# Research Article

# Treatment with Aqueous Extract from Croton cajucara Benth **Reduces Hepatic Oxidative Stress in Streptozotocin-Diabetic Rats**

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Croton cajucara Benth is a plant found in Amazonia, Brazil and the bark and leaf infusion of this plant have been popularly used to treat diabetes and hepatic disorders. The present study was designed to evaluate the oxidative stress as well as the therapeutic effect of Croton cajucara Benth (1.5 mL of the C. cajucara extract i.g.) in rats with streptozotocin-induced diabetes. Croton cajucara Benth was tested as an aqueous extract for its phytochemical composition, and its antioxidant activity in vitro was also evaluated. Lipid peroxidation and superoxide dismutase, catalase, and glutathione reductase activities were measured in the hepatic tissue, as well as the presence activation of p65 (NF-KB), through western blot. Phytochemical screening of Croton cajucara Benth detected the presence of flavonoids, coumarins and alkaloids. The extract exhibited a significant antioxidant activity in the DPPH-scavenging and the hypoxanthine/xanthine oxidase assays. Liver lipid peroxidation increased in diabetic animals followed by a reduction in the Croton-cajucara-Benth-treated group. There was activation of p65 nuclear expression in the diabetic animals, which was attenuated in the animals receiving the Croton cajucara Benth aqueous extract. The liver tissue in diabetic rats showed oxidative alterations related to the streptozotocin treatment. In conclusion the Croton cajucara Benth aqueus extract treatment effectively reduced the oxidative stress and contributed to tissue recovery.

## 1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eves, kidneys, nerves, heart, and blood vessels [1]. There are convincing experimental and clinical pieces of evidences that the generation of reactive oxygen species (ROS) is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress [2]. Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins [3].

Oxidative stress, the prevalence of oxidant factors over antioxidant mechanisms, plays a central role in the pathogenesis and progression of diabetes and its complications [4]. Enhanced formation of oxygen free radicals occurs in tissues during hyperglycemia [5]. The hyperglycemia can also activate transcription factors, such as nuclear factor NF- $\kappa$ B. This factor regulates the expression of a large number of genes including those who have deeper connection to the complications of diabetes [6]. Hyperglycemia also favors, through the activation of NF- $\kappa$ B, an increased expression of inducible nitric oxide synthase (iNOS), which is accompanied by increased generation of nitric oxide [7].

Traditional medicines and extracts from medicinal plants have been extensively used as alternative medicine for better control and management of DM [8]. *Croton cajucara* Benth (*C. cajucara*), commonly known as Sacaca, is a shrubby plant found in Amazonia, Brazil [9]. The bark and leaf infusions of this plant have been popularly used to treat diabetes, diarrhea, malaria, fever, gastrointestinal, renal, and hepatic disorders, as well as in the control of high levels of cholesterol [10, 11]. Antioxidant effects of *C. cajucara* leaf extracts was investigated both *in vitro* and *in vivo* models. Leaf extracts showed radical inhibitory scavenging activity against the stable radical DPPH and reduced oxidative stress in animals treated with paraquat [12].

The liver is the main organ of oxidative and detoxifying processes. In many diseases, biomarkers stress oxidative are elevated in the liver at an early stage [13]. Thus, the present investigation was carried out in order to study the possible antioxidant effect and the expression of NF- $\kappa$ B of *C. cajucara* aqueous extract in streptozotocin-diabetic rats.

#### 2. Materials and Methods

2.1. Plant Material and Preparation of C. cajucara Bark Aqueous Extract. Bark fragments of C. cajucara were collected in Amazônia-Santarém, Brazil. A voucher specimen (number 247) identified by Nelson A. Rosa was deposited in the IAN Herbarium (Belém, PA, Brazil).

The bark (5 g) was ground and mixed with boiling water (100 mL) to provide a 5% aqueous extract. After 10 minutes, the mixture was filtered with filter paper and the extract was administered to the rats. After cooling and filtering, the extracts were frozen and concentrated by lyophilization for five days overnight.

