

## Assessment of anti-diabetic activity of a novel hydrazine-thiazole derivative: *in vitro* and *in vivo* method

Marina Ferrara de Resende<sup>1</sup>, Cleudiomar Inácio Lino<sup>2</sup>, Elaine Maria de Souza-Fagundes<sup>3</sup>, João Vitor Paes Rettore<sup>4</sup>, Renata Barbosa de Oliveira<sup>2</sup>, Renata Adriana Labanca<sup>1\*</sup>

<sup>1</sup>Department of Foods, Pharmacy Faculty, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, <sup>2</sup>Department of Pharmaceutical Products, Pharmacy Faculty, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, <sup>3</sup>Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, <sup>4</sup>Department of Biology, Institute of Biological Science, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil

Diabetes mellitus is a chronic disease resulting in oxidative stress that promotes tissue damage. The appearance of this disease is highly related to lifestyle and food of the population, being of great interest to search for a dietary supplement that can also act by reducing oxidative alterations. Based on the broad range of biological activity of thiazole derivatives, this work aimed to evaluate the *in vitro* antioxidant activity of a novel hydrazine-thiazole derivative and studies *in vivo*. In *in vivo* experiments, the liver extracts of healthy and diabetic Wistar rats were used, with analysis to determine the enzymatic activity of SOD, CAT, GPx, and GR, and determination of lipid peroxidation. Finally, in the blood of these animals, biochemical parameters were evaluated. Statistical evidence of changes caused in liver enzymes and lipid peroxidation was not detected; however, these parameters were also not changed between control groups with and without diabetes. On the other hand, concerning biochemical parameters, significant differences were detected in uric acid, alkaline phosphatase, ALT, and urea, indicating a possible antioxidant protective role of such substances in the liver and kidney of diabetic animals that could be acting by means other than that commonly reported in the literature.

**Keywords:** Oxidative stress. Diabetes mellitus. Curcumin. Thiazole derivatives. Dietary supplement.

### INTRODUCTION

Human organism, by means of its normal biochemical functions, continuously produces reactive species, against which there is an antioxidant defense system. However, when there is an imbalance, either by increased production of these reactive species or from a decrease in the defense network, a condition known as oxidative stress is installed. Under such condition, reactive species generate cumulative damage to biomolecules such as lipids, proteins and DNA, being related to several chronic diseases, for example diabetes mellitus (Carocho, Ferreira, 2013; Yadav *et al.*, 2013; Rani *et al.*, 2016).

Diabetes mellitus (DM) is a metabolic disorder

characterized by chronic hyperglycemia, affecting the oxidative stress associated metabolism, which leads to serious health problems. The increasing number of such disease in world population, assigned not only to the increasing number of elderly, but mainly to the current lifestyle, causes diabetes to have a major impact on health expenditures. It is therefore important to control the worsening of such disease in order to both reduce the suffering of patients and contain social costs (Henriques *et al.*, 2010; WHO, 2016).

Thus, in recent years, it has grown the interest for antioxidant compounds naturally present in food or synthesized from them, that are effective in preventing the onset or worsening of diabetes, with minimal side effects (Hussein, Abu-Zinadah, 2010; Prabhakar *et al.*, 2012; Jeenger *et al.*, 2015).

In this context stands out the curcumin, a yellow-gold pigment which is the main active extracted from

\*Correspondence: R. A. Labanca. Departamento de Alimentos, Faculdade de Farmácia, Universidade Federal de Minas Gerais. Av. Antônio Carlos 6627, 31270-901 - Belo Horizonte, Minas Gerais, Brazil. E-mail: [renata@bromatologiaufmg.com.br](mailto:renata@bromatologiaufmg.com.br)

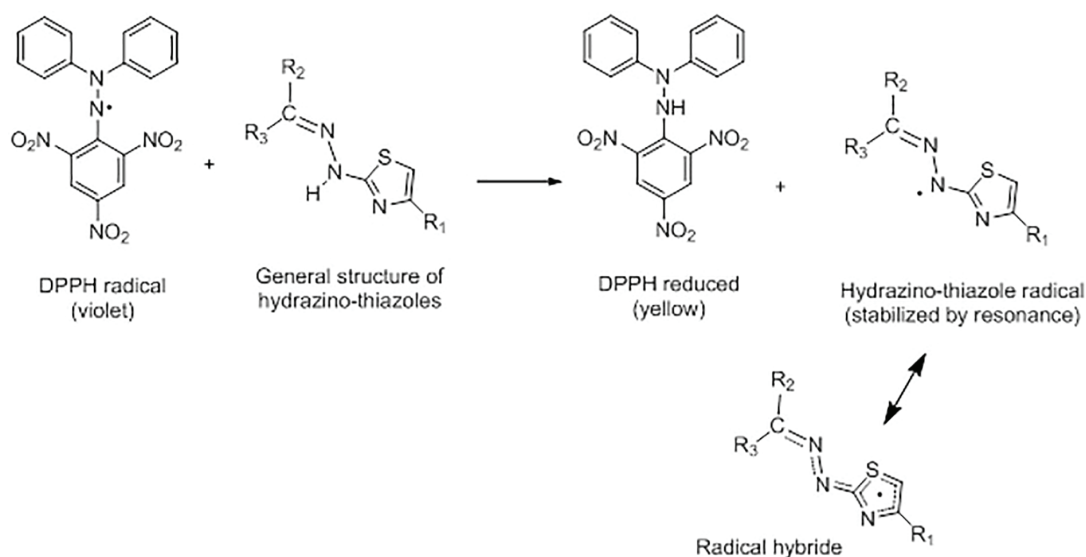
the rhizome of turmeric or saffron (*Curcuma longa*) being such plant traditionally used in Chinese and Indian cooking and medicine (Jeenger *et al.*, 2015). Curcumin is widely studied for its great antioxidant potential and can be used as a dietary supplement to act in the protection of biomolecules, being associated with improvements in the treatment of several diseases (Anand *et al.*, 2008; Volp, Renhe, Stringueta, 2009; Honorato *et al.*, 2013). Despite its important pharmacological properties and excellent safety profile, curcumin has problems as to its solubility, bioavailability and intense color, hindering its widespread use (Anand *et al.*, 2007; Prasad, Tyagi, Aggarwal, 2014).

Thus, the search for new compounds displaying antioxidant properties and that overcome the problems associated with curcumin is of interest for use as a food supplement. This type of supplement could be used, for example, to reduce damage caused by oxidative stress in diabetes (Anand *et al.*, 2008; Bhullar *et al.*, 2013; Jeenger *et al.*, 2015). The antioxidant activity *in vitro* of hydrazine-thiazole derivatives was described by Shih *et al.* in 2007, with interesting results (Shih, Su, Wu, 2007). According to these authors, the scavenging activity of hydrazine-

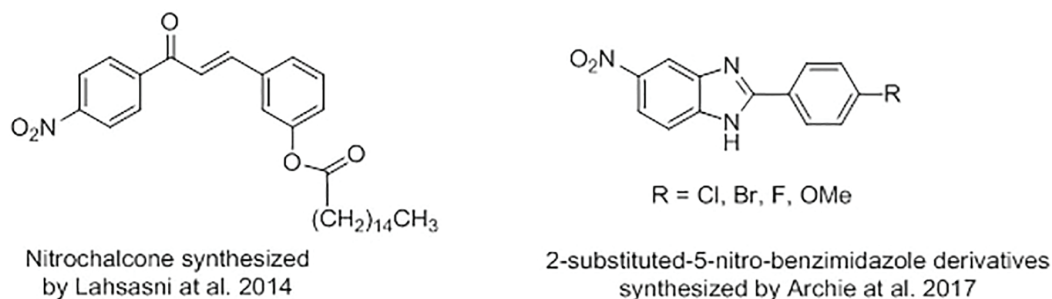
thiazoles can be related to the presence of N-H group in the hydrazine moiety, which has ability to donate a hydrogen atom and to reduce the DPPH radical (Figure 1).

