

**Hypoglycemic effect of *p*-hydroxycinnamic diesters extracted from carnauba wax powder (*Copernicia Prunifera*) in alloxan-induced diabetic animals**

**Efeito hipoglicêmico de diésteres *p*-hidroxicinâmicos extraídos do pó da cera de carnaúba (*Copernicia Prunifera*) em animais diabéticos induzidos por aloxano**

**Efecto hipoglucemiante de diésteres *p*-hidroxicinâmicos extraídos del polvo de la cera de carnauba (*Copernicia Prunifera*) en animales diabéticos inducidos por aloxano**

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**Abstract**

Diabetes is a chronic non-transmissible disease and the number of cases is increasing every year. Plants appear as an alternative therapy since ancient civilizations. Among the species that have promising pharmacological activities are carnauba (*Copernicia prunifera* (Miller) H. E. Moore). Carnauba wax powder consists of a mixture of heterogenic compounds, among them, *p*-hydroxycinnamic diesters (HCA). Therefore, this study aimed to investigate the hypoglycemic effect of HCA in alloxan-induced diabetic animals. Therefore, this study was performed with 50 female Swiss mice, distributed in 5 groups (n = 10). The animals of the negative and positive control groups were treated with water: healthy and diabetic mice, respectively; animals of the HCA groups 25 and 50 were diabetic animals and they were treated with the HCA solution at doses of 25 and 50 mg / Kg, respectively; and with metformin, standard drug, at the dose of 200 mg / kg. After 28 days of HCA treatment, a

significant hypoglycemic effect was observed in animals treated with HCA at the lowest dose tested (25 mg / kg). In the present study, HCA shown to be a promising compound with good scientific applicability due to the reduction of glycemia of diabetic animals induced by alloxan.

**Keywords:** Diabetes; *p*-hydroxycinnamic diesters; Carnauba.

### Resumo

O diabetes é uma doença crônica não transmissível e o número de casos estão aumentando a cada ano. As plantas aparecem como terapia alternativa desde civilizações antigas. Entre as espécies que apresentam atividades farmacológicas promissoras estão a carnaúba (*Copernicia prunifera* (Miller) H. E. Moore). A cera de carnaúba em pó consiste em uma mistura de compostos heterogênicos, entre eles os diésteres *p*-hidroxicinâmicos (HCA). Portanto, este estudo teve como objetivo investigar o efeito hipoglicemiante do HCA em animais diabéticos induzidos por aloxana. Portanto, este estudo foi realizado com 50 camundongos Swiss fêmeas, distribuídos em 5 grupos (n = 10). Os animais dos grupos controle negativo e positivo foram tratados com água: camundongos saudáveis e diabéticos, respectivamente; os animais dos grupos 25 e 50 HCA eram diabéticos e foram tratados com a solução de HCA nas doses de 25 e 50 mg / Kg, respectivamente; e com metformina, medicamento padrão, na dose de 200 mg / kg. Após 28 dias de tratamento com HCA, um efeito hipoglicêmico significativo foi observado em animais tratados com HCA na dose mais baixa testada (25 mg / kg). No presente estudo, o HCA mostrou-se um composto promissor e com boa aplicabilidade científica devido à redução da glicemia de animais diabéticos induzida por aloxano.

**Palavras-chave:** Diabetes; Diésteres *p*-hidroxicinâmicos; Carnaúba.

### Resumen

La diabetes es una enfermedad crónica no transmisible y el número de casos aumenta cada año. Las plantas aparecen como una terapia alternativa desde las civilizaciones antiguas. Entre las especies que tienen actividades farmacológicas prometedoras se encuentran la carnauba (*Copernicia prunifera* (Miller) H. E. Moore). El polvo de cera de carnauba consiste en una mezcla de compuestos heterogênicos, entre ellos, diésteres *p*-hidroxicinâmicos (HCA). Por lo tanto, este estudio tuvo como objetivo investigar el efecto hipoglucémico del HCA en animales diabéticos inducidos por aloxano. Por tanto, este estudio se realizó con 50 ratones suizos hembra, distribuidos en 5 grupos (n = 10). Los animales de los grupos de control negativo y positivo se trataron con agua: ratones sanos y diabéticos, respectivamente; los

