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Changes in the Oxidative Stress Biomarkers in Liver of Streptozotocin-diabetic Rats Treated with *Combretum lanceolatum* Flowers Extract

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Authors' contributions

This work was carried out in collaboration between all authors. Author CRPD conceived the project, carried out all the experiments, the literature search and the analysis of the data. Authors DLPS, JTS, MPP and RPA contributed to the laboratory work and the analysis of the data. Authors VCS, PTSJ, ILB, CMBA and NHK co-designed the experiments, discussed analyses and contributed to critical reading and writing of the manuscript. Author AMB designed the study, supervised the laboratory work, the analysis and interpretation of the data and wrote the paper. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study investigated the *in vivo* antioxidant activity and the *in vitro* radical scavenging capacity of the *Combretum lanceolatum* Pohl (*Combretaceae*) flowers ethanolic extract (ClEtOH) in streptozotocin-diabetic rats.
Place and Duration of Study: Department of Chemistry, Federal University of Mato Grosso, Cuiabá, Brazil; between February 2012 and December 2012.
Methodology: Male Wistar rats were divided into four groups: Normal rats treated with water/vehicle (N); diabetic rats treated with water (DC); diabetic rats treated with 250 mg/kg (DT_{250}) or with 500mg/kg (DT_{500}) of ClEtOH. After 21 days of treatment, liver samples were used for the analysis of the oxidative stress biomarkers and activity of antioxidant enzymes. *In vitro* radical scavenger capacity was investigated by the following methods: DPPH radical scavenging, ABTS radical cation decolorization and crocin bleaching assays.
Results: Significant oxidative stress was observed in liver of DC, since the malondialdehyde (MDA, biomarker of lipoperoxidation) levels were increased in comparison with N. Increased activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were also observed in DC, which could represent a compensatory mechanism against oxidative stress. Glutathione (GSH) levels were lower and similar between N and DC. The MDA levels were significantly decreased in liver of rats from DT_{250} and DT_{500}, reaching levels similar those of N, suggesting that ClEtOH prevented lipoperoxidation. The treatment of diabetic rats with ClEtOH also increased the GSH levels, as well as increased the GSH-Px activity, and did not change the SOD activity. The results of *in vitro* radical scavenging capacity indicated that ClEtOH is highly active.
Conclusion: These findings indicate that ClEtOH has antioxidant properties in liver of diabetic rats, decreasing lipoperoxidation and increasing the endogenous antioxidant responses. Both the antihyperglycemic effect and the capacity to scavenge free radicals may be related to the antioxidant activity of ClEtOH in diabetes.

Keywords: Combretum lanceolatum Pohl; streptozotocin-diabetic rats; malondialdehyde; reduced glutathione; antioxidant enzymes.

1. INTRODUCTION

Oxidative stress results from the imbalance between the oxidants production and the antioxidant capacity, a condition that favors the increase in the levels of radical species, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$) and peroxyl ($ROO^{\bullet}$) radicals and non-radical species, such as hydrogen peroxide (H_2O_2) and hypoclorous acid (HOCl). These species appear as the commonest reactive oxygen species (ROS) that react with lipids, DNA and proteins in cells, leading to the loss of biological function; in addition, ROS can also act as initiator effectors of intracellular processes that participate in the development of various pathologies.

It is well known the pathogenic role of the oxidative stress in the establishment of various complications observed in diabetes mellitus (DM) [1,2]. Chronic hyperglycemia appears as a crucial factor in the development of oxidative stress in various tissues, mainly those in which the glucose transport is partially independent of insulin, such as pancreas, vascular endothelium, retina, kidney, and liver. Therefore, in DM, the high intracellular glucose levels

culminate in the increased ROS generation via mitochondrial electron transport chain [3]. In addition to the elevated ROS production, reduction in the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) also contributes to the development of oxidative stress in DM [4].

In addition to the direct damage in the functional integrity of many biomolecules leading to cellular injuries, ROS (mainly $O_2^{\bullet-}$) have been also cited as initiating factors of several other processes that are involved in the installation of the micro- and macrovascular complications of DM, such as: i) The increased glucose flux through the polyol pathway, decreasing the intracellular levels of nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH) and impairing the regeneration of the reduced glutathione (GSH), one of the most important non-enzymatic endogenous antioxidants; ii) Changes in the cellular components by the advanced glycation end products, leading to function loss; iii) The increased diacylglycerol synthesis, which lead to the activation of protein kinase C isoforms; and *iv*) the increased glucose flux through the hexosamine pathway, leading to protein changes (via O-linked acetylglucosamine modification), these last two processes increase the expression and activity of many factors involved in the genesis of diabetes complications [1].

Recent studies are evidencing the deleterious consequences of oxidative stress in liver during DM, in both humans and rodents. Increased ROS generation in the liver of diabetic individuals has been involved in the progression of steatosis to worsening conditions, mainly nonalcoholic fatty liver disease (NAFLD) [5]. In rodents under experimental models of diabetes, increased ROS levels are related to morphological changes in hepatocytes and liver DNA damage [6]. Redox alterations, such as oxidation of thiols groups, nitrosylation, glutathionylation, and the formation of disulfide bonds have been impairing the activity of various components belonging to signal transduction pathways in the liver [7].