Lahsasni *et al.* (2014) evaluated the antioxidant activity of nitrochalcones using the 1,1-biphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method and, among the tested compounds, the (*E*)-3-(3-(4-nitrophenyl)-3-oxoprop-1-en-1-yl)phenyl palmitate was the most effective, showing higher antioxidant activity than the ascorbic acid (Lahsasni, Al Korbi, Aljaber, 2014). In addition, recently, the antioxidant activity of 2-substituted-5-nitro-benzimidazole derivatives was investigated by Archie *et al.* (2017) and these compounds showed a good activity with IC<sub>50</sub> values ranging from 3.17 to 7.59 µg/mL (Archie *et al.*, 2017) (Figure 2). These studies indicate that the presence of a nitro group can be important for the antioxidant activity.

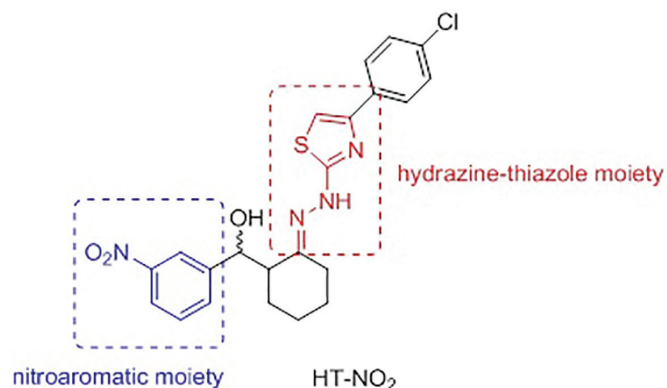
Based on the scavenging ability of hydrazine-thiazole derivatives and potential biological activity displayed by nitro compounds, we decided to synthesize a hydrazine-thiazole-nitrocompound (HT-NO<sub>2</sub>) hybrid and to evaluate its antioxidant potential activity (Figure 3).



**FIGURE 1** - Schematic representation of the reaction between DPPH radical and hydrazine-thiazole derivatives.



**FIGURE 2** - Examples of nitro compounds with antioxidant activity.



**FIGURE 3** - Chemical structure of the hybrid molecule synthesized in this work.

## MATERIAL AND METHODS

### Synthesis

Melting point was determined on a Microquímica MQAPF 301 apparatus and is uncorrected. A FT-IR spectrum was recorded using a Perkin Elmer Spectrum One infrared spectrometer and absorption is reported as wave numbers (cm<sup>-1</sup>). NMR spectrum was recorded on a BrukerAvance DPX 200 spectrometer (200 MHz). Chemical shifts are given in  $\delta$  (ppm) scale and J values are given in Hz. All reagents of analytical grade were obtained from commercial suppliers and used without previous purification.

Synthesis of 2-[(3-nitrophenyl)hydroxymethyl]cyclohexanone **1** (Singh, Pandey, Tripathi, 2008)

A solution of NaOH 0.2 mol/L (1 eq.) was added slowly to a mixture of cyclohexanone (2.0 eq.) and 3-nitrobenzaldehyde (1.0 eq.) and stirred at room temperature. The reaction was monitored by TLC (hexane/ethyl acetate 6:4). After 1 hour, the mixture was diluted with cool water and washed with ethyl acetate (3x 30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated. The product **1** was obtained as a yellow oil in 91% yield. IR (cm<sup>-1</sup>): 3472, 3090, 2940, 2865, 1697, 1524, 1347 1480, 1448.

Synthesis of 2-[(3-(nitrophenyl)hydroxymethyl)cyclohexylidene]hydrazine-carbothioamide **2**

Using the general procedure described in literature (Maccioni *et al.*, 2002), the thiosemicarbazone **2** was obtained as a white solid in 61% yield. Mp: 171.5-174.9 °C; IR (cm<sup>-1</sup>): 3392, 3238, 3153, 3026, 2985, 1603, 1521, 1346, 1471, 1434.

Synthesis of 2-[(2-(3-nitrophenyl)hydroxymethyl)cyclohexylidenehydrazo]-4-(4-chlorophenyl)thiazole (HT-NO<sub>2</sub>)

Using the general procedure described in literature

(Maccioni *et al.*, 2002), HT-NO<sub>2</sub> was obtained as an orange solid in 99 % yield. Mp: 182.1-182.9 °C; IR (cm<sup>-1</sup>): 3376, 3037, 2936, 2857, 1619, 1536, 1348, 1479, 1445, 737; <sup>1</sup>H NMR (200 MHz, DMSO-d<sub>6</sub>),  $\delta$ /ppm: 8.3 (1H, s); 8.2 (1H, d); 7.9-7.8 (3H, m); 7.6-7.6 (2H, m); 7.4 (2H, d); 7.3 (1H, s); 5.2 (1H, d); 2.6 (2H, m); 1.7-1.2 (8H, m); <sup>13</sup>C NMR (50 MHz, DMSO-d<sub>6</sub>),  $\delta$ /ppm: 169.6, 156.8, 149.3, 147.4, 143.2, 132.8, 132.5, 132.3, 128.4, 128.1, 126.5, 121.9, 121.4, 102.7, 74.2, 50.0, 30.0, 26.8, 24.7, 23.7; HRMS (*m/z*) 457.1096 [M+H]<sup>+</sup> calcd 457.1101 C<sub>22</sub>H<sub>22</sub>ClN<sub>4</sub>O<sub>3</sub>S<sup>+</sup>.

### *In vitro* assays

#### DPPH radical scavenging assay

The ability of curcumin and HT-NO<sub>2</sub> to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>) radical was measured using the method described by Lue *et al.* (2010) with modifications (Bhullar *et al.*, 2013), on microscale and triplicate. The absorbance was measured at 515 nm on a spectrophotometer with automatic microplate reader (Molecular Devices, VersaMaxProgram, Sunnyvale, California, USA) and the percentage of DPPH that has reacted was calculated by using Eq. 1:

$$\% \text{ antioxidant activity} = ((A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}) * 100 \quad (1)$$

being A = absorbance at 515 nm.

#### ABTS radical cation scavenging assay

Antioxidant activity of 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS<sup>•+</sup>) radical capture method was determined by the method described by Re *et al.* (1999) and modified by Dinkova-Kostova *et al.* (2007), on microscale and triplicate, measuring the absorbance at 734 nm in a spectrophotometer with automatic microplate reader (Molecular Devices, Versa Max Program, Sunnyvale, California, USA). The amount of radical scavenged by the samples was calculated using Eq. 2, and the result was expressed in percentage of antioxidant activity:

$$\% \text{ antioxidant activity} = ((A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}) * 100 \quad (2)$$

being A = absorbance at 734 nm.