animales de los grupos 25 y 50 de HCA eran animales diabéticos y se trataron con la solución de HCA a dosis de 25 y 50 mg / kg, respectivamente; y con metformina, fármaco estándar, a la dosis de 200 mg / kg. Después de 28 días de tratamiento con HCA, se observó un efecto hipoglucémico significativo en los animales tratados con HCA a la dosis más baja probada (25 mg / kg). En el presente estudio, el HCA demostró ser un compuesto prometedor con buena aplicabilidad científica debido a la reducción de la glucemia de los animales diabéticos inducida por el aloxano.

**Palabras clave:** Diabetes; Diésteres p-hidroxicinámicos; Carnauba.

## 1. Introduction

*Diabetes mellitus* (DM) is described as a metabolic disorder resulting from the lack of insulin and/or inability of insulin to efficiently perform its actions, characterized by chronic hypoglycemia and changes in the metabolism of carbohydrates, lipids and proteins (Brazilian Diabetes Society, 2016). In addition, the World Health Organization (WHO) and American Diabetes Association (ADA) subdivide this pathology into four classes: type 1 DM, type 2 DM, gestational DM and other specific types.

DM Type 2 (DM2) can be considered a deficiency relative referred to the action and/or excretion of insulin. Among the factors associated with the development of diabetes, environmental factors such as low physical activity and high-fat diets as well as genetic variations present in the pre-clinical phase of this disease stand out (Brazilian Diabetes Society, 2016). According to the report of the International Diabetes Federation, about 415 million of the population have DM. The global estimate is that the number of cases may increase and reach 642 million by 2040 (Rwegeram *et al.*, 2017). Among these, about 90% correspond to cases of DM2. Thus, it indicates the importance of studies on treatment and maintenance of control of blood glucose and comorbidities (Gomes *et al.*, 2019).

The hypoglycemic effect of some derivatives of natural products has increased the interest and application for their use today, since the manipulation of plants or compounds extracted from them and it is a practice used since the early days of civilization (Gurib-Fakim, 2006). This fact is due to the inherent benefits of plant derivatives compared to synthetic substances, besides being classified as less toxic (Huang *et al.*, 2015).

Carnauba (*Copernicia prunifera* (Miller) H. E. Moore) is a palm tree native of Brazil in the semi-arid region and it has great importance in the region due to its various functions both for the environment and for the economy (Dalva, 2004). Chemically, the carnauba wax

powder consists of a complex mixture of chemical compounds such as esters corresponding to about 80%, besides alcohols, triterpenoids and phenols among others. It is worth mentioning that the scientific literature denotes an interesting antioxidant effect on esters and phenols, both present in carnauba wax powder, commonly used in foods (Andrade *et al.*, 2018).

Previous studies with compounds extracted from carnauba wax powder and fruits, such as PCO - C (*p*-methoxycinnamic acid diesters) and pectin demonstrated relevant results in attenuation of deleterious effect of diabetes and dyslipidemias (Guedes *et al.*, 2011; Rodrigues *et al.*, 2014; Filho *et al.*, 2017; Paim *et al.*, 2017). Other chemical compounds that are worthy of note and are also found in carnauba wax powder are *p*-hydroxycinnamic diesters (HCA). Several studies report their antioxidant effects and their actions against reactive oxygen species (ROS) responsible for induces a powerful inflammatory process in the patients (Ambika; Saravanan; Thirumavalavan, 2013; Terpinc, 2011).

Therefore, the present research aimed to investigate the hypoglycemic effect of *p*-hydroxycinnamic diesters extracted from carnauba in alloxan-induced diabetic animals.

## **2. Materials and Methods**

### **2.1 Material vegetal**

The carnauba wax powder from *Copernicia prunifera* (Miller) H. E. Moore was provided by Company Pontes Industria de Ceras Ltda.