2.2. Phytochemical Screening. The phytochemical analysis (flavonoids, tannins, anthraquinones, alkaloids, saponins, coumarins, and cardiac glycosides) of *C. cajucara* bark was carried out according to the methods described by Harbone [14]. The thin layer chromatography analyses were performed following systems and developers indicated by Wagner and Bladt [15].

## 2.3. In Vitro Test for Antioxidant Activity of Bark Aqueous Extract

2.3.1. Hypoxanthine/Xanthine Oxidase Assay. The method employed to assay the hydroxyl radical (OH<sup>•</sup>) scavenging ability of *C. cajucara* aqueous extract or infusions was based on the method developed by Owen et al. [16]. Briefly, *C. cajucara* aqueous extract or infusions were dissolved in the assay buffer (hypoxanthine, Fe (III), EDTA, and salicylic

acid) at a concentration of 2.0 mg/mL and diluted appropriately (in triplicate) in assay buffer to a final volume of 1.0 mL, giving a range of 0.1-2.0 mg/mL for extract and volumes of 10-200 µL of aqueous infusions of C. cajucara bark. A 5  $\mu$ L aliquot of xanthine oxidase dissolved in 3.2 M  $(NH_4)_2SO_4$  was added to initiate the reaction. The sample tubes were incubated for 3 h at 37°C, and the reaction stopped afterwards by adding  $10 \,\mu\text{L}$  of HCl. A  $30 \,\mu\text{L}$  aliquot of the reaction mixture was analyzed by HPLC under chromatographic conditions as described by Owen et al. [17, 18] Chromatographic analysis was done using a gradient based on methanol/water/acetic acid with a µBondaPak C18 reverse phase column (Waters) and detection at 325 nm. The HPLC equipment had a 2695 separation module (Waters) and a UV detector 2487 (Waters). Hydroxylation of salicylic acid and hypoxanthine was monitored at A = 325 and A = 278 nm, respectively. The amount of dihydroxyphenols (2,5-dihydroxybenzoic acid and 2,3-dihydroxibenzoic acid) (2,5-DHBA and 2,3-DHBA) produced by (OH<sup>•</sup>) attack on salicylic acid was determined from standard curves prepared with the respective pure dihydroxyphenols.

2.3.2. DPPH Scavenging Assay. Scavenging of the DPPH free radical was measured using a modified Yamaguchi et al. [19] method in which *C. cajucara* aqueous extract or *C. cajucara* bark infusions were added to Tris-HCl (100 mM) buffer, pH 7.0, containing 250 mM DPPH dissolved in methanol. At least six different dilutions of *C. cajucara* aqueous extract or *C. cajucara* bark infusions were tested and allowed to stand for 20 min in the dark, before absorbance was measured at 517 nm using a Shimadzu spectrophotometer model UV-1602PC (Kyoto, Japan). The experiment was conducted in triplicate. Antioxidant activity was expressed as  $IC_{50}$ (inhibitory concentration in  $\mu$ g/mL of samples or positive controls necessary to reduce the absorbance of DPPH by 50%, as compared to the negative control). The lower the  $IC_{50}$ , the higher is the antioxidant activity.