By targeting various biochemical processes in several tissues, the treatment of DM has as the main objective the reduction of hyperglycemia to values close to the normality. If an antidiabetic therapy has the liver as a target tissue, it can be efficient in the hyperglycemia reduction if it stimulates the hepatic glycogen synthesis and/or it inhibits glycogenolysis and gluconeogenesis, both processes involved in the hepatic glucose production. So, the preservation of both the cell integrity and the adequate hepatocyte function is essential to the success of the antidiabetic therapy, which can be achieved with the association of an antioxidant therapy. Among the antioxidant options used together with the conventional therapy to DM, it can be highlighted the use of natural products preparations and/or isolated compounds with proved antioxidant properties. Various natural antioxidants preparations may reduce oxidative deleterious consequences directly, since they have antioxidant compounds that possess scavenging capacity against ROS. In addition, it has been attributed to natural antioxidants the capacity to promote changes in the rates of intracellular processes involved in ROS turnover, decreasing the generation of ROS in various cellular compartments and/or stimulating the expression of antioxidant enzymes [8].

Recent studies have shown that plants of the *Combretum* genus have antidiabetic activity [9,10], as well as *in vivo* antioxidant activity [11,12]. Recently, study of Dechandt and collaborators [13] observed a decrease in glycemia, glycosuria and urinary urea levels in streptozotocin-diabetic rats treated for 21 days with the ethanolic extract of the flowers of *Combretum lanceolatum* (ClEtOH). The treatment with ClEtOH also diminished the body weight loss typical of this experimental model, as a consequence of the minor weight loss of adipose and muscle tissues. In this study it was also observed that quercetin, a flavonoid with *in vivo* antioxidant action, is the major component of the extract. The mechanism of the

antidiabetic activity of ClEtOH can be attributed, at least in part, to its effects in liver, since diabetic rats treated with ClEtOH showed a decrease in the rate of hepatic glucose production through gluconeogenesis [14]. Increased glycogen content was also observed in liver of diabetic rats treated with the extract [13]. Therefore, liver seems to exert a crucial role in the beneficial effects of ClEtOH in the glucose metabolism of diabetic rats.

Considering that, *i*) the majority presence of quercetin in ClEtOH, and *ii*) the antidiabetic activity of the extract, partially recovering the normal functioning of metabolic processes in liver, the present study was undertaken to investigate the antioxidant properties of ClEtOH in liver of streptozotocin-diabetic rats.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Extract Preparation

Flowers of *Combretum lanceolatum* Pohl ex Eichler, *Combretaceae*, were collected in Porto Cercado, Poconé highway, km 18, Mato Grosso, Brazil (S 16°32'58.42"; W 56°64'42.72") in July 2010. The access to plant samples was authorized by the Conselho de Gestão do Patrimônio Genético of the Ministério do Meio Ambiente (license number 010457/2010-0). The identification of the plant material was made by Dr. Germano Guarim Neto, in the Central Herbarium, Universidade Federal de Mato Grosso (UFMT), where a voucher specimen was deposited (number 39,149). The flowers of *Combretum lanceolatum* were dried at room temperature and grounded in electric grinder; the powder (5,960g) was macerated with ethanol (13 L at each extraction) at room temperature under occasional shaking, in seven cycles of seven days. The mixture was then filtered and concentrated on rotary evaporator at reduced pressure and 38°C. The dry residue corresponds to the crude ethanolic extract of the flowers of *Combretum lanceolatum* (ClEtOH, 2,350kg; 39.43% w/w).

2.2 *In vitro* Radical Scavenging Assays

2.2.1 DPPH radical scavenging assay

The ability of ClEtOH to react with the stable 2,2-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich, USA) free radical was made according to Mensor et al. [15]. One milliliter of the methanolic solution of DPPH (0.004%) was incubated for 30 minutes at room temperature with 0.5mL of methanolic solutions of the extract (0.5-50µg/mL). Blank solution contained 1.0mL of methanol and 0.5mL of the extract at the cited concentrations. Methanolic solutions of ascorbic acid at the same concentrations of the extract were used as positive controls. The antioxidant activity was expressed as percentage of DPPH scavenger, monitored spectrophotometrically at 518nm. From these results it was also finding the concentration (µg/mL) of the extract or ascorbic acid needed to scavenge 50% of the DPPH radical, i.e., inhibiting by 50% the analytical signal (IC_{50}).

2.2.2 ABTS radical cation decolorization assay

The ability of ClEtOH to react with $ABTS^{\bullet+}$ radical was made according to Re et al. [16], with modifications. The $ABTS^{\bullet+}$ was generated by oxidation of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma-Aldrich, USA) (7mmol/L) with potassium persulfate (140mmol/L) in the dark at room temperature for 12 to 16 hours. The $ABTS^{\bullet+}$ stock solution was diluted in sodium phosphate buffer (10mmol/L, pH 7.0) to an absorbance

of 0.750±0.020, at 734 nm. Solutions of the extract (0.5-60 µg/mL) or ascorbic acid (0.5-8µg/mL) were incubated with $ABTS^{\bullet+}$ solution for 15 minutes in the dark and at room temperature, thereafter the absorbance was read at 734 nm. The results were expressed as mean ± standard error of the mean (SEM) of the 50% inhibitory concentration (IC_{50}).

