day, once it has other common constituents, such as caffeine, theophylline, saponins, tannins, and catechins, that in high doses may cause damage to the body (Heard et al., 2016).

Generally, caffeine is measured at outstanding levels in phytochemical analyses, which roughly comprises 2.5% to 5% of the guarana dry weight extract (Weckerle et al., 2013). Caffeine is a natural alkaloid that, depending on the dose and the frequency of use, has varied beneficial biological properties: it acts as an antioxidant, an antibacterial and an anti-inflammatory substance, besides acting as an antidepressant, anti-mutagenic, cyto-protective, and neuro-protective agent (Da Silva et al., 2018; Viana et al., 2020). Studies have suggested that most of the beneficial effects of guarana are due to the presence of caffeine (Yonekura et al., 2016; Majhenič et al., 2017). So much so that *P. cupana* stands out for being one of the most promising herbal medicines of the Brazilian flora (Ventura et al., 2018).

In this sense, some studies have already conducted with guarana by using human beings as experimental models (Kennedy et al., 2008; Costa et al., 2011; Zeidán-Chuliá et al., 2013). However, it is important to take into account the complexity in the control of human eating habits, mainly because of the ingestion of drugs or different herbs that can be consumed along with *P. cupana*, which may lead, hence, to misinterpretations of

biochemical analysis results (Marques et al., 2019). Thus, experimental laboratory research has been frequently performed through the use of alternative animal models. For instance, the ovoviviparous species *Nauphoeta cinerea*, popularly known as lobster cockroach, is widely used in biochemical and toxic-pharmacological assays since it has a rapid reproductive cycle and similar general biophysical principles of nervous system functioning compared to mammals (Adedara et al., 2015; Carrazoni et al., 2016; Adedara et al., 2020; Pereira et al., 2022).

Indeed, laboratory tests by using *N. cinerea* as an experimental model offer a direct approach to examine applicable biochemical interactions, remarkably regarding oxidative stress, a condition characterized by an increased production of reactive oxygen and nitrogen species, which leads to several damages in proteins, DNA and RNA (Forma; Zhang, 2021). Nevertheless, studies still need to be utterly carried out to better cover the mechanisms of action provided by *P. cupana*. In this study, it is evidenced that there are no considerable reports in the literature on the sensitivity of *N. cinerea* after dietary supplementation with *P. cupana* in order to evaluate either behavioral patterns or oxidative stress markers, aiming to report its stimulant and antioxidant activity. Therefore, this work focuses on evaluating the effects of *P. cupana* (Kunth) in the locomotor and exploratory patterns, as well as in biological marker levels of oxidative stress in lobster cockroach *N. cinerea*.

2. Materials and Methods

2.1. Plant Material

Guarana (*Paullinia cupana*) powder was manufactured at Arte Nativa Produtos Naturais LTDA (São José da Lapa/MG, Brazil). Each 2 gram of guarana powder contains roughly 60 milligram of caffeine, according to the manufacturer.

2.2. Preparation of Guarana (P. cupana) Powder for the Assays

The guarana (*P. cupana*) powder was diluted by decoction in distilled water (10 mL) for subsequent preparation of specific concentrations. The water was heated for 10 minutes at 100 °C. Then, the guarana powder was added to form a mixture (called decoction), which was filtered with filter paper to remove undiluted solid residues (Jedidi et al., 2020).

2.3. Treatment and Diet Formulation of Nauphoeta cinerea Cockroaches

In this study, the assays were performed by using cockroach nymphs obtained from the Biology and Toxicology Laboratory - BIOTOX of the Regional University of Cariri - URCA. It should be noted that Brazil does not have an ethics committee in force regarding the use of invertebrates in experimental tests (Rodrigues et al., 2013; Lira et al., 2016; Fisher; Santos, 2018). In order to properly carry out the experimental assays, the nymphs of *N. cinerea* were put in white polystyrene containers (15 cm of length × 15 cm of width × 7 cm of height), with a temperature between 23-25 °C and a relative humidity of 70%, in a 12h light and 12h dark cycle. The size of the containers was adequate so that the specimens could have enough space to move around without cluster.

In total, 150 cockroach nymphs of both sexes were divided into 5 groups with 30 individuals in each polystyrene container (5 boxes in total). To specify, the number of 30 cockroach nymphs in each group was considered in this study by taking into consideration the possibility of mortality of the specimens. In this way, it is guaranteed in stock of specimens to perform the biochemical assays.

In fact, the number of 5 cockroach nymphs used in each group in the behavioral assays provided enough statistics for data interpretation. This number is justified by the amount of homogenates that were used in each biochemical assay. Therefore, it was not expressly necessary to use all 150 cockroaches for behavioral and biochemical assays, even because studies that cover behavioral and biochemical analysis with *N. cinerea* vary in the number of specimens considered (Leal et al., 2020; Piccoli et al., 2020; Adedara et al., 2021).

The pharmacological doses consisted of increasing concentrations of 1, 25, 50 and 100 milligrams of guarana (*P. cupana*) powder decoction per gram of food (mg/g), which has, respectively, 30, 750, 1500 and 3000 μg of caffeine per gram of food (according to the manufacturer). These concentrations were measured and separately added to the diet of the cockroach nymphs (supplemented diet). The control group did not receive food supplemented by guarana powder decoction (non-supplemented diet). All groups tested were exposed to food and water *ad libitum* for 42 days.

Specifically, 10 g of dry food were used for each group, with a formulation of: 250 g of corn flour, 175 g of

wheat flour, 50 g of sugar, 2.5 g of commercial salt, 12.5 g of casein, and 10 g of powdered milk (Adedara et al., 2016). It is worth mentioning that it was not possible to measure the daily food consumption of the specimens due to limitations in the use of the equipment. Nevertheless, it was feasible to analyze whether the specimens were eating or not, insofar as the food was being replenished during the 42-day dietary exposure period.