2.3.3. Animals and Experimental Protocol. The experimental protocol used complied with the norms established by the Ethical and Health Research Committee of the Group of Research and Postgraduate Studies of the Hospital de Clínicas of Porto Alegre as well as with the Principles for Research Involving Animals. Only male Wistar rats were used, obtained from the breeding colony of the Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul (UFRGS). The mean weight of animals at the start of the study was 200-300 grams. They were kept under a 12:12 hours light/dark cycle (light from 7 am to 7 pm) in a temperature-controlled environment (22  $\pm$  4°C). The rats were randomly divided into 6 groups. In 3 groups, diabetes was induced by a single intraperitoneal (i.p) injection of streptozotocin (70 mg/kg body weight; Sigma Chemical) in freshly prepared 10 mmol/L sodium citrate, pH 4,5. Five days after the STZ injection, plasma glucose concentration was measured using retro orbital blood samples obtained from rats after overnight food deprivation. A plasma glucose level > 250 mg/dL was considered indicative of diabetes. The experimental groups comprised the following: (i) normal control group (CO: n = 10) that received 1,5 mL of distilled water administered intragastrically (i.g.); (ii) group treated with *C. cajucara* for 5 days (CO5D: n = 10) 1,5 mL of the *C. cajucara* extract i.g. during the last 5 days before being killed; (iii) group treated with *C. cajucara* for 20 days (CO20D: n = 10) 1,5 mL of the *C. cajucara* for 20 days (CO20D: n = 10) 1,5 mL of the *C. cajucara* extract i.g. for 20 days before being killed; (iv) diabetic group (DM: n = 10) 1,5 mL of distilled water i.g.; (v) diabetic group treated with *C. cajucara* extract i.g. during the last 5 days before killed; (vi) diabetic group treated with *C. cajucara* for 5 days (DM5D: n = 10), 1,5 mL of the *C. cajucara* extract i.g. during the last 5 days before killed; (vi) diabetic group treated with *C. cajucara* for 20 days (DM20D: n = 10), 1,5 mL of the *C. cajucara* extract i.g. for 20 days before being killed.

2.3.4. Biochemical Analyses of Oxidative Stress and Antioxidant Assay. The livers were homogenized with 9 mL of phosphate buffer (KCL 140 mM, phosphate 20 mM, pH 7.4) per gram of tissue. The protein concentration in these liver homogenates was determined using a standard solution of bovine albumin according to Lowry et al. [20]. Hepatics lipoperoxidation was determined by the method of thiobarbituric acid reactive substances (TBA-RS) [21]. Superoxide dismutase (SOD) activity in the liver tissue was determined using a technique based on the inhibition of adrenochrome formation in epinephrine autoxidation [22]. Catalase (CAT) activity in the liver tissue was determined as described elsewhere [23]. For Glutathione reductase (GSH) measurement, it was made according to adapted method by Kolberg et al. [24].

2.3.5. Western Blot for p65 NF-κB Subunit. 75 µg of nuclear extracts, prepared as described by Gutiérrez et al. [25], was loaded on an SDS/PAGE gel (10%), electroblotted, and p65 protein was detected using specific polyclonal antibodies (65 kDa) (NF-κB p65 (C22B4) Rabbit mAb-cell signaling). Bound primary antibody was detected, HRP—with Antirabbit IgG antibody Cell Signaling and blots were developed using an enhanced chemiluminescence detection system (ECL kit, Amersham Pharmacia, Uppsala, Sweden). The density of the specific bands was quantified with an imaging densitometer Software (Scion Image, Maryland, MA).

2.4. Statistical Analysis. The results were expressed as mean values  $\pm$  SEM. The data were compared by analysis of variance (ANOVA); when the analysis indicated the presence of a significant difference, the means were compared with the Student Newman Keuls test. The level of significance used was P < .05.

# 3. Results

3.1. *Phytochemical Analyses.* Phytochemical analyses of *C. cajucara* indicated the presence of flavonoids, coumarins, and alkaloids. Other secondary metabolites such as saponins, anthraquinones, cardiac glycosides, fenolic acids, and tannins were not detected.