2.2.3 Crocin bleaching assay

The crocin bleaching assay was performed according to Tubaro et al. [17], as a competitive kinetics procedure. The reaction was initiated by the addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, Sigma-Aldrich, USA), which generates peroxyl radicals at a constant rate by thermolysis at 40°C. It was used the molar extinction coefficient (ε) of crocin in DMSO: ε = 13,726 L mol^{-1} cm^{-1}, at 443nm [18]. Crocin (25µmol/L) in sodium phosphate buffer (0.12mol/L, pH 7.0) was mixed with various concentrations of the extract (20-100µg/mL) or ascorbic acid (0.3-3µg/mL). The reaction was started by adding AAPH (12.5mmol/L) and performed with constant stirring at 40°C. The rate of crocin bleaching was monitored at 443 nm for 10 minutes, except for the assay carried out with ascorbic acid, which was monitored at 443nm for 5 minutes. Reaction mixtures without crocin were prepared for the extract, ascorbic acid and Trolox, which were used as blank.

The rate of crocin bleaching by the generated peroxyl radical (v_0) decreases in the presence of an antioxidant, since it competes with the crocin for the peroxyl radical, and the new bleaching rate (v) is given by:

$$v = v_0 \, x \frac{kc\,[C]}{kc\,[C] + ka\,[A]}$$

Where $v_0 = k_1$ x $[ROO^{\bullet}]$ x [C]; kc = k_1 x $[ROO^{\bullet}]$; ka = k_2 x $[ROO^{\bullet}]$; $[ROO^{\bullet}]$ = concentration of peroxyl radical; v_0 = reaction rate between crocin and $ROO^{\bullet}$; k_1 = rate constant for the $ROO^{\bullet}$-crocin reaction; k_2 = rate constant for the $ROO^{\bullet}$-antioxidant reaction; [C] = crocin concentration; [A] = antioxidant (extract, ascorbic acid or Trolox) concentration.

The fall in crocin bleaching rate in the presence of an antioxidant can be modeled:

From the equation,

$$\frac{v_0}{v} = \frac{kc\,[C] + ka\,[A]}{kc\,[C]} = 1 + \frac{ka}{kc} x \frac{[A]}{[C]}$$

The coefficient ka/kc, calculated as the slope of the regression line for the v_0/v *versus* [A]/[C] plot, indicates the relative capacity of an antioxidant to interact with the peroxyl radicals. By dividing the slope for the extract or ascorbic acid by the slope for a standard antioxidant such as Trolox, the ratio of rate constants, and thus the relative antioxidant capacity, of the analyzed compound can be estimated, being expressed in Trolox equivalents.

2.3 Induction of Diabetes and Animals Treatment

Male Wistar rats weighing 180-210 g (38-40 days old) were housed under environmentally controlled conditions (24 ± 1°C) with a 12h light/dark cycle and had free access to water and

normal lab chow diet (Purina®Labina). All experiments were performed between 08:00 and 10:00 a.m.

Streptozotocin (STZ, 40mg/kg, Sigma-Aldrich, EUA) was dissolved in 0.01mol/L citrate buffer (pH 4.5) and then administered through a single intravenous injection in 15h fasted rats. Non-diabetic, normal animals were injected with citrate buffer. Five days after STZ administration, diabetic rats with post-prandial glycemia levels of approximately 450mg/dL were used in the experiments. Glycemia levels were determined by the glucose oxidase method [19] using commercial kit (Labtest Diagnostica SA, Brazil).

The rats were divided into four groups: N, normal, non-diabetic group, treated with vehicle (water); DC, diabetic rats treated with water; DT_{250}, diabetic rats treated with 250mg/kg of ClEtOH; DT_{500}, diabetic rats treated with 500mg/kg of ClEtOH. Groups received vehicle or freshly prepared extract once a day, by oral gavage, for 21 days. At the end of the treatment, the rats were euthanized and liver samples were immediately collected, washed in saline and stored at -80°C for the posterior analysis of the oxidative stress biomarkers and activity of the antioxidant enzymes.

2.4 Oxidative Stress Biomarkers and Antioxidant Enzymes Activity

Liver samples were homogenized in 0.1mol/L potassium phosphate buffer (pH 7.0) at 4°C. The homogenates were centrifuged at 10,000*g* for 20 min at 4°C and the supernatants were used for the analysis.

The malondialdehyde (MDA) levels, a lipoperoxidation (LPO) diene product, were determined through thiobarbituric acid (TBA) reaction [20]. The colored product was measured spectrophotometrically at 535 nm and 1,1,3,3-tetramethoxypropane was used as a standard. Results were expressed as mmol of MDA/g tissue.

Non-protein sulphydryl groups represent an indirect measurement of reduced glutathione (GSH) and were determined according to the method of Sedlak & Lindsay [21], which measures the reduction of 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm. Results were expressed as mmol of GSH/g tissue.