2.4. Sample Preparation for Locomotor and Exploratory Assays

At the end of the 42-day dietary exposure, 5 cockroaches (henceforward, adult cockroaches) from each group (control group and groups submitted to a diet supplemented separately with 1, 25, 50 and 100 mg of guarana powder decoction per gram of food) were transferred to a polystyrene container (12.5 cm width x 19 cm length x 5 cm height) in order to rightly record the individual behavior. For this purpose, a simple camera was used for a period of 10 minutes. In sequence, the videos were converted to AVI format and their resolution was reduced to 320 x 240 pixels by using the *Any Video Converter Software* (Pereira et al., 2022).

Subsequently, the videos were analyzed in the *ANY-maze Software*, Stoelting Co, USA, for that aiming at reading the behavior and exploration of the cockroaches in the recipients. In the interval between recording each group, the containers were cleaned to start filming the ensuing group. For the purpose of ensuring the same experimental conditions, all procedures were performed at the same time, during the light phase of the day.

Cockroaches that were not used in the behavioral (locomotor/exploratory) and biochemical assays were anesthetized on ice, they had their heads removed and, herewith the lower limbs, they were placed in 70% alcohol to guarantee the death of each individual. Lastly, they were properly disposed. As shown in the following table (Table 1), the behavioral patterns selected were:

Table 1. Behavioral patterns selected to perform the locomotor and exploratory activities of *N. cinerea*.

Total distance travelled (m)	Total immobile episodes (s)	Time at the zone edges
	Total mobile episodes (s)	Permanence time in the center (s)
Average speed (m/s)	Total time immobile (s)	Latency for the beginning of the first mobile episode
	Total time mobile (s)	Latency for the beginning of the first immobile episode
Maximum speed (m/s)	Entry number in the central zone	Absolute turning angle
	Entry numbers at the edges	

2.5. Sample Preparation for Biochemical Assays

After performing the behavioral and exploratory assays, 5 cockroaches from each group (control group and supplemented groups) were anesthetized on ice; the heads were removed, weighed, homogenized in ice-cold phosphate buffer (0.1 M), pH 7.4 (ratio of 1 mg of head / 40 µl buffer), and they were centrifuged at 10,000 rpm for 10 minutes. The supernatant was separate from the pellet and it was used for further biochemical assays (Adedara et al., 2020).

2.5.1. Cell viability assessment (MTT)

It was used a volume of 20 µL of the supernatant, 170 µL of potassium phosphate (0.1 M, pH 7.4), 10 µL of MTT [3-(4,5-dimethylthiazol)-2,5-diphenyl-20-tetrazolium bromide] prepared in ethanol. The material was incubated for 120 min. After that, 150 µL of the incubated mixture and 50 µL of DMSO were pipetted. Following 10 min of incubation at room temperature, the plates had their absorbance measured at 630 nm on an ELISA (Enzyme-Linked Immuno-Sorbent Assay) micro-plate reader (Da Silva et al., 2018).

2.5.2. Measurement of protein and non-protein thiol levels (NPSH)

Both measurements were estimated by using the Ellman method. Specifically for the determination of protein thiol levels, it was used 190 µL of potassium phosphate buffer (0.1 M, pH 7.4), 20 µL of the supernatant, and 10 µL of 5,5′-dithiobis-(2-nitrobenzoic acid) - DTNB. The reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 405 nm on an ELISA micro-plate reader. Glutathione (GSH) was used as standard curve for each concentration.

For the determination of non-protein thiol levels, it was used 50 µL of the homogenate, 25 µL of TCA 10% and the reaction was centrifuged at 10,000 rpm for 3 min. After centrifuging, the supernatant pellet was removed and then 150 µL of potassium phosphate buffer (1 M, pH 7.4), 40 µL of clear supernatant and 10 µL of DTNB (10 mM) were added. The absorbance was measured again at 405 nm on an ELISA micro-plate reader, and the results were expressed as µmol GSH/g of tissue (Da Silva et al., 2018).

2.5.3. Determination of Thiobarbituric Acid Reactive Species (TBARS)

For the determination of TBARS, a mixture containing 100 µL of the supernatant, 100 µL of trichloroacetic acid (TCA, 10%) and 100 µL of 2-thiobarbituric acid (TBA prepared in 0.1 M HCl) at 0.75% was used. The mixture was incubated at 95 ºC for 1 h. After cooling, the mixture was centrifuged at 10,000 rpm for 10 min. The micro-plate was read with an absorbance measured at 405 nm on ELISA micro-plate reader. For the standard curve, it was used malondialdehyde (MDA) obtained by the hydrolysis of 1,1,3,3-tetramethoxypropane (TMP). The results were expressed in Mol MDA/g of tissue (Filho et al., 2014).

2.5.4. Iron (II) Quantification

For free Fe^{2+} quantification, it was used 100 µL of saline solution (NaCl, 0.9%), 60 µL of 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris-HCl), 0.1 M (pH 7.4), 20 µL of the supernatant, 10 µL of 1,10-phenanthroline (25%). The reaction mixture was incubated for 60 min at room temperature. Subsequently, Iron (II) sulfate was used to create the standard curve, and the absorbance was measured at 492 nm by using ELISA (Da Silva et al., 2018).

2.6. Statistical Analysis

Data were expressed as the mean ± SEM (Standard Error of the Mean). All measured patterns were processed by a one-way analysis of variance (ANOVA) and they were followed by Dunnett's multiple comparison test (*post-hoc* test) when a significant difference was determined at $p < 0.05$. Statistical analysis of the data was performed with the *GraphPad Prism Software*, version 6.0.