3.2. Hypoxanthine/Xanthine Oxidase In Vitro Assay. The in vitro antioxidant activity of the extract was determined



FIGURE 1: Dose-dependent inhibition of reactive oxygen species attack on salicylic acid by the *C. cajucara* aqueous extract ( $\blacktriangle$ ) and of Trolox ( $\blacksquare$ ), as positive control, in the HPLC-based hypoxanthine/xanthine oxidase assay (mean of 3 assays). DHBA: dihydroxybenzoic acid.

by monitoring the production of hydroxyl benzoic acids (DHBA) as a product of the hydroxyl radical attack on salicylic acid in the hipoxanthine-xanthine oxidase assay. The reduction of total oxidation products as a function of the concentration of *C. cajucara* added to the assay is shown in Figure 1. The aqueous extract ( $IC_{50} = 1.61 \text{ mg/mL}$ ) displayed a pronounced *in vitro* antioxidant capacity in a dose-dependent manner, reducing the formation of both DHBA species to 45.49% in the highest concentration used (2 mg/mL), whereas Trolox ( $IC_{50} = 0.34 \text{ mg/mL}$ ), as positive control, could reduce both compounds totally at the same concentration.

3.3. DPPH Scavenging Assay. The free radical scavenging effect of extracts, as well as ascorbic acid and rutin, both acting as positive controls, was tested using the DPPH free radical scavenging assay. The free radical scavenging effect of *C. cajucara* showed a concentration-dependent activity, based on a 91.38% inhibition at the concentration of 1000  $\mu$ g/mL and of 57.32% at 100  $\mu$ g/mL (Table 1). The respective IC<sub>50</sub> value for the extract was 63.34  $\mu$ g/mL. In comparison, the results of the free radical scavenging effect of ascorbic acid (IC<sub>50</sub> = 4.03  $\mu$ g/mL) and trolox (IC<sub>50</sub> = 15.52  $\mu$ g/mL) were respectively, 99.62% and 99.03% inhibition at the concentration of 1000  $\mu$ g/mL and 99.40% and 91.27% at 100  $\mu$ g/mL.

3.4. Biochemical Analysis and Oxidative Stress. C. cajucara administration failed to reduce glycemia in diabetic rats. The treatment with C. cajucara during 5 (DM5D) and 20 (DM20D) days significantly reduced the lipoperoxidation. Normal animals treated with the aqueous extract during 20 days (CO20D) had the lipoperoxidation increased, although significantly less than the diabetic animals. SOD activity was higher in diabetic animals if compared to the controls. This effect was partially abolished by administration of the bark extract. Animals that received only bark extract for 20 days

Sample	Inhibition (%)				$IC_{ro}(\mu g/mL)$
concentration	1 μg/mL	10 µg/mL	100 µg/mL	1000 µg/mL	
Ascorbic acid	9.70	98.11	99.40	99.62	$4.03\pm0.16$
Trolox	8.94	45.82	91.27	99.03	$15.52\pm2.64$
Rutin	5.22	31.83	82.86	97.89	$18.64\pm0.94$
C. cajucara	2.61	13.74	57.32	91.38	$63.34 \pm 29.28$

TABLE 1: Inhibition of DPPH, IC<sub>50</sub> values for the DPPH assay of the *C. cajucara* aqueous extract, Trolox, rutin, and ascorbic acid, as well as the AEAC\*\*.

Mean  $\pm$  standard deviation of three individual determinations. Results were based on the values measured at 20 min. Ascorbic acid, vitamin E, and rutin were used as positive controls.

TABLE 2: Biochemical analyses of oxidative stress in liver tissue.

Group	TBARS (nmoles/mg of protein)	SOD (U/mg protein)	CAT (pmoles/mg protein)	GSH (nmoles/mg of protein)
СО	$0.31\pm0.04$	$2.38\pm0.29$	$0.34\pm0.04$	$4344.01 \pm 433.64$
CO5D	$0.38\pm0.06$	$3.39\pm0.19$	$0.42\pm0.07$	$5188.72 \pm 233.98$
CO20D	$0.54 \pm 0.05^{*}$	$5.57\pm0.67^*$	$0.5\pm0.07$	$4654.82 \pm 480.51$
DM	$0.92 \pm 0.05^{**}$	$8.34 \pm 1.05^{**}$	$0.57\pm0.05$	$2851.94 \pm 378.76^{**}$
DM5D	$0.55 \pm 0.06^{***}$	$4.14 \pm 0.48^{***}$	$0.49\pm0.07$	$4094.46 \pm 293.00^{***}$
DM20D	$0.48 \pm 0.08^{***}$	$3.93 \pm 0.44^{***}$	$0.48\pm0.08$	$3020.94 \pm 151.43$