SOD activity was determined using a commercial kit (Randox Laboratories, UK) that uses the method described by McCord & Fridovich [22]; the xanthine oxidase reaction generates $O_2^{\bullet-}$, which in turns reduces 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to a formazan product. The assay is based on the SOD inhibition of INT reduction, monitored at 505nm. Results were expressed as U/mg protein. One unit of SOD is defined as the enzyme amount required to inhibit the rate of INT reduction by 50%.

GSH-Px activity was determined according to method of Paglia & Valentine [23], using a commercial kit (Randox Laboratories, UK). GSH-Px catalyzes the oxidation of GSH in the presence of cumene hydroperoxide. In the presence of gluthatione reductase, the oxidized gluthatione is reduced to GSH with concomitant oxidation of NADPH to $NADP^+$. NADPH disappearance was monitored at 340nm. Results were expressed as U/mg protein. One unit of GSH-Px is defined as one μmol of NADPH oxidized in one minute.

CAT activity was measured according method of Aebi [24] by monitoring the disappearance of H_2O_2 at 230nm. Results were expressed as U/mg protein. One unit of CAT is defined as mmol of H_2O_2 decomposed in one minute.

Protein levels in liver supernatants were determined according to the Bradford method [25] using bovine serum albumin as standard.

2.5 Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad InStat 3.05. One-way analysis of variance (ANOVA) followed by Student Newman-Keuls test was used to compare the data from the different groups. The data for each test were normally distributed and the differences were considered significant at $P<0.05$.

3. RESULTS AND DISCUSSION

Our data clearly show the establishment of oxidative stress in STZ-diabetic rats, because even with the increased activity of the antioxidant enzymes SOD and GSH-Px, the major biomarker of lipid peroxidation (LPO), the malondialdehyde (MDA), was increased in liver of diabetic rats when compared with normal rats. LPO comprises many chain reactions initiated by $^{\bullet}OH$ radical, the most reactive ROS, which attacks polyunsaturated fatty acids in phospholipid membranes, yielding lipid hydroperoxides (LOOH) and many cytotoxic products, including MDA. Besides being a biomarker of LPO and an indicative of the *in vivo* oxidative status [26], MDA may also cross-link with proteins and nucleic acids, altering their biological properties [27]. According our data, the levels of MDA were 81% increased in liver of diabetic rats when compared with values of normal rats (Fig. 1), consistent with the literature findings [28,29]. It has been observed that hyperglycemia leads to an increase in $^{\bullet}OH$ production in rat liver [30], due to increased mitochondrial generation of $O_2^{\bullet-}$, the precursor of $^{\bullet}OH$. In addition, the low insulin levels have been associated with the increased hepatic expression of fatty acyl coenzyme A oxidase, an enzyme that participates in the fatty acid oxidation [31]. Altogether, the increased oxidation of both glucose and fatty acids in liver contributes to the increased ROS generation and LPO in diabetes.

Oxidative stress in diabetes may be also a consequence of the diminished levels of both non-enzymatic and enzymatic antioxidants. The primary line of defense against ROS is the activity of SOD, which catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2; although it is not a radical, H_2O_2 diffuses through cell lipid membranes, reaching sites with Fe^{2+} or Cu^{+} ions and generates $^{\bullet}OH$, this last causing cellular damage. Antioxidant enzymes CAT and GSH-Px both reduce H_2O_2 to water and O_2. In animals, CAT is found in several tissues, but its activity is higher in liver, erythrocytes and kidney. The selenium-containing enzyme GSH-Px shares with CAT the capacity to reduce H_2O_2, but it also reduces lipoperoxides and other organic hydroperoxides in a reaction system that involves the oxidation of GSH [32]. In the present study, it was observed an increase in the activities of SOD (6-fold) and GSH-Px (22%) in liver of diabetic rats when compared with normal rats (Table 1), which can be interpreted as a compensatory mechanism against the increased ROS generation, commonly described in DM [33,34]. However, it must be highlighted that this compensatory antioxidant response was not sufficient to prevent the oxidative stress, since we observed increase in LPO (Fig. 1). Increased $O_2^{\bullet-}$ production induces SOD activity [33,35], which in turn lead to increased H_2O_2 production. Mates et al. [36] found that the expression of GSH-Px is upregulated by

H_2O_2 and other ROS. Both the high quantity and molecular catalytic capacity of CAT may explain the unchanged activity of this enzyme in liver of diabetic rats (Table 1). Finally, it must be cited that the extent of the increase in the SOD activity was higher than that of GSH-Px activity; it has been described that the increased SOD activity seems to be important to protect both CAT and GSH-Px against inactivation by $O_2^{\bullet-}$ when increased levels of this radical are observed [37,38].