#### Ferric reducing antioxidant power (FRAP) assay

To assess the antioxidant capacity based on ferric reduction, the method described by Benzie and Strain (1996) was used with modifications (Bhullar *et al.*, 2013), in triplicate. After reading the absorbance at 593 nm in a spectrophotometer with automatic microplate reader

(Molecular Devices, Versa Max Program, Sunnyvale, California, USA), the antioxidant potentials of the analyzed substances were calculated based on Trolox standards at concentrations of 50, 100, 150, 200, 250, 300 and 350  $\mu\text{M}$ . The results were expressed as Trolox equivalents, or TE, in  $\mu\text{M}$  Trolox/ 100  $\mu\text{M}$  sample.

#### Cytotoxicity against Vero cells

The cytotoxicity of HT-NO<sub>2</sub> was tested using healthy cells model (Vero cells, isolated from kidney of an African green monkey), donated by Dr Erna Kroon (Federal University of Minas Gerais, Brazil). The lineage was maintained in the logarithmic phase of growth in DMEM (Dulbecco's Modified Eagle Medium, Sigma Aldrich, St. Louis, Missouri, USA) supplemented with 100 U mL<sup>-1</sup> penicillin and 100 mg mL<sup>-1</sup> streptomycin (GIBCO BRL, Grand Island, NY) enriched with 5% of fetal bovine serum (GIBCO BRL, Grand Island, NY). Cells were maintained at 37 °C in a humidified incubator with 5% CO<sub>2</sub> and 95% air. The media were changed twice weekly and they were regularly examined.

Cytotoxicity assay was performed using MTT to measure cell viability, as previously described (Monks *et al.*, 1991). Compound was tested at a range of concentrations from 0.1 to 100  $\mu\text{M}$ . Cells were seeded at density of 10,000 cell/well (96 well-plates, Sarstedt, Germany) and pre incubated for 24 hours to allow stabilization. Cells were seeded at density of 10,000 cell/well (96 well-plates, Sarstedt, Germany) and pre incubated for 24 hours to allow stabilization. The different concentrations of compound was added to cells and incubated for 48 hours in an atmosphere of 5% CO<sub>2</sub> and 100% relative humidity. Twenty microliters of MTT solution (5 mg.mL<sup>-1</sup> in phosphate-buffered saline, Sigma Aldrich, St. Louis, Missouri, USA) were added to each well 4 h before the end of period incubation. The supernatant was removed and 200  $\mu\text{L}$  of 0.04 M HCl in isopropyl alcohol (Vetec, Brazil) were added to dissolve the formazan crystals. The optical densities (OD) were evaluated in a spectrophotometer at 595 nm (Molecular Devices, Versa Max Program, Sunnyvale, California, USA). Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as control (blank) and was treated in the same way as the drug-containing media. Results were expressed as percentage of cell proliferation, comparing with 0.5% DMSO control and were calculated by Eq. 3:

$$\text{viability (\%)} = (\text{mean OD treated} - \text{mean OD background}) / (\text{mean OD untreated cultured, i.e. 0.5\% DMSO} - \text{mean OD blank wells}) \times 100. \quad (3)$$

## In vivo assays

### Animal procedures

Forty-eight male Wistar rats, near 30 days old, weighting 80-170 g, were obtained from animal house of Faculty of Pharmacy, UFMG. The rats were individually housed in a room with monitored temperature ( $23.6 \pm 1.6$  °C) and humidity ( $61.6 \pm 8.4\%$ ), with a lighting schedule of 12 h light and 12 h dark. Before the experiment, the rats were submitted to a period of 12 days of adaptation. Diabetes was induced in 40 rats by a single intraperitoneal injection of streptozotocin (50 mg/kg body weight). After 3 days, capillary blood glucose (CBG) was assessed, and the animals with fasting glycemia higher than 200 mg/dL were considered diabetic, being chosen for the experiment. The proposed experimental protocol was prepared in compliance with the Ethical Principles of Animal Experimentation, and approved by the Ethics Committee on Animal Use (CEUA/UFMG) under registration number 273/2013.

The 48 animals were distributed into six groups of 8 animals each, as follows:

- Control Group: Non-diabetic, vehicle (Carboxymethyl cellulose – CMC – 0.5%) by oral gavage;
- Diabetic Control Group: Diabetic, CMC 0.5% by oral gavage;
- Group Curc-50: Diabetic, curcumin 50 mg/kg of body weight by oral gavage;
- Group Curc-100: Diabetic, curcumin 100 mg/kg of body weight by oral gavage;
- Group HTNO<sub>2</sub>-50: Diabetic, HT-NO<sub>2</sub> 50 mg/kg of body weight by oral gavage;
- Group HTNO<sub>2</sub>-100: Diabetic, HT-NO<sub>2</sub> 100 mg/kg of body weight by oral gavage.

A standard pellet diet and tap water were supplied *ad libitum* for all the animals. The oral gavage was performed once a day in the morning, for 31 days, using CMC 0.5% as vehicle. After that, animals were euthanized, blood was collected and liver was dissected out for analyses.

Lipid peroxidation assessment by formation of thiobarbituric acid reactive substances (TBARS)

For evaluation of lipid peroxidation, it was used a method based on Buege and Aust (1978), on microscale and triplicate, by measuring the thiobarbituric acid (TBA) reactivity of malondialdehyde (MDA), an end product of fatty acid peroxidation (Buege, Aust, 1978). Readings at 530 nm were performed (Molecular Devices, Versa Max Program, Sunnyvale, California, USA) and a standard curve was prepared using MDA. TBARS measure was expressed as  $\mu\text{M}$  of MDA/ gram of the liver.



## Evaluation of liver antioxidant enzymes activity

Superoxide Dismutase (SOD). SOD activity was determined as proposed by Marklund and Marklund (1974), with modifications, being measured the inhibition of pyrogallol autoxidation (Marklund, Marklund, 1974). The assay was performed on microscale and triplicate, performing readings at 570 nm (Molecular Devices, Versa Max Program, Sunnyvale, California, USA). The results were expressed as units of SOD per milligrams of total protein (U SOD/mg total protein).

Catalase (CAT). CAT activity was determined by a method based on Aebi (1984) (30), in triplicate (Aebi, 1984). The difference between 240 nm absorbance (Molecular Devices, Versa Max Program, Sunnyvale, California, USA) on time 1 minute and time 0 minute give the result, expressed as  $\Delta E$ . Result of CAT activity was expressed in arbitrary units, as Eq. 4:

$$\text{CAT activity} = \Delta E \times \text{min}^{-1} \times \text{g}^{-1} \text{ of total protein} \quad (4)$$

Glutathione Peroxidase (GPx). GPx activity was assessed following Flohé e Gunzler (1984) with modifications, on microscale and triplicate. Readings were performed on time 0 minute and time 1 minute, at 340 nm, in a spectrophotometer with automatic microplate reader (Molecular Devices, Versa Max Program, Sunnyvale, California, USA). Results were expressed in arbitrary units, as Eq. 5:

$$\text{GPx activity} = \Delta E \times \text{min}^{-1} \times \text{g}^{-1} \text{ of total protein} \quad (5)$$

Glutathione Reductase (GR). GR activity was determined by the method described by Carlberg e Mannervick (1975) with modifications, on microscale and triplicate. Readings were performed on time 0 minute and time 1 minute, at 340 nm, in a spectrophotometer with automatic microplate reader (Molecular Devices, Versa Max Program, Sunnyvale, California, USA). Results were expressed in arbitrary units, as Eq. 6:

$$\text{GR activity} = \Delta E \times \text{min}^{-1} \times \text{mg}^{-1} \text{ of total protein} \quad (6)$$

## Biochemical analysis

Biochemical analysis were performed by means of Bioclin® standardized diagnostic kits (Bioclin – Quibasa Química Básica Ltda) for spectrophotometric assessments (spectrophotometer UV/Vis AJX-1900, Micronal S.A., São Paulo, São Paulo, Brazil). The following blood biochemical parameters were analyzed: uric acid,

alanine aminotransaminase (ALT), albumin, amylase, aspartate aminotransaminase (AST), total cholesterol, creatinine, HDL cholesterol, alkaline phosphatase (ALP), hemoglobin, magnesium, total protein, triglycerides and urea. Kits were utilized according to manufacturer's specifications.