3. Results

3.1. Behavioral Analysis of N. cinerea after Supplementation with Guarana (P. cupana) Powder Decoction

In overall, there was no mortality of the *N. cinerea* cockroach nymphs at any concentration tested of guarana (*P. cupana*) powder decoction. Figure 1 represents the lines of the path travelled by *N. cinerea* cockroaches after being submitted to a 42 day-dietary exposure during the nymph stage. It becomes amenable to observe a stimulus in the locomotor activity of the cockroaches exposed to all concentrations tested: when increasing the concentrations, there is also an increase in cockroaches' mobility compared to the control group (Figure 1A) *($p < 0.05$)*. The points represented by the blue and red colors in Figure 1 correspond, respectively, to the beginning and to the end of the cockroaches' mobility in the polystyrene container during 10 min of recording. There is an overlap of these points in Figure 1 A (control group).

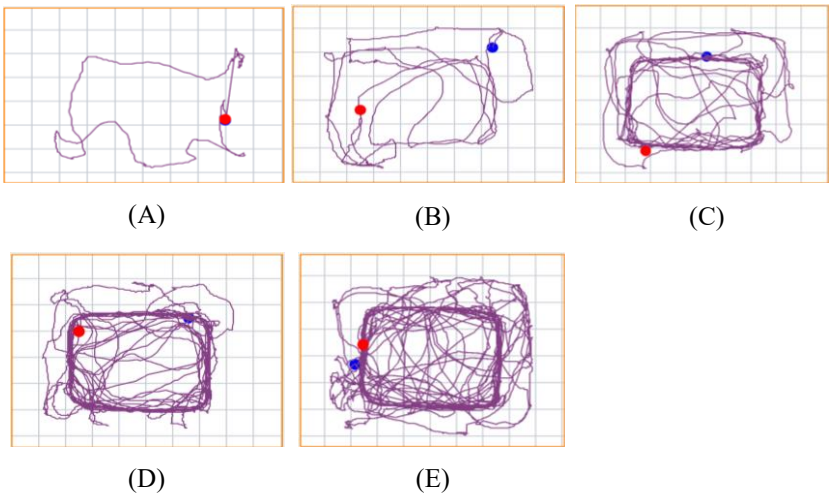


Figure 1. Line representations of the locomotor behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction during the 10-minute test.

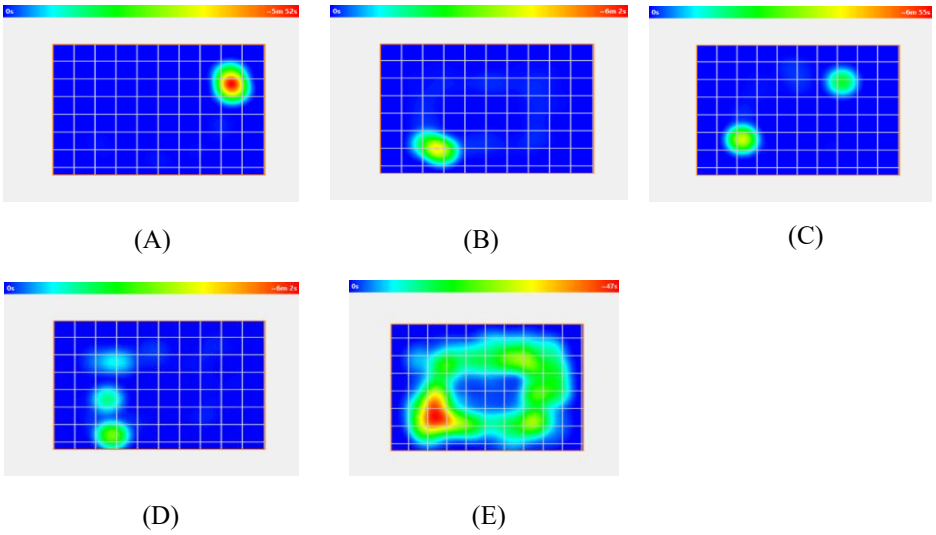
A - Control, B – 1 mg/g, C – 25 mg/g, D – 50 mg/g, E – 100 mg/g. The blue spot represents the beginning of the route and the red spot represents the end of the route.

From the line representation of *N. cinerea* cockroaches' locomotor behavior (Figure 1), it was possible to obtain the thermal representation of the individual behavior (heat map) (Figure 2). It provides the time that cockroaches remain at a given point, which is shown by using a linear color scale from blue ("cool colors") to red ("warm colors"). Thus, the area represented in red is the warmest area where the organism spent the most time, and the area represented in blue is the coldest area, where the organism spent less time. As depicted in Figure 2, the exploratory behavior of cockroaches increased with increasing concentrations tested of guarana (*P. cupana*) powder decoction (1, 25, 50 and 100 mg/g, Figure 2B-E).

Note that the red color shown in the control group (Figure 2A) and the one exposed to the concentration of 100 mg/g of guarana powder (Figure E) is easily detected, which means a longer time spent by cockroaches at certain points in the container. On the other hand, it can be seen that at concentrations of 1, 25, and 50 mg/g (Figure 2B-D) there is predominance in the frequency of blue and green colors, which means a shorter time spent at a given point in the container. Still of importance, there was a greater exploration of the environment (polystyrene container) by the cockroaches submitted to a diet with the highest concentration tested (100 mg/g). This means a shorter time spent in the areas indicated by the colors blue and green (Figure 2).

Precisely, the cockroaches of the control group remained in a given portion of the container for a time period of 5 min and 52 seconds. During this time, they passed through the edges of the plots, but they did not remain for a long period (indicated by "cool colors"). The red color ("warm colors") is located in the center of the plot and it corresponds to the longest permanence time of cockroaches in the container (review Figure 2).

As highlighted in Table 2, the permanence time in the respective container plots was the highest for both the control group and the groups submitted separately to a diet supplemented with increasing concentrations of 1, 25 and 50 mg/g of guarana (*P. cupana*) powder decoction (Table 2 A-D). Cockroaches exposed to the concentrations of 1, 25, and 50 mg/g remained in the indicated locations for an approximate time (6 min 2 s, 6 min 55 s and 6 min 2 s, respectively), which was slightly longer than the permanence time of cockroaches in the control group (Table 2). The portion indicated by the red color (Figure 2) provides a permanence time of 47 s spent by cockroaches at a certain point in the container (Table 2E). This time was shorter compared to the permanence time of the cockroaches in the control group (5 min and 52 s) (Table 2).