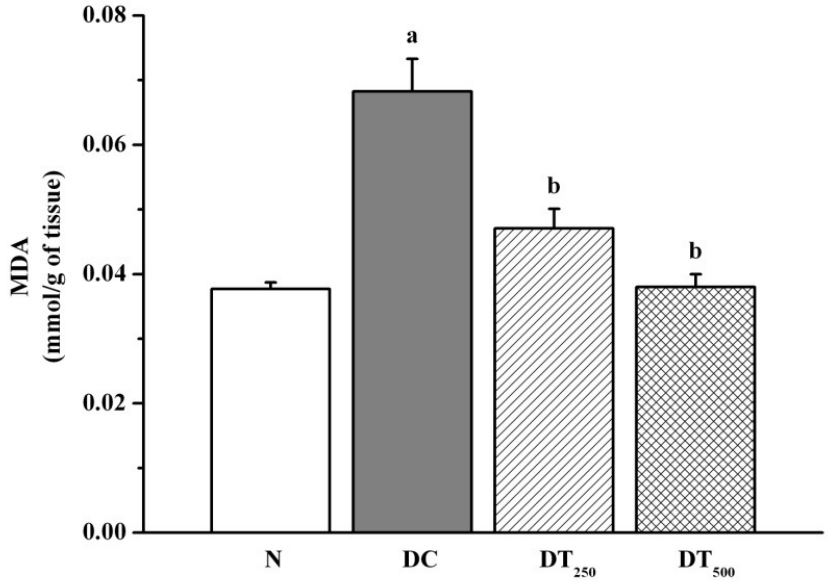

Fig. 1. Malondialdehyde (MDA) levels in liver of diabetic rats treated with ClEtOH. Values are given as mean ± SEM of 6-7 animals per group. N: normal rats; DC: non-treated diabetic rats; DT_{250}: diabetic rats treated with 250mg/kg of ClEtOH; DT_{500}: diabetic rats treated with 500mg/kg of ClEtOH. [a] *P*<0.05 *vs* N; [b] *P* < 0.05 *vs* DC (ANOVA followed by Student Newman-Keuls)

GSH is one of the most important non-enzymatic endogenous antioxidants and it is a crucial substrate for the antioxidant activity of GSH-Px. Decreased levels of GSH have been observed in diabetes, which can be mainly attributed to the increased flux of glucose through the polyol pathway, consuming NADPH in the sorbitol formation, a condition that impairs the regeneration of GSH [39]. Diminished levels of GSH contribute to the increase in the MDA levels in diabetes, since GSH is an important inhibitor of ROS-mediated LPO [40]. Interestingly, our study shows that the GSH levels in liver of diabetic rats were similar to those observed in normal rats (Fig. 2). Mclennan et al. [41] also observed that the hepatic levels of GSH were similar between normal and diabetic rats, and the authors suggested that the increased activity of hepatic gamma-glutamyl transferase lead to a decrease in the biliary excretion of gluthatione, representing a compensatory mechanism to conserve glutathione; therefore, this response could be masking the expected fall in the GSH levels in liver of diabetic rats. Finally, in the present study, it can be inquired if, even with the increased GSH-Px activity, the GSH regeneration is sufficient to maintain their levels; however, the association between increased GSH-Px activity and maintenance of GSH levels was not sufficient to prevent LPO in diabetic rats.

Our results demonstrate that the administration of *C. lanceolatum* extract produced a marked decrease in the oxidative stress in liver of STZ-diabetic rats. The hepatic MDA levels were significantly diminished (31%) in diabetic rats treated with 250mg/kg of ClEtOH and even decreased (44%) with 500mg/kg of the extract, reaching values similar to those found in normal rats (Fig. 1). So, our data showed that *C. lanceolatum* protects diabetic rats against liver LPO in a dose-dependent manner. Previously, Dechandt et al. [13] observed that STZ-diabetic rats treated with the extract also showed reduction in the glycemia levels in a dose-dependent manner. The capacity of the *C. lanceolatum* extract to protect against LPO can be attributed, at least in part, to its ability to reduce hyperglycemia.

Table 1. Activities of SOD, GSH-Px, and CAT in liver of diabetic rats treated with ClEtOH

Groups	N	DC	DT_{250}	DT_{500}
SOD (U/mg protein)	0.129±0.014	0.783±0.115 [a]	0.831±0.257 [a]	0.705±0.165 [a]
GSH-Px (U/mg protein)	0.284±0.010	0.346±0.008 [a]	0.395±0.012 [a,b]	0.505±0.027 [a,b,c]
CAT (U/mg protein)	63.41±2.286	62.86±2.849	64.60±2.464	66.21±4.889

Values are given as mean ± SEM of 6-7 animals per group. N: normal rats; DC: non-treated diabetic rats; DT_{250}: diabetic rats treated with 250mg/kg of ClEtOH; DT_{500}: diabetic rats treated with 500 mg/kg of ClEtOH. [a] P<0.05 vs N; [b] P<0.05 vs DC; [c] P<0.05 vs DT_{250}
(ANOVA followed by Student Newman-Keuls)

Corroborating the MDA results, the present study also shows that GSH levels were increased in liver of diabetic rats treated with *C. lanceolatum* extract, in a dose-dependent manner (Fig. 2). Once again, the antihyperglycemic effect of the extract may have a relationship with the increase in the liver GSH, since the reduction in the glucose flux through the polyol pathway may avoid the excessive NADPH consumption, allowing an increased regeneration of GSH.