### Statistical analyses

After removal of outliers by the Grubbs test, the comparison of means was performed by analysis of variance (ANOVA), following Tukey's test. Differences were considered significant when the probability was  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Synthesis

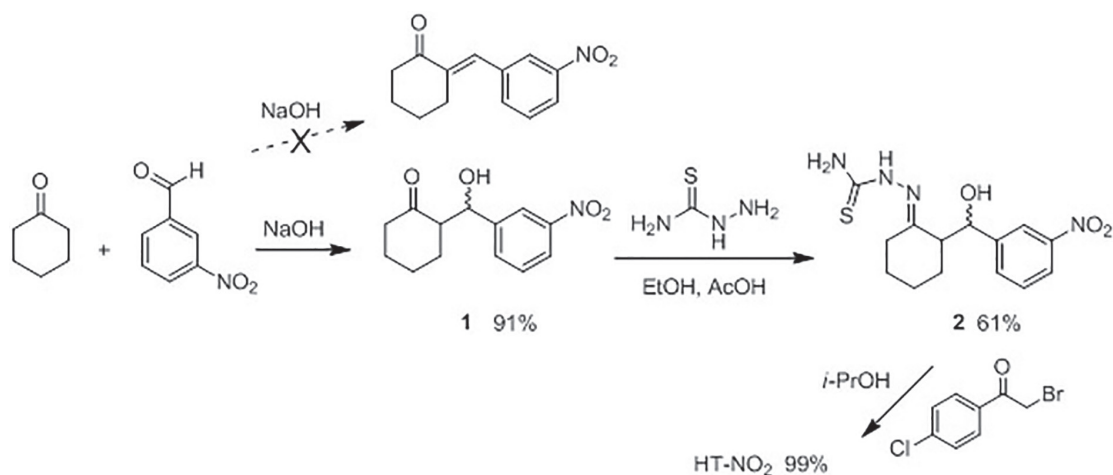
The hydrazine-thiazole TH-NO<sub>2</sub> was synthesized in three steps as depicted in Scheme 1. All the attempts to obtain the product resulting from the aldol condensation between cyclohexanone and 3-nitrobenzaldehyde failed, even though different temperature conditions were tested. In all cases, the only product obtained was the product of aldol addition **1**, which was then used in the next steps of synthesis. The mixture of isomers of **1** was converted to the thiosemicarbazone **2** using a classic methodology by reacting the ketone with thiosemicarbazide (Maccioni *et al.*, 2002). Then, the thiosemicarbazone **2** was subjected to a cyclization reaction in the presence of 4-chloro-2'-bromoacetophenone.

### DPPH, ABTS and FRAP assays

*In vitro* antioxidant activity results are shown in Table I. Curcumin and the synthetic antioxidant Trolox were used as positive control.

In the DPPH assay for antioxidant activity assessment, curcumin showed a lower capability for capturing such radical ( $40.10 \pm 1.36\%$ ) when compared to trolox ( $47.43 \pm 1.48\%$ ). The opposite occurred in the ABTS assay, in which curcumin ( $30.18 \pm 2.01\%$ ) showed better results than trolox ( $19.51 \pm 0.98\%$ ). In the FRAP assay, 100  $\mu\text{M}$  of curcumin was equivalent to 102.55  $\mu\text{M}$  of trolox, showing a slightly superior antioxidant capacity of curcumin.

In all of these assays, it was observed that HT-NO<sub>2</sub> showed better results than that of the positive control (curcumin). Regarding IC<sub>50</sub>, the hydrazine-thiazole derivative showed lower value than that of curcumin (IC<sub>50</sub> = 167.7  $\mu\text{M}$ ), thus being more active: HT-NO<sub>2</sub> (IC<sub>50</sub> = 139.2  $\mu\text{M}$ ).



**SCHEME 1** - Synthetic route for the preparation of hydrazine-thiazole HT-NO<sub>2</sub>.

**TABLE I** - Comparison of the results obtained for the *in vitro* antioxidant assays: DPPH radical scavenging assay; ABTS radical cation scavenging assay and ferric reducing antioxidant power (FRAP) assay

Sample	DPPH (%)	ABTS (%)	FRAP ( $\mu\text{M TE}/100 \mu\text{M sample}$ )
Curcumin	40.10 $\pm$ 1.36	30.18 $\pm$ 2.01	102.55 $\pm$ 0.80
Trolox	47.43 $\pm$ 1.48	19.51 $\pm$ 0,98	-
HTNO <sub>2</sub>	38.91 $\pm$ 0.95	18.27 $\pm$ 3.09	64.12 $\pm$ 2.41

Before the *in vivo* evaluation, the substance HT-NO<sub>2</sub> was tested for its cytotoxicity against Vero cell lines. Vero cell lines are cell lines derived from African Green Monkeys and are commonly used to investigate the cytotoxicity of the substances against mammalian cells (Lavrado *et al.*, 2010). Substance HT-NO<sub>2</sub> showed no cytotoxicity against Vero cells at 100  $\mu\text{M}$  since no reduction on cell viability was observed using MTT assay, when compared with the control (DMSO, 0.5%). Therefore, it was considered suitable for use in the animal model of diabetes mellitus.

### Physiological parameters of animals

*In vivo* studies were conducted using normal rats and rats with streptozotocin-induced diabetes. The capillary blood glucose of rats was checked weekly, and the mean values are presented in Figure 4. Table II shows the statistical analysis of glucose levels in the last week of the experiment in order to assess the real impact of the treatments.