### **2.2 Extraction of p-hydroxycinnamic diesters from carnauba wax powder**

The carnauba wax powder was weighted (100g) and mixed with 1L of ethyl acetate and hexane solution (3:7) and keep under agitation during 1 hour. Then the contents were filtered through a paper filter and the retain material was situated in a drying oven at 50° C overnight. After this, the residual powder was mixed with 1L of chloroform, remain under agitation for 1 hour, and filtered. The filtered solution was concentrated in a vacuum evaporator TE-211 (Tecnal®) at 60° C and it was obtained a solid light green material that was denominated as HCA. Subsequently, it was followed the protocol of purification appointed by Vandenburg & Wilder, (1967) and Silva *et al.*, (2019).

The HCA was added in tween 80 (3%) and heated for 5 minutes. Posteriorly, it was added hot water and adjust the doses used in the present study.

### **2.3 Animals**

Fifty adult female mice (*Mus musculus*) weighting of  $26 \pm 5$ g, age varying 8 to 12 weeks were obtained from Federal University of Ceará. They were kept in a collective cage at controlled temperature between  $22 \pm 2^\circ\text{C}$  in light-dark cycles of 12/12 hours for 30 days. All animals received water and feed *ad libitum*.

### **2.4 Induction of diabetes**

For diabetes induction, the animals were fasted for 12 hours. Posteriorly, they received an injection with alloxan solution (Sigma®) diluted in saline solution (0.9%) at a dose of 150mg/Kg via intraperitoneal (Sousa *et al.*, 2015). Blood samples were collected to determine serum glucose levels and after the induction period animals that presented glucose above 200 mg/d were considered diabetic (Rodrigues *et al.*, 2014). In addition, the animals had their weights, water consumption and diet measured weekly.

### **2.5 Experimental design**

The animals were divided into 5 groups (n=10) and the treatment was performed by gavage during 28 days. The experiment began after the ratification of the DM and it was called initial time.

The groups were divided into negative control (NC), where healthy mice received only water with tween (3%) as a vehicle. The positive group (CP) had diabetic mice that received only water and tween (3%), as a vehicle. The third group of diabetic mice received p-Hydroxycinnamic diesters (HCA 25) dissolved in water with tween (3%), the dose was 25 mg / kg. The fourth group of diabetic mice received p-Hydroxycinnamic diesters (HCA 50) dissolved in water with tween (3%), the dose was 50 mg / kg. Finally, the last group of diabetic mice (MET 200) received a dose of 200 mg / kg of metformin.

### **2.6 Assessment of body mass of animals**

Animals of all groups were weighed weekly during the treatment in electronic balance (fiziola) and the results were expressed in grams (g).

## **2.7 Assessment of water and food consumption of animals**

Animals of all groups had their water and food intake measured weekly and the results were in milliliters (ml) and grams (g), respectively.

## **2.8 Determination of biochemical profile**

The biochemical analysis was performed on blood samples collected from the sinus of mice, which were kept fasting for 8 hours. The samples were centrifugated: thirty minutes after collection at 11000 rpm for 3 minutes, posteriorly, all the supernatant were collected and they were submit to another centrifugation at 11000 rpm for 1 minute. The supernatant was collected and analyzed by the ELISA (Enzyme-Linked Immunosorbent Assay) method in the Biochrom® Anthos Multiread 400 device. The serum was analyze according to the guidelines described by the manufacturer of the reagents (BIOCLIN®) for determination of glucose, aspartate aminotransferase, alanine aminotransferase, urea, creatinine and albumin.

## **2.9 Statistical analysis**

Data were express as mean  $\pm$  standard error of the mean (SEM). To analyze the significance of the differences between the animals of the groups, ANOVA was used followed by the Newman-Keuls test, in which the level of significance considered was  $p < 0.05$ . Statistical analyses were perform in the Prisma 5.0 program to present the graphs and apply the tests.

## **2.10 Ethical aspects**

All protocols described in this research were submitted to the Ethics Committee for Animal Research of the State University of Ceará and it was approved under the number 0527541/2018.