(A) (B) (C)

(D) (E)

Figure 2. Heat map representation of the exploratory behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction during the 10-minute test. A - Control, B – 1 mg/g, C – 25 mg/g, D – 50 mg/g, E - 100 mg/g.

Table 2. Permanence time of cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana powder decoction during the 10-minute test shown in Figure 2 as heat maps. "min" - minutes; "s" - seconds.

Groups	Concentrations	Permanence time
A (Control)	-	5 min 52 s
B	1 mg/g	6 min 2 s
C	25 mg/g	6 min 55 s
D	50 mg/g	6 min 2 s
E	100 mg/g	47 s

As illustrated in Figure 3, the group of cockroaches that received a diet supplemented with *P. cupana* powder decoction at concentration of 100 mg/g showed an increase in both the total distance traveled (m) (Figure 3A) and in the maximum speed (m / sec) (Figure 3C). Furthermore, there was an increase in the average speed (m/s) of the cockroaches submitted to a diet supplemented at concentrations of 50 and 100 mg/g (Figure 3B) when compared to the control group, which undeniably demonstrates an increase in the values of these patterns that is directly proportional to the concentration tested of *P. cupana* powder decoction.

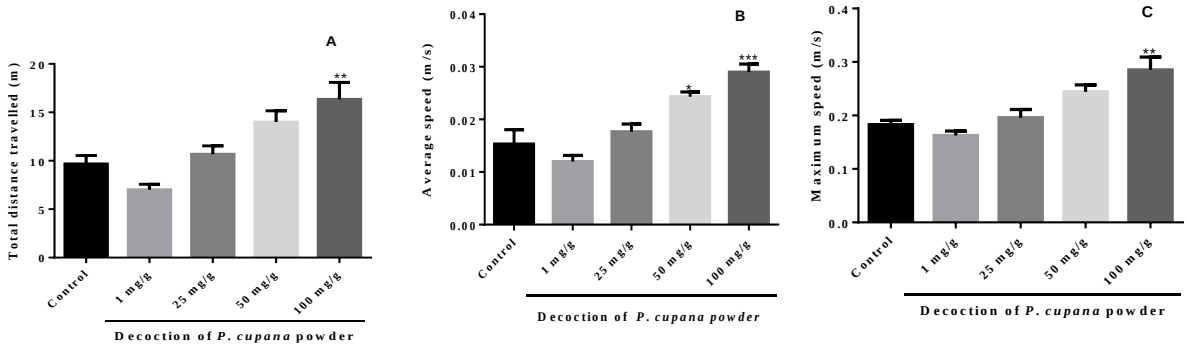


Figure 3 A-C. Locomotor behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of *P. cupana* powder decoction performed after a period of 42-day dietary exposure. Five (5) cockroaches were filmed for 10 minutes.

(A) Total distance traveled (m), (B) Average speed (m/s), (C) Maximum speed (m/s). *indicates significant difference compared to control (*P <0.05; **P <0.01; ***P <0.001).

Analyzing the Figure 4A, it is noticeable that the total immobile episodes dwindle as a result of a positive influence of the guarana (*P. cupana*) powder decoction on the cockroaches' mobility, especially for the supplemented group at concentration of 100 mg/g in comparison to the control group. In the total mobile episodes, there was an increase in the number of periods in which the animals moved, for all concentrations tested (1, 25, 50 and 100 mg/g) in relation to the control group (Figure 4B). The total immobile time of the control group was the greatest (Figure 4C), what can indicate longer periods of immobility when compared to the groups of cockroaches whose diet was supplemented with guarana (*P. cupana*) powder decoction at different concentrations. As expected, it is remarkable the variation in the total mobile time between the control group and the group that received a diet supplemented with the concentration of 100 mg/g (Figure 4D).

In containers that were filmed during the behavioral analysis of *N. cinerea* cockroaches, there was a greater entry number in the central zone for the group submitted to the concentration of 100 mg/g when compared to the groups that received the other concentrations tested and also to the control group during the 42-day dietary exposure period (Figure 5A). Despite the fact that there were many entries in the central zone, cockroaches submitted to a diet supplemented at concentration of 100 mg/g did not remain in this area for a long time period in comparison to the groups submitted to the concentrations of 1, 25 and 50 mg/g (Figure 5B).

In its turn, Figure 6A shows the entry numbers of *N. cinerea* cockroaches at the edges of the containers. The cockroaches exposed to a diet supplemented at concentration of 100 mg/g demonstrated a large number of entries and a longer permanence time at the edges of the containers in relation to the groups summited to diets separately supplemented with the other concentrations tested (Figure 6B). As it is appropriate to suggest, the specimens were constantly moving around the edges.

The latency for the beginning of the first mobile episode, that is, the moment when cockroaches started to move, is represented in Figure 7. The concentration of 100 mg/g stands out in the latency when it is compared to the control group as well as the groups which were submitted to the other concentrations tested (1, 25 and 50 mg/g). The opposite is observed in the latency for the beginning of the first immobile episode (Figure 7B). The control group exhibited more latency periods for immobile episodes when compared to the other concentrations tested. Gradually, the absolute turning angle increased in relation to these concentrations (Figure 7C).