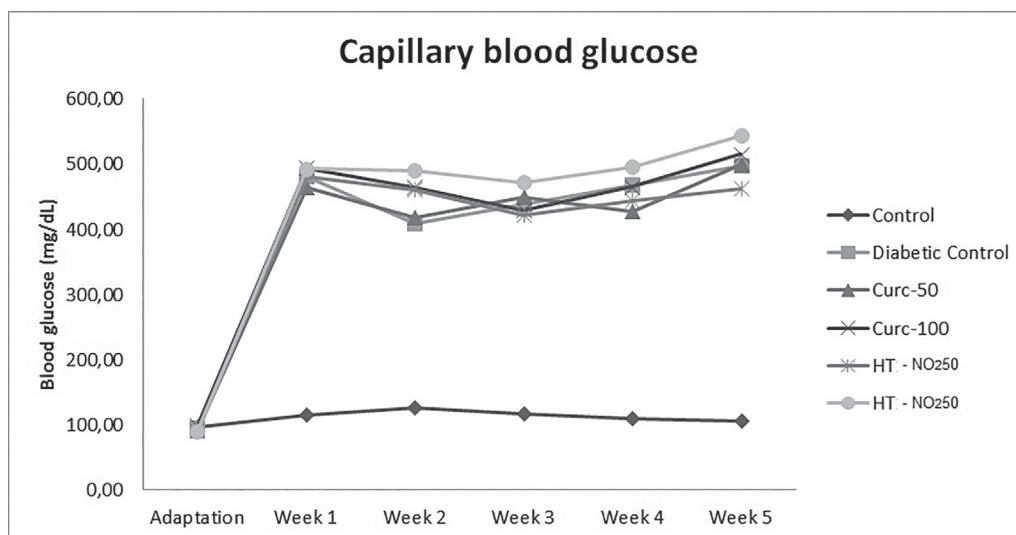
All experimental groups showed initial average blood glucose levels within the normal range (around 95 mg/dL), just as observed by Najafian (2014) and Palma *et al.* (2014). After three days of diabetes induction, the groups submitted to STZ administration at the dose of

50 mg/kg and had a significant increase in blood glucose levels (around 480 mg/dL), characterizing them as diabetics. Chronic hyperglycemia, a characteristic found in diabetic groups treated or not, results from the failure in production or effect of insulin, leading to abnormalities in carbohydrate, protein and lipid metabolism (Prabhakar *et al.*, 2013).

At the end of the experiment, it was observed that the glycemia of the non-diabetic control group maintained normal levels, in contrast to the diabetic rats, either treated or not. Treatments with either curcumin or HT-NO<sub>2</sub> showed no changes in the altered glycaemic profile of diabetic rats. Such result may be due in part to the high glycaemic rates resulting from altered insulin production, whose control requires inclusion of insulin in the treatment.

Food intake for each group, as well as the evolution of the animals' body weight, were assessed every three days throughout the test. Results are shown in Figures 5 and 6.

Figures analysis showed a significant increase in food intake for diabetic mice when compared to the control group without diabetes. However, treatments with curcumin and HT-NO<sub>2</sub> showed no influence on food intake when compared to the diabetic animals with no treatment. The non-diabetic control group differed from others with a greater weight gain, which lasted throughout the experimental period. At the end of the experiment, a



**FIGURE 4** - Changes in capillary blood glucose levels of animals throughout the experimental period. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.

**TABLE II** - Capillary blood glucose levels of animals at the end of experiment (week 5)

Group	Blood glucose (mg/dL) ± S.D.
Control	106.6 ± 6.8 <sup>b</sup>
Diabetic Control	496.9 ± 102.0 <sup>a</sup>
Curc-50	499.0 ± 92.4 <sup>a</sup>
Curc-100	514.9 ± 80.9 <sup>a</sup>
HTNO <sub>2</sub> -50	462.2 ± 73.9 <sup>a</sup>
HTNO <sub>2</sub> -100	543.7 ± 51.7 <sup>a</sup>

Mean ± standard deviation followed by different letters in the column differ from each other by Tukey’s test, with 95% confidence. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc 100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HT-50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.

minor body weight gain was observed in the diabetic rats when compared to the control group without diabetes. Among the groups treated with curcumin and HT-NO<sub>2</sub>, the animals treated with HT-NO<sub>2</sub> at 100 mg/kg showed a similar weight gain to the diabetic control group. Animals treated with curcumin 50 mg/kg followed by HT-NO<sub>2</sub> 50 mg/kg and curcumin 100 mg/kg showed a greater weight gain when compared to the diabetic control group,

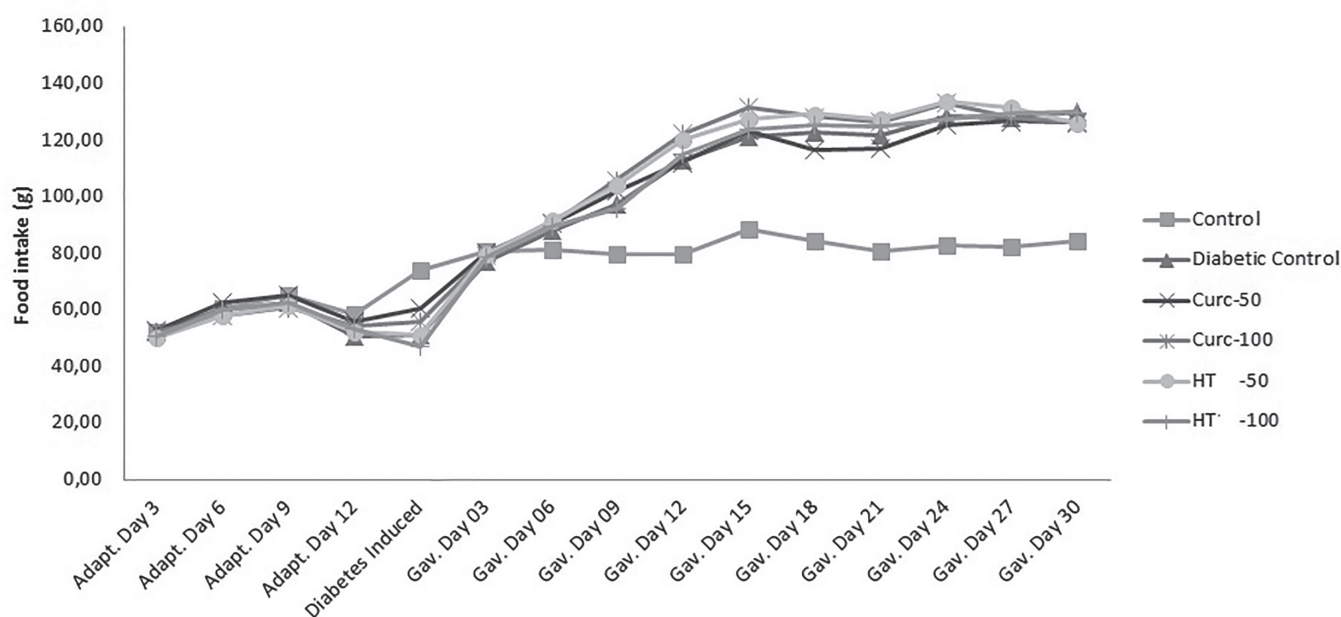
showing evidence of a protective role of these substances in animals’ weight gain.

Thus, treatment with curcumin and HT-NO<sub>2</sub> had no influence on animal food intake, but prevented at least partially the loss of body weight when compared to diabetic animals with no treatment.