### 3. Results and Discussion

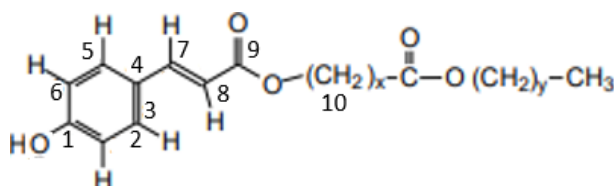
Carnauba (*Copernicia prunifera* (Miller) H. E. Moore) is a plant native to northeastern Brazil and known as the tree of life due to its various commercial properties that include cosmetic, automotive, food and pharmacological applications. Carnauba wax, considered the main input extracted from this plant, has several relevant chemical compounds such as esters, hydrocarbons, phenols, alcohols, triterpenoids, among others (Sova *et al.*, 2012).

Through carnauba wax, Guedes *et al.*, (2011) extracted a chemical compound rich in kinemics esters that was named PCO - C (*p*-methoxycinnamic diesters).

This compound has promising pharmacological activities such as hypoglycemic effect (Rodrigues *et al.*, 2014), hypolipemiant (Filho *et al.*, 2017) and antioxidant (Freitas *et al.*, 2016).

The present research extracted a compound similar to PCO-C and it was named as HCA (Figure 1). The HCA was previously characterized (Silva *et al.*, Unpublished results) and showed promising antioxidant activity, hypoglycemic and antihyperlipidemic effects.

**Figure 1.** Chemical structure of *p*-hydroxycinnamic diesters extracted from *Copernicia prunifera* (Miller) H. E. Moore



Source: Authors.

Chemically, both compounds (HCE and PCO - C) have similarities such as the presence of aromatic rings and esters, however they differ in the hydroxyl radical (-OH) present in the HCA and the methoxyl radical (-OCH<sub>3</sub>) present in the PCOC, where both are linked in the *para* position to the aromatic ring of their chemical structures (Vandenburg & Wilder, 1967).

The extraction of HCA, obtained from carnauba wax powder (type 1), resulted in a mixture of chemical compounds with light green coloring and the yield obtained was 12.3%. Vandenburg & Wilder (1967) reported that the HCA has the same coloring obtained and corresponds to 75% of the aromatic acids. Guedes *et al.*, (2011), Rodrigues *et al.*, (2014), Freitas *et al.*, (2016), and Filho *et al.*, (2017) extracted *p*-methoxycinnamic diesters from



carnauba wax powder (type 1) and they reported lower yields of 7%, 7%, 5.13%, and 4.2%, respectively.

### 3.1 Effect of hce on glycemic levels

Recently, increased significantly research using chemical compounds extracted from *Copernicia prunifera* (Miller) H. E. Moore. Studies developed by our group point out several benefits attributed to these various compounds against chronic non-transmissible diseases, such as dyslipidemias and alloxan-induced diabetes (Guedes *et al.*, 2011; Rodrigues *et al.*, 2014; Filho *et al.*, 2017; Paim *et al.*, 2017).