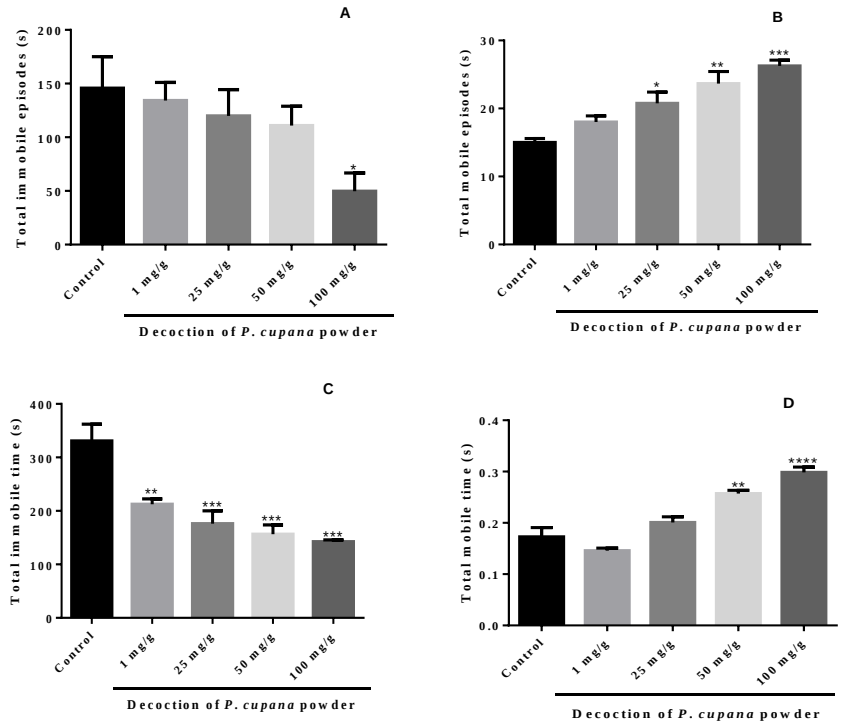


Figure 4 A-D. Locomotor behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction performed after a period of 42-day dietary exposure. 5 (five) cockroaches were filmed for 10 minutes.

(A) Total immobile episodes, (B) Total mobile episodes, (C) Total immobile time, (D) Total mobile time. *indicates significant difference compared to control (*P <0.05; **P <0.01; ***P <0.001; ****P <0.0001).

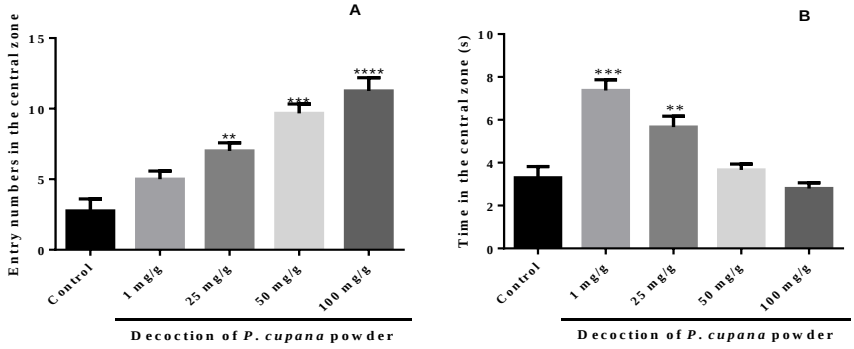


Figure 5. Locomotor behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction performed after 42-day dietary exposure. 5 (five) cockroaches were filmed for 10 minutes.

(A) Entry numbers in the central zone, (B) Permanence time in the central zone (s). *indicates significant difference compared to the control group (**P <0.01; ***P <0.001; **** P <0.0001).

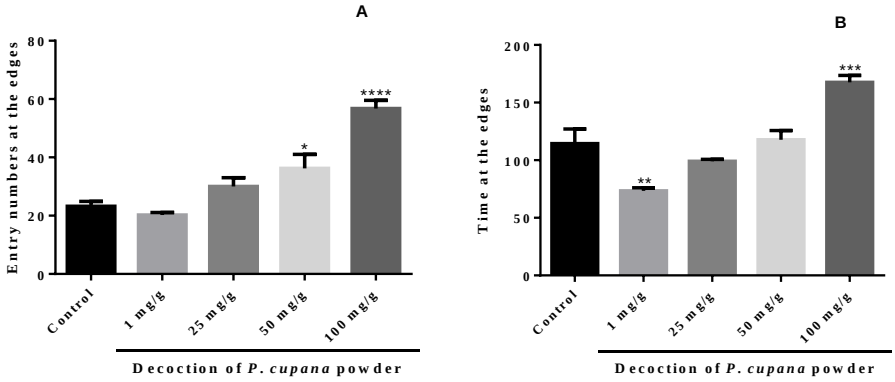


Figure 6. Locomotor behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction performed after a period of 42-day dietary exposure. 5 (five) cockroaches were filmed for 10 minutes.

(A) Entry numbers at the edges, (B) Permanence time at the edges (s). *indicates significant difference compared to control (*P <0.05; **P <0.01; ***P <0.001; ****P <0.0001).