CO: control (n = 10); CO5D: control treated with *C. cajucara* for 5 days (n = 10); CO20D: control treated with *C. cajucara* for 20 days (n = 10); DM: diabetes mellitus(n = 10); DM5D: diabetes mellitus treated with *C. cajucara* for 5 days (n = 10); DM20D: Diabetes Mellitus treated with *C. cajucara* for 20 days (n = 10). *C. cajucara* 5% aqueous extract.

Data appear as mean  $\pm$  SEM.

\**P* < .05 CO20D, versus CO, CO5D.

\*\**P* < .05 DM versus CO, CO5D, CO20D, DM5D, DM20D.

\*\*\**P* < .05 DM versus CO, DM.

(CO20D) also had SOD activity increased. However, activity of antioxidant enzyme CAT did not show any differences between the groups. GSH activity was significantly higher in liver. The treatment with *C. cajucara* during 5 days decreased significantly GSH activity (Table 2).

3.5. Western Blot for  $p65 NF-\kappa B$  Subunit. To study the effects on  $p65 NF-\kappa B$  subunit expression, liver nuclear extracts were studied by western blot. As shown in Figure 2 *C. cajucara* affects NF- $\kappa B$  binding activity in CO20D. Experimental diabetes markedly induced NF- $\kappa B$ , an effect that was abolished by *C. cajucara* treatment.

#### 4. Discussion

The potential mechanism underlying the antioxidant property of *C. cajucara* extract was investigated using the *in vitro* assay hypoxanthine/xanthine oxidase, which revealed an expressive reduction in the concentration of DHBAs, the byproducts of the reaction between the hydroxyl radical and salicylic acid, as shown in Figure 1. Therefore, it can be suggested that the compounds present in *C. cajucara* extract may act as scavengers of the hydroxyl radical generated by the Haber-Weiss/Fenton reaction, acting as  $O_2^{\bullet-}$  and/or OH<sup>•</sup> scavenger, and that the protection provided by extract is probably due to its ability to quench free radicals. Several plant extracts and secondary metabolites derived from plants show *in vitro* antioxidant activity by quenching hydroxyl radicals, evaluated using this assay [26]. In addition, the results of the antioxidant evaluation using DPPH reduction confirm the dose-response manner pattern of the antioxidant effect of this extract (Table 1). When the DPPH solution is mixed with some hydrogen donor compound, it is reduced to diphenylpicrylhydrazine and loses its violet color [27]. The degree of coloration loss bears a direct correlation with the activity of elimination of free radicals in the compound evaluated [28]. Several series of chemical compounds have been studied and presented a close correlation between DPPH sequestering activity and antioxidant activity in biological and nonbiological models [29, 30]. The differences of the C. cajucara extract IC<sub>50</sub> values for both antioxidant assays might be explained due to different antioxidant compounds responsible for radical-scavenging in both tests. Through of the phytochemical screening were detected alkaloids, flavonoids, and coumarins. Based on the antioxidant activity reported in the literature for alkaloids, flavonoids, and coumarins we can link the presence of these secondary metabolites to the antioxidant activity detected [31, 32].

Oxidative stress is produced under diabetic condition and it is likely to be involved in progression of pancreatic  $\beta$ -cell dysfunction [33]. High levels of free radicals, due to insufficiency of the antioxidant defense system, may lead to disruption of cellular function, oxidative damages to membranes, and enhance their susceptibility to lipoperoxidation [34]. In recent years, dietary plants with antioxidative property have been the center of focus. It is believed that these plants can prevent or protect tissues by antiobesity and hypoglycaemic effects [35]. The protection of hepatocyte cells from the effects of oxidative stress by treatment with



FIGURE 2: Effect of streptozotocin-induced diabetes and *C. cajucara* aqueous extract on liver in p65 NF- $\kappa$ B protein by Western blot analysis.

vitamin E was evidenced in a recent study [36]. In addition, it has been shown that dietary supplementation with natural antioxidants such as vitamins C and E, melatonin, and flavonoids attenuates the oxidative stress and diabetic state induced by STZ [37, 38].