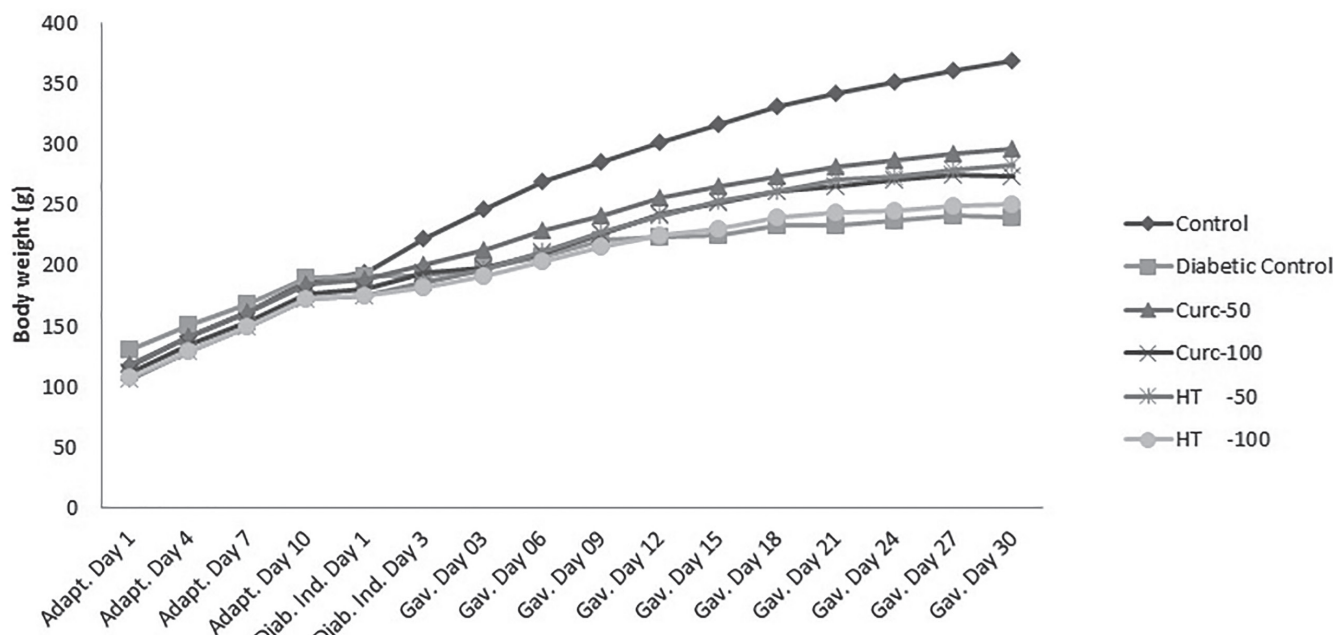
Weight loss, which is a characteristic symptom of uncontrolled diabetes, and which can be observed from the induction model used in this study, may be explained by the absence of insulin in DM, compromising glucose uptake by cells of the organism, making such carbohydrate unavailable for energy gain. Thus, the organism comes into a state of high catabolism, leading to degradation or loss of structural proteins responsible for rigidity, consistency and elasticity of tissues, as well as increased lipolysis, both affecting reduction of body weight in DM, together with dehydration by polyuria (Sarkhail *et al.*, 2007).

The increase in food intake that occurs in DM can be linked to disturbances in the processes of hunger regulation in the hypothalamus. After a meal, insulin is present at high plasma concentrations, being transported across the blood-brain barrier, acting as an anorectic agent with action mainly in the arcuate nucleus of the hypothalamus, causing satiety. However, in DM, with no insulin, the central system is not inhibited and therefore indicates a lack of glucose, and hunger, stimulating increased food intake (van de Sande-Lee, Velloso, 2012).

In the present study, it was also observed that the animals of diabetic groups showed other typical symptoms of decompensated DM beyond polyphagia and poliastenia,



**FIGURE 5** - Food intake of the animals throughout experimental period. Adapt. = Adaptation period, measurement of food intake on the indicated days; Gav. = Oral gavage period, measurement of food intake on the indicated days. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.



**FIGURE 6** - Animals' body weight evolution throughout experimental period. Adapt. = Adaptation period, measurement of body weight on the indicated days; Diab. Ind. = Diabetes induced period, measurement of body weight on the indicated days; Gav. = Oral gavage period, measurement of body weight on the indicated days. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.



such as polyuria and polydipsia, consistent with symptoms found in other experiments that used the same STZ-induced diabetes model in rats (Najafian, 2014; Gutierrez *et al.*, 2012). Such symptoms are widely known in DM and are related to hyperglycemia, which is the main factor responsible for the development of polyuria, polydipsia, and polyphagia.

The animals' liver was removed and its weight measured, as shown in Table III. Values are presented as percentage of animal's body weight  $\pm$  standard deviation, followed by a mean comparison test.

**TABLE III** - Mean values for animals liver weight. Data compared to total body weight, divided in experimental groups

Group	Liver (% of body weight) $\pm$ S.D.
Control	3.7 $\pm$ 0.2 <sup>b</sup>
DiabeticControl	4.9 $\pm$ 0.7 <sup>a</sup>
Curc-50	4.9 $\pm$ 0.4 <sup>a</sup>
Curc-100	4.8 $\pm$ 0.4 <sup>a</sup>
HTNO <sub>2</sub> -50	4.9 $\pm$ 0.5 <sup>a</sup>
HTNO <sub>2</sub> -100	5.4 $\pm$ 0.7 <sup>a</sup>

Mean  $\pm$  standard deviation followed by different letters in the column differ from each other by Tukey's test, with 95% confidence. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.

It is observed that only the non-diabetic control group was statistically different from the others. All diabetic groups, both with and without treatment, showed an increased ratio of liver to body weight when compared to the control group without diabetes. Such fact can be explained by the lack of insulin production in this experimental model of DM, causing disturbances in the metabolism of carbohydrates, proteins, and lipids. In this pathogenesis, adipose tissue begins to release stored fat in order to be used as energy by the body. Lipids pass through the liver, which starts to accumulate an excess of available lipids (Sousa, Navarro, 2013).

#### *Thiobarbituric acid reactive substances (TBARS)*

Table IV shows the means of each group for TABRS carried out in animal liver samples. The values are expressed as  $\mu$ M of MDA/gram of the liver  $\pm$  standard deviation, followed by mean comparison test.

**TABLE IV** - Mean values obtained on formation of thiobarbituric acid reactive substances (TBARS) assay in liver samples for lipid peroxidation assessment

Group	TBARS ( $\mu$ M MDA/g liver)
Control	32.7 $\pm$ 8.7 <sup>a</sup>
DiabeticControl	36.4 $\pm$ 8.1 <sup>a</sup>
Curc-50	49.7 $\pm$ 13.2 <sup>a</sup>
Curc-100	44.0 $\pm$ 8.1 <sup>a</sup>
HTNO <sub>2</sub> -50	48.9 $\pm$ 18.4 <sup>a</sup>
HTNO <sub>2</sub> -100	52.4 $\pm$ 23.8 <sup>a</sup>

Mean  $\pm$  standard deviation followed by different letters in the column differ from each other by Tukey's test, with 95% confidence. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HT 100 mg/kg of body weight by oral gavage.

Based on the results of this study, changes in levels of thiobarbituric acid reactive substances between the control and treated groups were not observed. It is known that hyperglycemia in DM is responsible for the increased production of reactive species, thereby causing oxidative stress, which in turn leads to impairment of antioxidant defenses and increased susceptibility to lipid peroxidation. Although diabetic groups showed high glycemic rates in this assessment, increased lipid peroxidation (measured by TBARS) was not observed in such groups. Such fact can be related to the state of the antioxidant system, which played an important role in the elimination of reactive species and may have been protecting the body against lipid peroxidation during the 31 days of study.

#### **Antioxidant enzyme activity**

Results obtained in testing the activity of SOD, CAT, GPx, and GR are shown in Table V, followed by  $\pm$  standard deviation and mean comparison test.