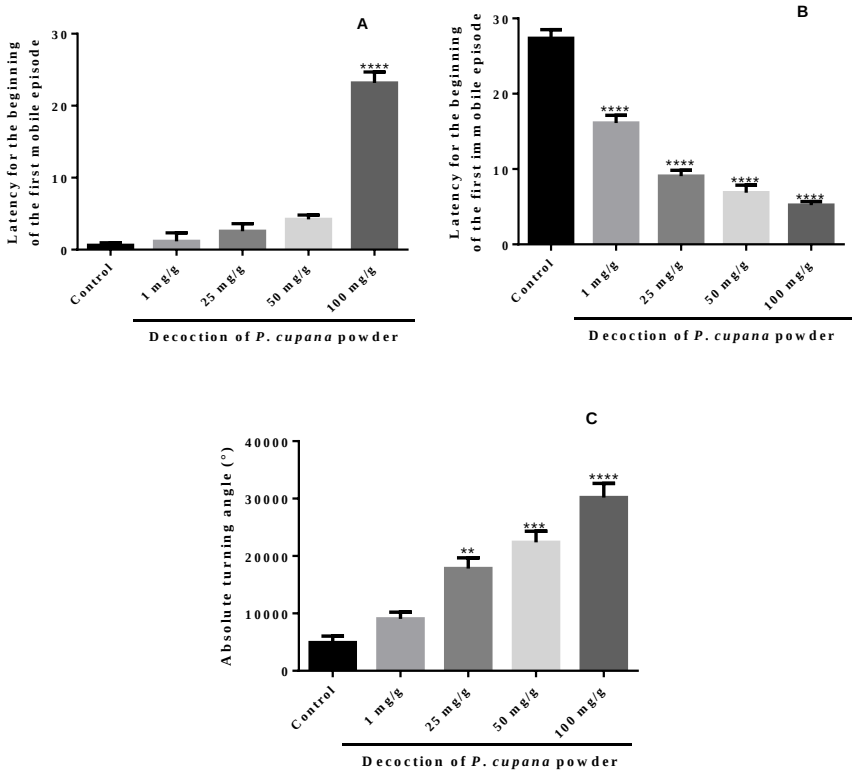


Figure 7. Locomotor behavior of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana powder decoction performed after a period of 42-day dietary exposure. 5 (five) cockroaches were filmed for 10 minutes.

(A) Latency for the beginning of the first mobile episode, (B) Latency for the beginning of the first immobile episode, (C) Absolute turning angle (°). *indicates significant difference compared to control (**P <0.01; ***P <0.001; ****P <0.0001).

3.2. Biochemical Assays

3.2.1. Cell Viability Evaluation of N. cinerea Homogenates

This assay was used to measure the cell viability of *N. cinerea* head homogenates and it is performed by using MTTP [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium], a dye that is reduced from yellow to formazan purple color due to the action of the dehydrogenase enzymes that are present in the mitochondria of viable cells. Analyzing Figure 8, there was a significant increase in the mitochondrial activity of the cells of *N. cinerea* cockroach head homogenates exposed to all concentrations tested (1, 25, 50 and 100 mg/g) of the guarana powder decoction, which indicates the direct action of *P. cupana* on cell viability.

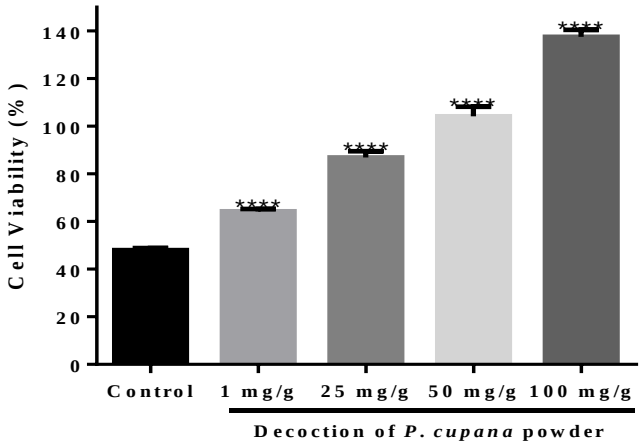


Figure 8. Evaluation of the cell viability (%) at the absorbance of 630 nm in the homogenates obtained from the heads of the *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and supplemented diet with the decoction of guarana (*P. cupana*) powder.

****indicates significant difference when compared to control ($P < 0.0001$).

3.2.2. Measurement of protein and non-protein thiol levels

The measurement of protein and non-protein thiol levels is analyzed as a final product of oxidative changes in sulfhydryl groups (-SH) of proteins and peptides present in the supernatant. It is possible to observe in Figure 9A that there is an increase in protein thiol levels at all concentrations tested, especially 25, 50 and 100 mg/g that showed significance in relation to the control group. Similarly, there was an extended increase in non-protein thiol levels at all concentrations (1, 25, 50 and 100 mg/g) compared to the control group (Figure 9B). These results indicate a decrease in lipid peroxidation (Figure 9).

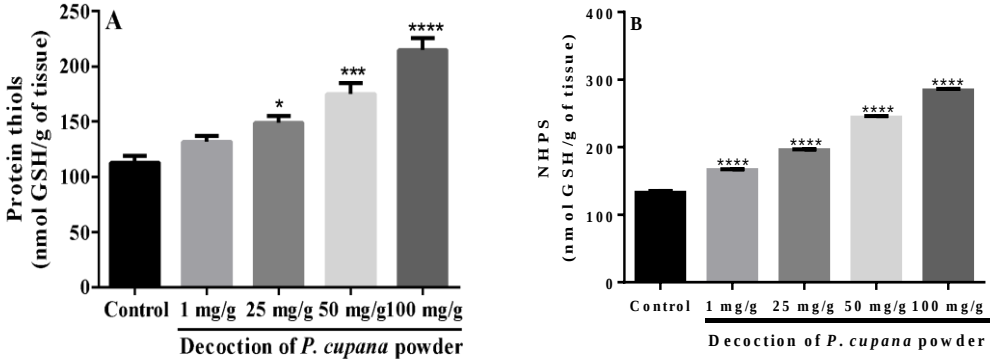


Figure 9. Measurement of protein thiol levels (nmol GSH/g of tissue) (A) and non-protein thiol levels (nmol GSH/g of tissue) (B) in the homogenates of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction.

indicates significant difference when compared to control (P <0.05; *** P <0.001; **** P <0.0001).

3.2.3. Determination of thiobarbituric acid reactive species (TBARS)

Malondialdehyde (MDA) is formed as a result of lipid peroxidation, and it is measured by the formation of Thiobarbituric Acid Reactive Species (TBARS). As depicted in Figure 10, the MDA levels were reduced in all concentrations, with a significant reduction at concentration of 100 mg/g when making a comparison with the control group. This result demonstrates that *P. cupana* is capable of reducing the lipid peroxidation in the cells.