Increased levels of TBARS, an end-product of lipoperoxidation, were found previously in the liver of streptozotocininduced diabetic rats [39–41]. In this study, the TBARS increase confirms this finding, which indicates an overall oxidative stress increase in diabetic rats. Treatment with *C. cajucara* bark extract suggests an amelioration of oxidative stress. *trans-dehydrocrotonin* (t-DCTN), the main component of the bark extract, showed hypoglycemic, anti-inflammatory, analgesic, antiulcerogenic, and antilipidemic effects [42].

Oxidative stress is the result of a redox imbalance between the generation of ROS and the compensatory response from the endogenous antioxidant network. Although some studies which measured activities of SOD, CAT, and glutathione peroxidase in diabetes mellitus showed reduction in the levels of theses enzymes [43], other authors reported an increase in SOD and CAT activities in streptozotocininduced diabetic rats [44]. These apparently contradictory results could be due to tissue specificity, variation in severity and duration of the disease, or other experimental conditions. Hyperglycemia results in increased enzymatic conversion of glucose to the polyalcohol sorbitol with concomitant decreases in NADPH and glutathione [43, 45]. The resulting loss of antioxidant reducing equivalents results in enhanced sensitivity to oxidative stress associated with intracellular ROS. In the present study, SOD activity increased in diabetic rats. This effect may thus be an adaptive response for increased oxidative stress in the liver tissue; C. cajucara bark extract by scavenging ROS prevents the elevation of this antioxidant enzyme activities in diabetic rat liver. This suggests the possibility of a radical superoxide scavenger activity. In our study, CAT activity remained unchanged. One the consequences of hyperglycemia is increase the metabolism of glucose by sorbitol pathway. Besides this, other pathways, such as fatty acid and cholesterol biosynthesis, also compete for NADPH with GSH. We also observed the decreased in GSH in the liver of streptozotocin-induced diabetic rats. Under in vivo condition, GSH acts as an antioxidant and its decrease is reported in diabetes mellitus studies in [46]. showed that GSH level was significantly lower in diabetic rats than normal rats. In the present study we found that treatment with C. cajucara bark extract significantly increased the glutathione when compared to diabetic control rats where the levels were significantly decrease. This increased GSH in rats treated with C. cajucara bark for 5 days extract may be one of the factors responsible for the inhibition of lipoperoxidation.

The NF- $\kappa$ B has been proposed to form a critical bridge between oxidant stress and the cellular response [47, 48]. One mechanism by which hyperglycaemia-induced oxidative stress might alter cellular functions is activation of the transcription factor NF-kB [39]. Rodrigues et al. [49] showed than glucose concentration in the blood plasma of streptozotocin-treated rats was significantly higher than in the normal control group and in the animals treated with C. cajucara. In our study, to evaluate the liver tissue of diabetic animals found significant increase in the activation of NF- $\kappa$ B. In contrast, the treatment with C. cajucara prevented NF- $\kappa$ B activation [49]. However, a significant increase in oxidative stress and in the expression of p65 NF- $\kappa$ B was found in the group that underwent treatment for 20 days. Although the previous study did not demonstrate genotoxic effects using the same period, other studies show reports of fulminant hepatitis related to the indiscriminate use of the plant [50].

In summary, the results show that in situations where there is no oxidative stress the extended use of *C. cajucara* behaves acts as a prooxidant and in situations where there is oxidative stress the treatment with *C. cajucara* may be an antioxidant scavenger of free radicals. Furthermore, the results seem to support the hypothesis that the oxidative stress in DM stimulates NF- $\kappa$ B expression and that *C. cajucara* aqueus extract administration reduces this expression.

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