It is interesting to note that GSH-Px activity was further increased in liver of diabetic rats treated with *C. lanceolatum* extract, a response dose-dependent; the activity of this antioxidant enzyme was further increased by 39% (DT_{250} group) and 78% (DT_{500} group), in liver of diabetic rats treated with ClEtOH, when compared with N. (Table 1). Considering that GSH-Px activity was increased in liver of non-treated diabetic rats (DC), it can be hypothesized that this further increase in its activity after *C. lanceolatum* treatment may be due to the increase in the levels of GSH, a substrate of GSH-Px. A direct relationship between increased GSH levels and increased GSH-Px activity in liver was previously described [42]. This hypothesis seems to be reasonable if we consider the following: the increased GSH-Px activity observed in liver of diabetic rats may be a consequence of an upregulation in its gene expression as a compensatory response against oxidative stress, which could be occurring in the initial stage of diabetes, where both hyperglycemia and oxidative stress were not yet corrected by the extract. The further increase in the GSH-Px activity in liver of diabetic rats treated with *C. lanceolatum* may be a consequence of the association between the increase of both GSH-Px expression and GSH levels that occurred in the later periods of diabetes, after the hyperglycemia reduction by the treatment with ClEtOH. This hypothesis is corroborated by the fact that the GSH-Px activity was increased after *C. lanceolatum* treatment in a dose-dependent manner, as well as the increase in the GSH levels. In addition to the antihyperglycemic effect, the increase in the GSH-Px activity is

certainly an important mechanism of *C. lanceolatum* extract to combat the oxidative stress in liver of diabetic rats, since GSH-Px detoxifies H_2O_2 but also organic hydroperoxides [43]. The generation of organic hydroperoxides is increased in LPO, and their exert toxicity through ROS formation in an iron-mediated process. Since CAT is not involved in the clearance of organic hydroperoxides, these compounds are detoxified essentially via GSH-Px activity, which uses GSH as electron donor. Liddell et al. [44] observed that both increased GSH-Px activity and high GSH levels are crucial for the effective removal of organic hydroperoxides. Therefore, the detoxification of organic hydroperoxides seems to explain the importance to increase the hepatic GSH-Px activity in rats treated with *C. lanceolatum*, and is corroborated by the fact that CAT activity was unchanged (Table 1).

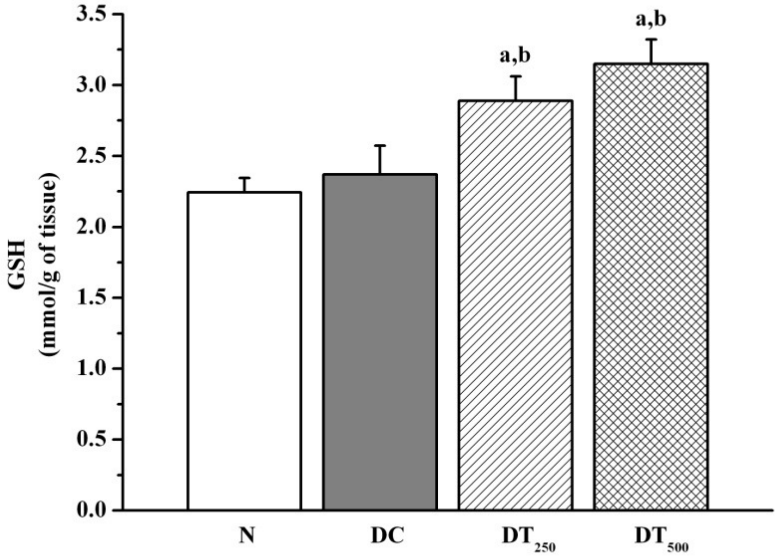

Fig. 2. Reduced glutathione (GSH) levels in liver of diabetic rats treated with ClEtOH. Values are given as mean ± SEM of 6-7 animals per group. N: normal rats; DC: non-treated diabetic rats; DT_{250}: diabetic rats treated with 250 mg/kg of ClEtOH; DT_{500}: diabetic rats treated with 500 mg/kg of ClEtOH. [a] *P*<0.05 *vs* N; [b] *P*<0.05 *vs* DC (ANOVA followed by Student Newman-Keuls)

In addition to the changes observed in the glutathione antioxidant system, another mechanism by which *C. lanceolatum* could be exerting its beneficial effects against oxidative stress is related to the presence of antioxidant compounds in the extract, such as quercetin, 3-O-methyl quercetin, dillenetin, and isorhamnetin [45]. We observed that *C. lanceolatum* extract was able to bleach the purple DPPH to a pale yellow color, representing a scavenging capacity against this radical, however it was minor when compared with ascorbic acid: the extract showed an IC_{50} of 10.58µg/mL, while ascorbic acid had IC_{50} of 2.95µg/mL (Fig. 3). These values were very similar those found by Aderogba et al. [46] with the methanolic extract of *Combretum apiculatum* (IC_{50}=2.4 and 14.5µg/mL, ascorbic acid and extract, respectively, for DPPH assay). The lower scavenging activity of *C. lanceolatum* extract against DPPH can be attributed to the steric obstruction of the 3',4'-catechol structure, which affect a significant proportion of the antioxidant capacity [47] and corroborates the presence of methoxyl groups on 3' and 4' positions of the flavonoids found in *C. lanceolatum* [45]. Another test screening widely used for the *in vitro* scavenging capacity of food and plant samples and/or isolated compounds is the $ABTS^{\bullet+}$ decolorization assay, which provides a good estimation for the antioxidant activity. This assay is based on

the radical decolorization at 734nm, which is proportional to both the concentration and the scavenging capacity of tested sample. The *C. lanceolatum* extract showed to be an effective antioxidant, with IC_{50} of 28.25µg/mL, while ascorbic acid had IC_{50} of 3.93µg/mL, proving to be even more effective (Fig. 4). Kilic et al. [48] also used ascorbic acid as antioxidant standard in the $ABTS^{\bullet+}$ scavenging assay and they found that 50% of the radical scavenging capacity for ascorbic acid was close to 5µg/mL, a value very similar that found in our study.