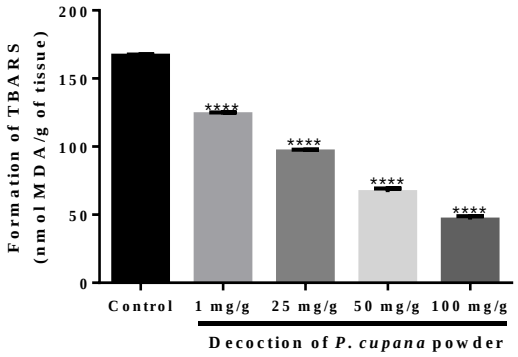


Figure 10. Determination of Thiobarbituric Acid Reactive Species (TBARS) (nmol MDA g^{-1} of tissue) in the homogenates of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet with the decoction of guarana (*P. cupana*) powder. ****indicates significant difference when compared to the control group (P <0.0001).

As seen in Figure 11, there was a substantial reduction in the amount of Fe^{2+} ions in the cockroach groups submitted to a diet supplemented with the decoction of guarana (*P. cupana*) powder at all concentrations tested (1, 25, 50 and 100 mg/g) compared to the control group (non-supplemented diet). As it is widely known, iron is a necessary element for oxygen transport, cellular respiration and enzyme activity. However, as it is considered a marker element of oxidative stress, it can trigger a Fenton reaction through the production of hydroxyl radicals, which leads to the condition of oxidative damage in the cells (Barbosa et al., 2010). By analyzing the graph, the concentration of 100 mg/g was the one that showed the greatest potential for reducing the amount of Fe^{2+} ions in the homogenates, that is, a reduction in the condition of oxidative stress of the cells.

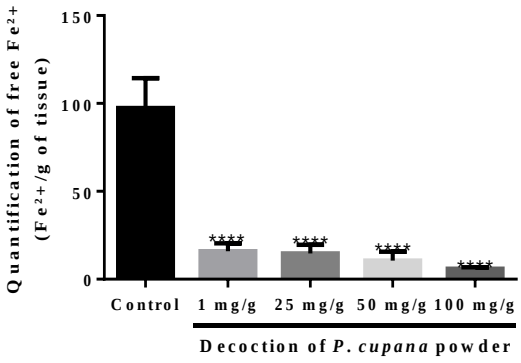


Figure 11. Quantification of free Fe^{2+} ions (Fe^{2+}/g of tissues) in the homogenates of *N. cinerea* cockroaches submitted to a non-supplemented diet (control) and separately supplemented diet at different concentrations of guarana (*P. cupana*) powder decoction.

****indicates significant difference when compared to control ($P < 0.0001$).

4. Discussion

The species *P. cupana* (guarana) contains high levels of caffeine (1,3,7-trimethylxanthine) in its chemical composition, especially the seed to which the stimulant properties of guarana are best attributed (Schimpl et al., 2013; Tfouni et al., 2017). In order to estimate, the caffeine content in guarana is 4 times greater than in coffee (*Coffea arabica* L.) and 10 times greater than in tea (*Camellia sinensis* L.) (Weckerle et al., 2013). Namely, Caffeine is one of the most ingested alkaloids whose effects on humans have been the subject of studies for some decades because it presents several pharmacological actions to the body (Viana et al., 2021a), mainly related to an increase in alertness (Nawrot et al., 2003), a reduction in fatigue (Espinosa; Sobrino Mejía, 2017), as well as an improvement in the performance of different activities (Temple et al., 2017). Notwithstanding, caffeine can lead to certain types of alterations in the central nervous system depending on the dose of consumption and the frequency of use, such as decrease in motor control, poor sleep quality and irritability in individuals with anxiety (McLellan et al., 2016; Viana et al., 2021a).

As previously mentioned, *N. cinerea* cockroaches share general biophysical principles of nervous system functioning similar to those present in mammals. In this context, it can be assumed that the stimulus observed in the locomotor activity of the cockroaches submitted to a diet supplemented with different concentrations of guarana (*P. cupana*) powder decoction is attributed to the stimulating effects of caffeine on the nervous system of the organisms. This was confirmed through the inference of the results gathered in those behavioral patterns heretofore listed (back to Table 1). Similar patterns were analyzed by Da Silva et al. (2018) when testing supplemented diet with isolated caffeine in *N. cinerea* cockroaches. They observed an increase in the total distance travelled and in the average speed of the supplemented cockroach groups at concentrations of 2.5 mg/g and 10 mg/g. There was a good locomotor performance in these analyzed patterns, with no mortality of the organisms as reported in our study.

As a matter of inference, the locomotor behavior of *N. cinerea* cockroaches submitted to a diet supplemented by guarana powder decoction at the highest concentration (100 mg/g) demonstrated an increase in the average speed (0.3 m/s, that is, equivalent to 30 cm/s), as well as in the maximum speed (0.4 m/s, equivalent to 40 cm/s). Interesting, Bender et al. (2011) reported that when the cockroaches move faster than normal they can get a speed of 30 cm/s, which corroborates with our results when it comes to emphasizing that *P. cupana* powder acted directly in the locomotor behavior of the cockroaches. The contrary was observed by Adedara et al. (2016) when exposing *N. cinerea* cockroaches to different concentrations of the insecticide Chlorpyrifos (CPF). There was a decrease in the total distance travelled (m) and an increase in the immobile time (s).

As it was affirmed earlier, it is prone to infer that the spur in behavioral patterns of *N. cinerea* cockroaches perhaps occur due to a stimulation proportionated by the presence of caffeine in guarana powder when it acts on the nervous system of these organisms. Indeed, Eberle et al. (2020) stated that there is a spur in the central nervous system when caffeine is ingested. As a result, it may dwindle the fatigue and, likewise, swell the alertness. In this sense, exploratory behavior is important for the survival of the specimens in their habitat mostly because total

exploration of the environment assists in obtaining food and escaping predators (Adedara et al., 2020).