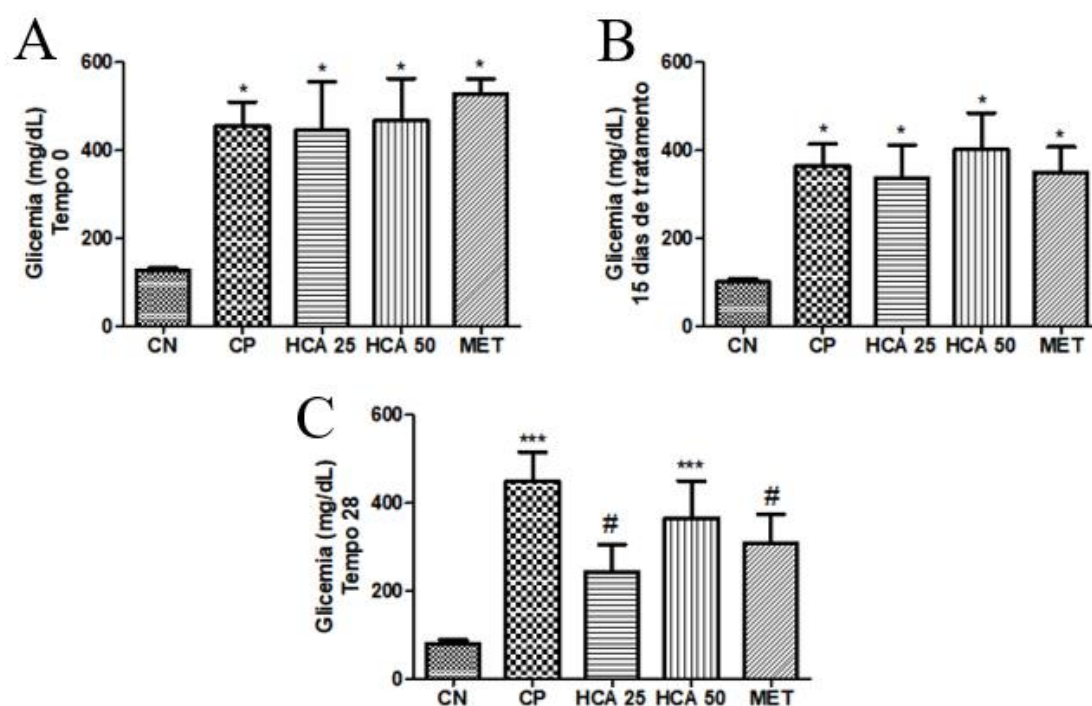
The induction protocol used in this study significantly increased ( $p < 0.05$ ) the glycemic levels of animals and it can be observed in Figure 2A.

This result is similar to finds reported by Rodrigues *et al.*, (2014), where they used alloxan to induce diabetes. Moreover, these animals presented classical symptoms of diabetes, such as hyperglycemia, increased water intake and feed consumption.

After 15 days of treatment (Figure 2B), it was possible observe that the treatments tested did not present statistically significant reductions ( $p > 0.05$ ), when compared to PC group. However, after 28 days of treatment, a significant hypoglycemic effect ( $p < 0.05$ ) was observed in animals treated with both HCA at a dose of 25 mg / kg and in the metformin group (200 mg / kg), when compared with the PC group (Figure 2C).

The esters present in HCE may have contributed to the reduction of glycemic levels in diabetic animals (Choi *et al.*, 2011). These authors attributed antioxidant and hypoglycemic activity to the esters present in *Sanguisorba officinalis* L induction of DM by streptozotocin (120 mg/Kg). The effects were related to the suppression of lipid peroxidation and the generation of hydroxyl radicals, in addition to increasing the serum level of antioxidant enzymes (Choi *et al.*, 2011).

**Figure 2.** Effect of HCA on glycemic levels of mice.



NC, Negative control; PC, Positive control; MET, metformin (200 mg/Kg/day, i.g.); HCA 25, HCA50 (*p*-hydroxycinnamic diesters solution at doses de 25 e 50 mg/Kg/day, i.g.); The values were presented as mean  $\pm$  standart error mean (SEM). To analyze the significance of differences between groups it was used ANOVA followed by Newman-Keuls test, \*  $p < 0,05$  vs NC; #  $p < 0,05$  vs PC. Source: Authors.

### 3.2 Effect of hca on renal and hepatic profiles in mice

One of the main contributions to the worsening of DM in experimental models is the increase in oxidative stress induced by toxins, since chronic hyperglycemia caused by alloxan causes increased production of free radicals, mitochondrial dysfunctions, release of metabolites and cellular apoptosis. However, these effects may be suppress by the use of natural compounds that are rich in cinnamic acids and esters (Di Marco *et al.*, 2016).

In the present study it was observed that important parameters such as albumin, AST and ALT did not present significant elevations ( $p > 0.05$ ) in relation to the control groups (NC and PC), during the experiment. However, the renal parameters presented variations throughout the study ( $p < 0.05$ ) (Table 1).