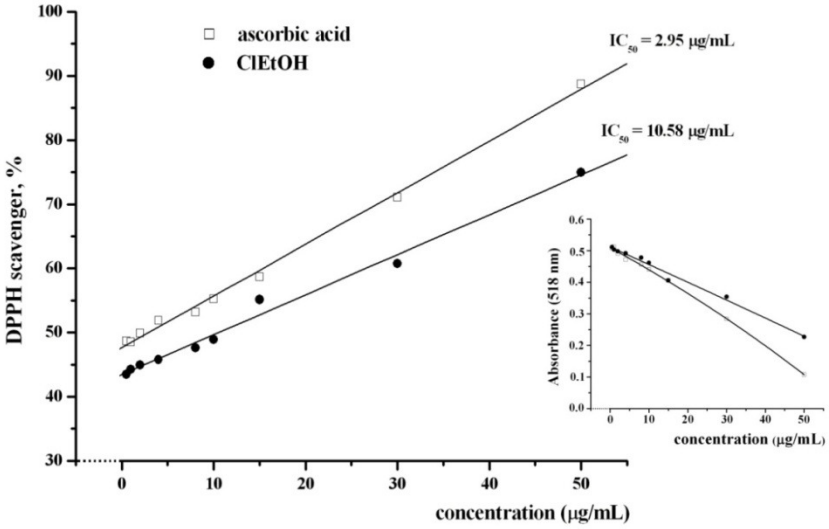

Fig. 3. DPPH scavenging capacity of ClEtOH. Values are given as % of DPPH radical scavenger. The inset represents the absorbance of DPPH at 518 nm in the presence of various concentrations of ascorbic acid or ClEtOH

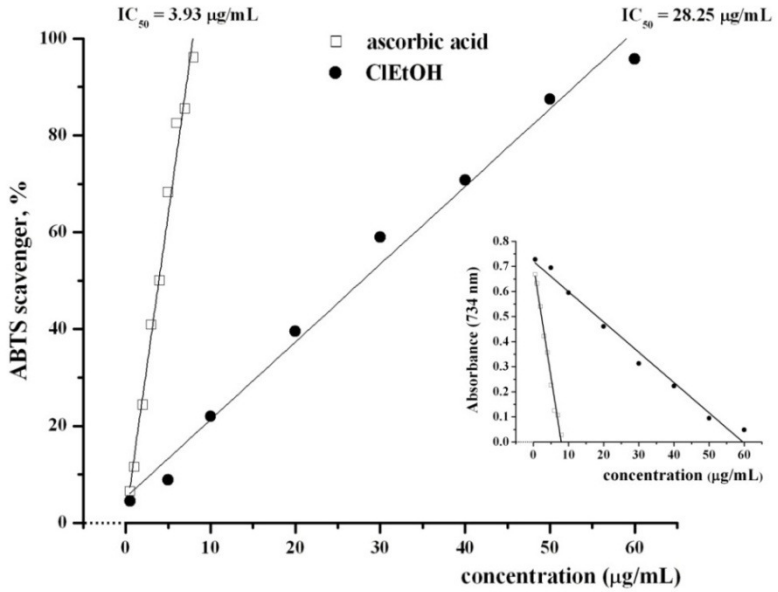

Fig. 4. ABTS scavenging capacity of ClEtOH. Values are given as % of ABTS radical scavenger. The inset represents the absorbance of ABTS at 734nm in the presence of various concentrations of ascorbic acid or ClEtOH

During LPO, peroxyl ($ROO^{\bullet}$) radicals are formed; some *in vitro* assays simulate this reaction to assess the capacity of antioxidants to scavenge $ROO^{\bullet}$, including the protection against the bleaching of crocin. In the crocin bleaching assay, antioxidants compete with crocin for the $ROO^{\bullet}$ generated by thermolysis of AAPH; therefore, the inhibition of the crocin oxidation depends on the antioxidant capacity to capture this radical generated *in situ*. For this assay it was used as the antioxidant standard the Trolox, an analogue of vitamin E, which has shown significant antioxidant activity and beneficial effects against oxidative damage in LPO. We used the values of slope of the linear regression of Trolox (Fig. 5A) and of *C. lanceolatum* extract (Fig. 5B) to calculate the equivalence to Trolox (obtained by the quotient of rate constants, ka/kc). Both samples decreased the velocity of crocin bleaching, *C. lanceolatum* extract showing activity of 0.5 Trolox equivalents, therefore Trolox showed a better antioxidant activity when compared with the extract.