Table 5

The assessment of SOD, CAT, GPx, and GR in liver tissue revealed that the activity of such enzymes was not statistically different between the control groups both with and without diabetes. Among the diabetic groups treated with curcumin and HT-NO<sub>2</sub>, the administration

**TABLE V** - Evaluation of liver enzymes activity – SOD, CAT, GPx, GR

Assay	Control	Diabetic Control	Curc-50	Curc-100	HTNO <sub>2</sub> -50	HTNO <sub>2</sub> -100
SOD (U SOD/mg total protein)	25.4 ± 6.7 <sup>b</sup>	34.5 ± 6.9 <sup>ab</sup>	35.5 ± 6.2 <sup>ab</sup>	51.3 ± 25.7 <sup>ab</sup>	29.3 ± 1.8 <sup>ab</sup>	54.2 ± 24.4 <sup>a</sup>
CAT (ΔE.min <sup>-1</sup> .mg <sup>-1</sup> total protein)	0.3 ± 0.1 <sup>b</sup>	0.5 ± 0.1 <sup>ab</sup>	0.5 ± 0.1 <sup>ab</sup>	0.7 ± 0.4 <sup>ab</sup>	0.4 ± 0.0 <sup>ab</sup>	0.7 ± 0.4 <sup>a</sup>
GPx (ΔE.min <sup>-1</sup> .mg <sup>-1</sup> total protein)	0.7 ± 0.2 <sup>ab</sup>	0.6 ± 0.3 <sup>ab</sup>	0.4 ± 0.2 <sup>b</sup>	0.6 ± 0.2 <sup>ab</sup>	1.1 ± 0.2 <sup>a</sup>	0.8 ± 0.5 <sup>ab</sup>
GR (ΔE.min <sup>-1</sup> .mg <sup>-1</sup> total protein)	2.1 ± 0.6 <sup>b</sup>	2.6 ± 0.4 <sup>ab</sup>	3.1 ± 0.4 <sup>ab</sup>	4.6 ± 2.1 <sup>ab</sup>	2.8 ± 0.2 <sup>ab</sup>	5.1 ± 2.7 <sup>a</sup>

Mean ± standard deviation followed by different letters in the column differ from each other by Tukey's test, with 95% confidence. SOD = superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; GR = glutathione reductase. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HT-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.

of HT-NO<sub>2</sub> at 100 mg/kg provided high levels of SOD, CAT, and GR, which did not differ statistically from the other diabetic groups, but differed from the control group without diabetes. Regarding the levels of GPx, there was no difference between the control groups both with and without diabetes and the groups treated with curcumin and HT-NO<sub>2</sub>. Only the Curc-50 and HTNO<sub>2</sub>-50 groups were statistically different from each other, the former presenting a higher value (1.06 ± 0.18 ΔE.min<sup>-1</sup>.mg<sup>-1</sup> protein) than the latter (0.44 ± 0.20 ΔE.min<sup>-1</sup>.mg<sup>-1</sup> protein). Thus, the enzyme levels in the liver presented no decrease in diabetes, and treatment with curcumin did not exert statistically proven effects on the studied enzymatic activity rates.

The effect of diabetes on enzyme activity may vary according to sex, animal species, time in which the animals remained diabetic, or studied tissue, being this activity increased, decreased, or unchanged (Martim, Sanders, Watkins, 2003). However, the connection between altered antioxidant enzymes and increased oxidative stress is not straightforward, as changes (increase or decrease) in the activities of antioxidant enzymes are not always unidirectional (Suryanarayana *et al.*, 2007).

In general, regarding the treatments with curcumin and HT-NO<sub>2</sub> in this work, no different effects were observed when analyzing the activity of liver enzymes after 31 days of administration of the substances, despite there being a clear tendency of increasing the concentration of such enzymes.

The apparent discrepancy between the studied molecules' considerable antioxidant activity detected *in vitro* and no change in liver enzymes and lipid peroxidation

test, used as indicators of antioxidant activity *in vivo*, may be due to the fact that each individual responds in a unique way to stimuli to which it is submitted, and there may be a wide variation in individual response. While it is possible to exercise control over the vast majority of variables involved in an *in vitro* test, the same does not occur with the animal testing where each individual shows itself as a complex web of variables dependent on the particularities of each one.

### Biochemical parameters

A total of 14 biochemical assays were performed in animal blood samples. A statistical difference in uric acid values between the control group and the HTNO<sub>2</sub>-100 group; alkaline phosphatase (ALP) among the control group, the diabetic control group, and the CURC-100 group; alanine aminotransaminase (ALT) and urea between the control group and the diabetic control group. The mean values found are presented in Table VI, followed by ± standard deviation and mean comparison test.

Uric acid, the final product of purine metabolism, is formed in the liver and excreted by the kidneys, and its blood level elevation may be an indicator of kidney disease, DM, cardiovascular disease, or inflammation. However, the level of this marker was not found statistically increased in the diabetic control group when compared to the control group without diabetes.

The parameters AST, ALT, and ALP are considered to be markers of liver function and elevated serum levels of such enzymes have been reported in diabetic animals, being reduced after treatment with insulin (Palma *et al.*, 2014).

**TABLE VI** - Results of biochemical assays of rats blood samples

Assay	Control	Diabetic Control	Curc-50	Curc-100	HTNO <sub>2</sub> -50	HTNO <sub>2</sub> -100
Uricacid (mg/dL)	0.8 ± 0.2 <sup>b</sup>	1.8 ± 1.0 <sup>ab</sup>	1.2 ± 0.4 <sup>ab</sup>	1.0 ± 0.1 <sup>ab</sup>	0.8 ± 0.2 <sup>ab</sup>	1.8 ± 1.1 <sup>a</sup>
ALP (U/L)	87.5 ± 5.8 <sup>c</sup>	280.5 ± 44.9 <sup>b</sup>	236.1 ± 104.1 <sup>b</sup>	402.8 ± 31.3 <sup>a</sup>	304.2 ± 29.8 <sup>b</sup>	304.9 ± 62.2 <sup>b</sup>
ALT (U/mL)	72.0 ± 27.2 <sup>b</sup>	147.9 ± 43.6 <sup>a</sup>	111.4 ± 15.5 <sup>ab</sup>	99.3 ± 14.1 <sup>ab</sup>	99.3 ± 42.1 <sup>ab</sup>	131.2 ± 29.2 <sup>a</sup>
Urea (mg/dL)	35.2 ± 3.9 <sup>b</sup>	71.3 ± 27.2 <sup>a</sup>	65.6 ± 21.9 <sup>ab</sup>	71.7 ± 20.4 <sup>a</sup>	54.2 ± 5.5 <sup>ab</sup>	74.2 ± 13.6 <sup>a</sup>
Albumin (g/dL)	2.3 ± 0.1 <sup>a</sup>	2.0 ± 0.2 <sup>a</sup>	2.3 ± 0.2 <sup>a</sup>	2.31 ± 0.2 <sup>a</sup>	2.4 ± 0.2 <sup>a</sup>	2.3 ± 0.2 <sup>a</sup>
Amylase (U/dL)	708.9 ± 23.8 <sup>a</sup>	711.8 ± 20.2 <sup>a</sup>	704.8 ± 3.2 <sup>a</sup>	694.4 ± 23.0 <sup>a</sup>	698.4 ± 21.7 <sup>a</sup>	701.3 ± 4.6 <sup>a</sup>
Total cholesterol (mg/dL)	105.3 ± 21.3 <sup>a</sup>	93.5 ± 16.2 <sup>a</sup>	106.7 ± 13.3 <sup>a</sup>	92.1 ± 12.2 <sup>a</sup>	86.2 ± 9.5 <sup>a</sup>	107.8 ± 21.4 <sup>a</sup>
HDL cholesterol (mg/dL)	88.9 ± 20.3 <sup>a</sup>	71.5 ± 12.5 <sup>a</sup>	88.5 ± 12.7 <sup>a</sup>	73.8 ± 5.1 <sup>a</sup>	73.7 ± 6.1 <sup>a</sup>	80.6 ± 9.7 <sup>a</sup>
Creatinine (mg/dL)	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.1 <sup>a</sup>	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.1 <sup>a</sup>	0.4 ± 0.0 <sup>a</sup>	0.4 ± 0.1 <sup>a</sup>
Hemoglobin (g/dL)	16.4 ± 1.8 <sup>a</sup>	14.3 ± 1.7 <sup>a</sup>	16.3 ± 1.6 <sup>a</sup>	16.1 ± 0.8 <sup>a</sup>	15.5 ± 1.1 <sup>a</sup>	15.4 ± 1.4 <sup>a</sup>
Magnesium (mg/dL)	2.5 ± 0.3 <sup>a</sup>	2.9 ± 1.0 <sup>a</sup>	2.1 ± 0.1 <sup>a</sup>	2.2 ± 0.2 <sup>a</sup>	2.1 ± 0.1 <sup>a</sup>	2.7 ± 0.8 <sup>a</sup>
Total protein (g/dL)	5.9 ± 0.3 <sup>a</sup>	5.7 ± 0.3 <sup>a</sup>	5.6 ± 0.4 <sup>a</sup>	5.5 ± 0.3 <sup>a</sup>	5.5 ± 0.1 <sup>a</sup>	5.6 ± 0.2 <sup>a</sup>
AST (U/mL)	149.5 ± 47.1 <sup>a</sup>	179.9 ± 10.4 <sup>a</sup>	171.3 ± 19.4 <sup>a</sup>	156.9 ± 15.8 <sup>a</sup>	133.1 ± 44.2 <sup>a</sup>	160.7 ± 27.1 <sup>a</sup>
Triglycerides (mg/dL)	107.8 ± 60.2 <sup>a</sup>	63.5 ± 27.1 <sup>a</sup>	94.6 ± 43.2 <sup>a</sup>	112.9 ± 47.0 <sup>a</sup>	72.6 ± 19.3 <sup>a</sup>	98.5 ± 48.5 <sup>a</sup>