No changes in albumin values in treated diabetic animals are a relevant finding, because changes in this parameter may indicate albuminuria and microproteinuria, considered as clinical markers of diabetic nephropathy and/or due to increased protein catabolism (Sancheti *et al.*, 2011).

The ALT and AST data found in the present study are relevant, as increases in these parameters are indicative of skeletal muscle necrosis and liver necrosis that reflect a liver dysfunction (Son *et al.*, 2015).

Two other important parameters in DM cases are urea and creatinine. Both are view as important renal biomarkers, as they may indicate the presence of lesions caused by decompensated DM. Their concentration may be regulated through glycemic control and, consecutively, can reduce the progression of diabetic nephropathy (Carvalho *et al.*, 2016).

Creatinine showed an increase in serum levels ( $p < 0.05$ ) in the animals of the group treated with the standard medication (metformin), in relation to the control groups (NC and PC). It is noteworthy that the animals treated with HCA, in both doses, did not present this increase in the period after induction. After 28 days of treatment, it was possible to observe that the HCA 25 group showed an improvement ( $p < 0.05$ ) in creatinine concentration, when compared to the control groups mentioned above (Table 1).

Table 1 shows that all groups presented a significant increase in urea ( $p < 0.05$ ) in the period after induction and the same was observe after 28 days of treatment.

Therefore, in this parameter, both HCA and MET doses were not able to reduce statistically the serum urea concentration of the present study. Although not statistically significant ( $p > 0.05$ ), a trend can be observed in the improvement of serum urea concentration in animals that received HCA in both doses, when compared to the PC group.

According to Sayed *et al.*, (2013), the data cited above, referring to creatinine and urea, suggest a reduction in the glomerular filtration rate, in addition to the suggestive of increased relative weight of organs, considered as common factors in diabetes.

**Table 1.** Effect of HCA on renal and hepatic profiles of mice.

GRUPOS	NC	PC	MET	P 25	P 50
<b>ALBUMIN</b>					
Initial time	3.17 ± 0.18	2.98 ± 0.20	2.78 ± 0.14	3.56 ± 0.12	3.39 ± 0.05
28 days of treatment	4.86 ± 0.18	4.46 ± 0.10	4.27 ± 0.23	4.16 ± 0.14	3.85 ± 0.23
<b>ALT (U / L)</b>					
Initial time	21.17 ± 2.3	38.76 ± 6.0	36.14 ± 7.8	25.98 ± 4.2	34.25 ± 3.8
28 days of treatment	20.92 ± 1.6	29.76 ± 3.6	29.07 ± 6.7	12.21 ± 3.6	32.55 ± 8.4
<b>AST (U / L)</b>					
Initial time	25.2 ± 0.9	23.7 ± 2	28.9 ± 3	27.7 ± 3	24.8 ± 4
28 days of treatment	42.6 ± 3	42.1 ± 8	44.6 ± 12	46.1 ± 3	44.0 ± 12.5
<b>CREAT (mg / dL)</b>					
Initial time	1.31 ± 0.27	1.21 ± 0.35	1.67 ± 0.2*#	1.26 ± 0.14	1.3 ± 0.21
28 days of treatment	1.29 ± 0.41	1.59 ± 0.3*	1.83 ± 0.8*#	0.9 ± 0.23*#	2.1 ± 1.26
<b>UREA (mg / dL)</b>					
Initial time	47.4 ± 2.4	87.2 ± 9.6*	71.1 ± 6.1*	85.5 ± 5.3*	91.1 ± 7.1*
28 days of treatment	31.1 ± 2.3	73.3 ± 7.0*	73.1 ± 8.5*	65.9 ± 10.0*	62.3 ± 7.8*

NC, Negative control; PC, Positive control; MET, metformin (200 mg/Kg/day, i.g.); HCA 25, HCA50 (*p*-hydroxycinnamic diesters solution at doses de 25 e 50 mg/Kg/day, i.g.); ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CREAT, Creatinine. The values were presented as mean ± standart error mean (SEM) of 10 animals per group. To analyze the significance of diferences between groups it was used ANOVA followed by Newman-Keuls test, \*  $p < 0,05$  vs NC; #  $p < 0,05$  vs PC. Source: Authors.