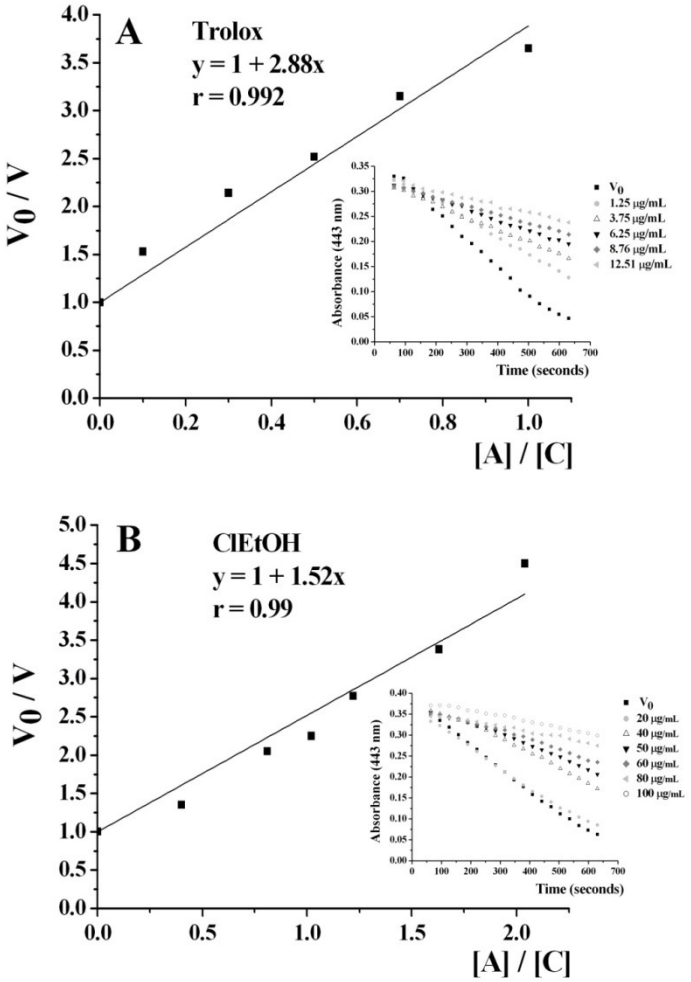

Fig. 5. Reaction velocity ratios plotted against samples concentrations in the crocin bleaching assay. A. Trolox; B. ClEtOH. v_0, velocity in the absence of sample; v, velocity in the presence of sample; [C], crocin concentration; [A], Trolox or ClEtOH concentration. The insets represent the decrease in the absorbance of crocin at 443 nm during 10 minutes, in the absence (v_0) and presence (v) of Trolox or ClEtOH

The assay was also performed in the presence of ascorbic acid, however the crocin bleaching was monitored for 5 minutes, since ascorbic acid showed a biphasic kinetic (lag phase) in this assay, as previously described [49]. Considering that the rate of crocin bleaching in the presence of ascorbic acid was y=1+ 88.17x, and in the presence of Trolox was y=1+3.10x, it can be concluded that ascorbic acid is 28-fold more effective to protect crocin against oxidation when compared with Trolox. In agreement with our results, Tubaro et al. [17] also observed that the antioxidant efficiency of ascorbic acid in the crocin bleaching assay is higher than Trolox. Another way to assess the antioxidant capacity is through the determination of the percent inhibition of crocin bleaching (% In) [50], thus obtaining the IC_{50} value. The *C. lanceolatum* extract showed an IC_{50}=56.81µg/mL, while Trolox and ascorbic acid had IC_{50} values of 4.10µg/mL and 0.85µg/mL, respectively. Therefore, for the lower IC_{50} value, the more efficient antioxidant capacity, while in the slope of linear regression, for the higher the value, the more effective the antioxidant capacity.

Finally, it must be highlighted that the presence of antioxidants and their effective *in vitro* scavenging capacities are also positively correlated with the protective effects of plant extracts against various disturbances, such as prevention of cell damage caused by UV light exposure [51], antineoplastic effects on tumor cell lines [52,53], reduction of the oxidative stress in animals models of hyperlipidemia [54,55] and liver injuries [56,57], among others.

4. CONCLUSION

Data from this study showed that the extract of *C. lanceolatum* flowers has antioxidant properties in liver of diabetic rats, since lipid peroxidation was decreased and the endogenous antioxidant capacity was increased in comparison with non-treated diabetic rats. Both the antihyperglycemic effect and the capacity to scavenge free radicals may be related with the antioxidant activity of the extract. Considering that the liver is an essential tissue controlling energy metabolism, the prevention of oxidative damage in this tissue is of extreme importance. Finally, considering that *C. lanceolatum* has both antidiabetic and antioxidant activities, this plant specie has showing great potential to be used in herbal formulations to treat diabetes mellitus, since both hyperglycemia and oxidative stress are conditions that play a crucial role in the establishment of the long-term complications of this disease.

CONSENT

Not applicable.

ETHICAL APPROVAL

Experimental procedures were made according to the Brazilian College of Animal Experimentation (COBEA) and received prior institutional approval by Committee for Ethics in Animal Experimental from UFMT (protocol n° 23108.029613/09-3).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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