Mean ± standard deviation followed by different letters in the column differ from each other by Tukey's test, with 95% confidence. ALP = alkaline phosphatase; ALT = alanine aminotransaminase; AST = aspartate aminotransaminase. Control = control group, non-diabetic; Diabetic Control = diabetic control group, diabetic; Curc-50 = group Curc-50, diabetic, curcumin 50 mg/kg of body weight by oral gavage; Curc-100 = group Curc-100, diabetic, curcumin 100 mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-50 = group HTNO<sub>2</sub>-50, diabetic, HTNO<sub>2</sub> 50mg/kg of body weight by oral gavage; HTNO<sub>2</sub>-100 = group HTNO<sub>2</sub>-100, diabetic, HTNO<sub>2</sub> 100 mg/kg of body weight by oral gavage.

AST is widely present in the heart, liver, skeletal muscles, and kidneys; ALT is found in a number of different tissues, with liver being the main one; and FA isoforms found in blood are originated mostly from the liver or skeletal muscle. Thus, injuries or disorders which entail cellular damage in the liver can result in the release of such enzymes into the bloodstream, acting as a marker of hepatocellular injury (Prabhakar *et al.*, 2013; Gutierrez *et al.*, 2012). In this work, no significant difference was found between the groups regarding AST levels. ALT and FA rates were higher in the diabetic control group when compared to the control group without diabetes, corroborating several studies showing association of DM with increased activity of liver enzymes (Hussein, Abu-Zinadah, 2010; Palma *et al.*, 2014; Gutierrez *et al.*, 2012). Regarding the FA enzyme, diabetic groups treated with curcumin or HT-NO<sub>2</sub> showed no protective effect on the liver. However, with regard to ALT, treatment with curcumin 50, curcumin 100, and HTNO<sub>2</sub>-50 showed statistically similar levels to those of the control group without diabetes, which may provide evidence of a partial protective action of these substances in the liver based on the assessment of such enzyme.

Urea and creatinine parameters were used for evaluation of the renal function of the animals. Urea is a

waste product of protein digestion formed in the liver, and creatinine is a residue of creatine phosphate metabolism by skeletal muscle tissues, both filtered in the kidneys (Prabhakar *et al.*, 2013). Assessed urea rates were higher in diabetic animal groups when compared to the control group without diabetes; however, it was observed that the CURC-50 and HTNO<sub>2</sub>-50 groups were not statistically different from the control group without diabetes, suggesting the protection of the renal function in these two groups. Regarding the level of creatinine, there was no significant difference between the groups.

Since in diabetic conditions protein synthesis is decreased in all tissues and muscle proteolytic activity is increased, there is a greater influx of amino acids to the liver, raising serum levels of nitrogenous compounds such as urea and creatine and increasing renal work excretion (Prabhakar *et al.*, 2013). In this study, statistical differences were evident in urea levels, but not creatinine, which may be related to the trial period since urea is more responsive to primary changes in renal conditions (Prates *et al.*, 2007).

Although DM is frequently associated with dyslipidemia, with high levels of total cholesterol, LDL, and triacylglycerols, and low levels of HDL, there were

no statistical differences between the groups regarding serum levels of total cholesterol, HDL cholesterol, and triacylglycerols.

This study found no significant differences in albumin and total protein levels (indicator of alteration in protein metabolism or renal dysfunction), amylase (indicator of pancreatic disease or renal dysfunction), hemoglobin (indicative of anemia, a common disease in patients with DM) and magnesium (lower rates associated with DM).

It was observed then, in this study, alteration caused by DM in hepatocellular injury markers (ALT and ALP) and renal function (urea, more sensitive to primary alterations of the kidneys, and uric acid). Treatments with curcumin 50 and HTNO<sub>2</sub>-50 showed similar results to those of animals without diabetes for ALT and urea parameters, suggesting a partial protective action of these substances in the liver and kidney of diabetic animals.

## CONCLUSION

Evaluation of *in vitro* antioxidant activity of TH-NO<sub>2</sub> showed superior activity to that of curcumin. Overall, in this study it was observed that treatment with curcumin and HT-NO<sub>2</sub> showed no effects on analyzed liver enzymes, although there was a clear trend to increase activity of such enzymes in the treated groups. Similarly, no changes were detected in lipid peroxidation when assessed after treatment of animals with tested substances. It should be pointed out, however, that statistically significant changes were also not detected in both parameters when comparing control groups with and without diabetes, showing that perhaps those systems were not altered in this study model.

Evaluation of blood biochemical parameters, moreover, allowed the observation that the treatments with curcumin and HT-NO<sub>2</sub>, both at 50 mg/kg, suggest a partial protective effect of these substances in the liver and kidney of diabetic animals, also demonstrating the *in vivo* antioxidant potential of this class of substances. This highlights its promising action toward diabetes-related complications, opening doors for new studies to be conducted.

In addition, the protective role of this substance *in vivo*, demonstrated by blood biochemical parameters, indicates that its antioxidant activity may be related to routes other than that of liver enzymes, indicating the need for further studies.

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