### 3.3 Assessment of body mass, water and food consumption of mice

In the present study, it was possible to observe a significant increase ( $p < 0.05$ ) in the body mass of the animals after the period of DM induction (Table 2). After 28 days of treatment, the animals of the MET and HCA 50 groups presented significant reductions ( $p < 0.05$ ) in body mass, in relation to the animals of the CN and PC groups (Table 2). In addition, the animals of HCA 25 group maintained body mass during the experiment and corroborates the blood glucose data observed previously.

Regarding the average feed consumption, it can be observe that, after the induction period, all the groups that received alloxan presented an increase ( $p < 0.05$ ). After 28 days of treatment, the animals treated with HCA and MET did not present statistical differences ( $p > 0.05$ ), although a tendency in the reduction of this parameter can be observed, when compared to the initial time and after 28 days of treatment (Table 2).

The water consumption presented significant alterations ( $p > 0.05$ ) in the groups that received alloxan, in relation to the animals of the CN group in the post-induction period. After 28 days of treatment, the animals that received HCA did not reverse the increase in water consumption observed in the previous time, in relation to the animals of the CN group (Table 2). However, it was observed that, in relation to the metformin group, the results of HCA showed more attenuated.

The main characteristics of diabetes are polydipsia, polyuria and polyphagia. Our results showed that HCA was not able to statistically revert the increase in water and feed consumption, although it acted in the attenuation of glycemic levels of diabetic mice induced by alloxan, as previously mentioned. According to Schwartz *et al.* (2000), the regulation of weight and water and food consumption in diabetic mice can be influenced by inhibiting the expression of neuropeptide Y in the hypothalamus.

**Table 2.** Effect of HCA on body mass, water and food consumption of mice.

GRUPOS	NC	PC	MET	HCA 25	HCA 50
<b>Body mass (g)</b>					
Initial time	32.93 ± 1.4	27.88 ± 2.8*	26.51 ± 0.6*	27.31 ± 0.9*	27.01 ± 0.6*
28 days of treatment	32.23 ± 1.5	33.39 ± 0.9	29.69 ± 0.7*#	30.96 ± 1.8	28.94 ± 1.5*#
<b>Food consumption (g/animal/day)</b>					
Initial time	8.5 ± 0.38	16.2 ± 1.9*	19.8 ± 2.1*	22.5 ± 4.0*	23.9 ± 4.5*
28 days of treatment	6.1 ± 0.84	10.7 ± 1.1*	11.5 ± 1.4*	12.5 ± 2.8*	15.9 ± 1.9*
<b>Water consumption (mL/animal/day)</b>					
Initial time	6.2 ± 0.9	27.5 ± 4.3*	28.3 ± 6.0*	28.8 ± 11.7*	15.2 ± 3.8*
28 days of treatment	10.5 ± 1.6	33.5 ± 5.7*	51.6 ± 13.2*#	39.8 ± 16.8*	28.8 ± 4.0*

NC, Negative control; PC, Positive control; MET, metformin (200 mg/Kg/day, i.g.); HCA 25, HCA50 (*p*-hydroxycinnamic diesters solution at doses de 25 e 50 mg/Kg/day, i.g.); The values were presented as mean ± standart error mean (SEM). To analyze the significance of diferences between groups it was used ANOVA followed by Newman-Keuls test, \*  $p < 0,05$  vs NC; #  $p < 0,05$  vs PC. Source: Authors.

The approach of tests, analyzes and preclinical results is an important limitation of this study, considering that for more concrete results it would be necessary to extrapolate this study to clinical tests in humans.

#### 4. Conclusion

With the results abovementioned in the present study, it can be conclude that HCA commonly found in carnauba wax powder showed significant hypoglycemic effects. It is worth mentioning that these data are relevant and may assist in the development of a nutraceutical or herbal medicine as an auxiliary treatment for diabetes of a compound present in food industry.

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