In our study, in spite of being in low concentrations, the presence of caffeine in guarana (*P. cupana*) powder might have affected the behavior patterns of *N. cinerea* cockroaches, which will be noteworthy to confirm by carrying out further accurate molecular experimental studies. The investigation conducted hitherto in this work revealed that the exposure to *P. cupana* stimulated the behavior patterns of cockroaches, basically through greater exploratory activity in the peripheral area of the containers. This is a behavioral mode escape response known by the term of thigmotaxis, necessary for any organism to adapt as soon as it is realized that the environment is dangerous and it would be better to avoid this (Rosemberg et al., 2012). The thigmotaxis phenomenon was demonstrated here through line representations of the locomotor pattern of *N. cinerea* cockroaches by evidencing a certain type of attraction to container wall (review Figure 1).

To illustrate this, a study was developed through analysis of the behavioral and biochemical patters of *N. cinerea* after an exposure to a diet supplemented with ciprofloxacin and atrazine (Adedara et al., 2021). The authors observed that the exposure of cockroaches to these compounds resulted in locomotor and exploratory deficits intrinsically related to the phenomenon of thigmotaxis. In this aspect, for an escape manifestation to occur, the organism develops greater body locomotion and this response was evidenced here when observing the exploitation of cockroaches in the vertical zone of the polystyrene container. On the other hand, organisms that do not show signs of escape manage to explore the inside zone of the environment (polystyrene containers) (Lipkind et al., 2004; Rosemberg et al., 2012).

Regarding the analysis of biochemical assays involving cell viability (MTT assay), lipid peroxidation, TBARS and quantification of Fe^{2+} ions, different studies reported a decrease in oxidative stress markers after treatment with antioxidant substances (Vrailas-Mortimer et al., 2012; Ayala et al., 2014; Kurutas, 2016; Niveditha et al., 2017; Viana et al., 2020; Viana et al., 2021b). Da Silva et al. (2018) noticed a considerable increase in cell viability of *N. cinerea* cockroaches after testing caffeine (natural antioxidant) in isolated concentrations of 0.5, 1, 2.5, 5 and 10 mg/g. Likewise, our results showed a significant increase in cell viability of cockroach head homogenates at all concentrations of *P. cupana* (1, 25, 50 and 100 mg/g), with the control group already showing a cell viability of approximately 50% (see results section).

In addition to cell viability, lipid peroxidation is a parameter that ought to be considered as it provides valuable indices for the evaluation of toxicity in biochemical assays (Adedara et al., 2021). As a rule, the increase in lipid peroxidation is indicated by a decrease in protein thiol levels (Lepedda et al., 2013). Mostly present in proteins, thiol groups are generally reactive and they can be oxidized by reactive species, which causes conformational changes in proteins, such as unfolding and degradation of the protein structure; consequently, for the proper maintenance of proteins and enzymes functions, as well as for an adequate cellular redox state, there must be a balance between oxidized thiols and free thiols in the cells (Trivedi et al., 2019). Of importance, our results evidenced a substantial increase in protein thiol levels after dietary exposure of *N. cinerea* cockroaches with the guarana (*P. cupana*) powder decoction at concentrations of 25, 50 and 100 mg/g, which means a decrease in lipid peroxidation and, hence, in the oxidative stress.

In a related way, Bittencourt et al. (2016) validated in an *in vitro* study that the *P. cupana* extract at concentrations of 1, 10, 100 and 1000 µg/ml was able to protect any oxidative damage caused to lipids in cell membranes. Not only guarana, but also its most predominant natural compound caffeine, is capable of inhibiting or decreasing lipid peroxidation. Experimental assays by using isolated caffeine at different concentrations showed a raise in protein thiol levels of homogenates obtained from the heads of *N. cinerea*, thereby indicating a reduction in lipid peroxidation (Da Silva et al., 2018). Still of relevance, the results gathered so far in this study also demonstrated a significant increase in non-protein thiol levels (NPSH) for all concentrations tested of *P. cupana* (1, 25, 50 and 100 mg/g). As expected, it led to an overall reduction in TBARS levels, especially at the concentration of 100 mg/g compared to the control group (review Figure 10).

Finally, in addition to lipid peroxidation, it was observed here depletion in free Fe^{2+} ion contents in *N. cinerea* head homogenates for all concentrations of guarana powder decoction (Figure 11), which might as well infer the potential antioxidant capacity of *P. cupana*. Indeed, substances capable of chelating free iron are categorized as antioxidant compounds, broadly used in toxic-pharmacological studies (Karthik et al., 2016; Schneider et al., 2019). Similar to our results, Pereira et al. (2022) reported a significant reduction in Fe^{2+} ion content in connective tissue homogenates of *N. cinerea* after treatment with Thiazolidine compounds NW05 (10 mM) e NJ20 (10 mM). The authors justified that certain thiazoles have excellent free Fe^{2+} chelating capacity and moderate toxicity, which corroborates our findings with regard to the antioxidant properties of *P. cupana*, whose powder decoction

did not show toxicity at any concentration tested here.

5. Conclusions

The lobster cockroaches *Nauphoeta cinerea* submitted to diets separately supplemented with different concentrations of guarana (*P. cupana*) powder decoction showed an overall increase in behavioral patterns (locomotor and exploratory activities). The biochemical assays evidenced an increase in cell viability, in protein and non-protein thiol levels, associated with a decrease in malondialdehyde (MDA) content, in addition to a significant reduction in Fe^{2+} ion contents. Thus, guarana (*P. cupana*) powder decoction stimulated the locomotor and exploratory activities of *N. cinerea* cockroaches, as well as exhibited a potential antioxidant capacity by regulating the oxidative stress markers, especially at higher concentrations. Appositely, our work highlighted *N. cinerea* as a viable model for carrying out toxicological assessments, not only biochemical, but also behavioral patterns. Certainly, these findings encourage further laboratory analyzes in order to better enlighten the specific mechanisms of stimulant and antioxidant properties attributed to guarana.

6. Acknowledgments

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7. Conflicts of Interests

The authors declare that there is no conflict of interests.

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