Full Length Research Paper

Effect of hydroethanolic extract from *Calophyllum brasiliense* Cambess on streptozotocin induced diabetic rats

Helison de Oliveira Carvalho¹, Grace Suzan Lopes Lacerda¹, Adriana Maciel Ferreira¹, Larissa Daniele Machado Góes¹, Beatriz Martins de Sá¹, Mona Saout³, Didier Bereau³, Jean-charles Robinson³, Rafael Lima Resque¹, Caio Pinho Fernandes¹ and José Carlos Tavares Carvalho¹,²*

¹Laboratory of Research in Pharmaceuticals, Pharmacy Course, Department of Biological Sciences and Health, Federal University of Amapá, Rod. JK, KM 02, 68902-280, Macapá, Amapá, Brazil.
²Postgraduate Program in Pharmaceutical Sciences, Department of Biological Sciences and Health, Federal University of Amapá, Macapá, Amapá, Brazil.
³Université de la Guyane Francesa, UMR QUALITROP, Campus Universitaire de Troubiran, P. O. Box 792, 97337 Cayenne Cedex, French Guiana, France.

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*Calophyllum brasiliense* (Cb) belongs to the Clusiaceae family and it is generally used for diabetes treatment. The aim of this study was to evaluate the anti-diabetic effect of Cb’s hydroethanolic extract (CBE) on diabetic induced rats by streptozotocin and evaluate the content of polyphenols and tannins of the extract. The polyphenols and tannins evaluation in the extract were determinated by a spectrophotometer in 760 nm and isocratic HPLC system and reverse phase column (C18). The induction of diabetes was performed by intraperitoneal injection of streptozotocin (55 mg/kg) and was confirmed by a histopathological analysis. The total content of polyphenols and tannins (gallic acid) in CBE was 0.025 ± 0.0028 mg/mL (4.77%) and 8.262 ± 0.417 µg/mL, respectively. According to the oral glucose tolerance test that was performed both in normal and diabetic mice, the treatment with 500 mg/kg of CBE appeared to significantly reduce the blood glucose levels compared to the untreated group (P < 0.001). The treatment of diabetic mice with 500 mg/kg of CBE for 30 days significantly improved diabetes clinical symptoms (polydipsia, polyuria, polyphagia and weight loss) (P < 0.001). After the urinary glucose analysis, it was found that the treatment with 500 mg/kg of CBE significantly decreased the urinary glucose levels at an average of 177.55 ± 17.8 mg/dL (32.40%) (P < 0.001). In relation to the blood glucose measurements, it was shown that the groups treated with 500 mg/kg CBE and 3 mg/kg of Glibenclamide had significantly lower levels of blood glucose when compared to non-treated group (P < 0.001) 24.09% (143.36 ± 19.6 mg/dL) and 36.04% (200.08 ± 14.9 mg/dL), respectively. The histopathological analysis revealed an increase in the number of endocrine cells in the islets of Langerhans in the groups treated with CBE500 and insulin. Therefore, it was concluded that the treatment with 500 mg/kg of CBE exhibited anti-diabetic activity.

**Key words:** *Calophyllum brasiliense*, polyphenols, diabetes, clinical parameters.
INTRODUCTION

Diabetes mellitus (DM) is a syndrome that interferes directly in the metabolism of carbohydrates, fats and proteins, due to deficiency in the insulin production by the β cells of the pancreas or reduced tissues’ sensitivity to insulin. The DM can be confirmed by symptoms such as hyperglycemia, glycosuria, polyuria, polyphagia, and polydipsia. The chronic hyperglycemia present in DM patients can result in microvascular complications, predominantly retinopathy, nephropathy and neuropathy, but also macrovascular complications, such as stroke and coronary disease. These complications make DM the seventh cause of death in developed countries (Sartori et al., 2002).

During the last decades, several studies about new hypoglycemic agents have been conducted, with special focus on well-known medicinal plants. A good example is the plant Galega officinalis, which led to the development of the oral hypoglycemic drug "Metformin" (Noel et al., 1997).

Calophyllum brasiliense Camb. (Cb) species belongs to Clusiaceae family, and can be spontaneously found in Latin America, and mainly in the Amazon and Atlantic Forest. Cb is known by several common names, including "jacareúba", "guanandi”, “guanandi-carvalho”, "cedro-do-pântano”, and “landim”, among others (Pereira, 1966).

According to phytochemical analyses, Cb contains a variety of substances with biological activities, such as xanthines, triterpenes, benzofurans, polyphenols (coumarins, flavonoids and tannins); at the same time, no presence of alkaloids and quinones was found (Sartori et al., 1999; Carvalho et al., 2013).

A previous study showed that many plant species are used as in alternative therapies for DM control in Brazil, and the Cb is among them (Silva et al., 2015). Cb is also used for the treatment of other diseases, such as bronchitis, liver and gastrointestinal disorders, pain, inflammation, hypertension and rheumatism (Silva et al., 2001).

Pharmacological studies about Cb revealed that it possesses antiretroviral activity (Huerta-Reyes et al., 2004), antiparasitic (against Trypanosoma cruzi, the etiological agent of Chagas disease) (Abe et al., 2004), antimicrobial (Cottiglia et al., 2004), gastroprotective and cytotoxic (Sartori et al., 1999) and anti-neoplastic (Ito et al., 2003) properties.

Many studies have already proven the pharmacological activities attributed to Cb, and have identified a wide variety of chemical compounds present in this plant. The objective of this study was to evaluate the anti-diabetic effect in diabetic streptozotocin-induced rats and to determine the total content of polyphenols and tannins.

MATERIALS AND METHODS

Plant material obtaintment

Plant material (bark of the stem) of Cb species was collected in Ferreira Gomes City, in the state of Amapá, Brazil. The fertile material was identified in the Herbarium of the Institute of Studies and Research of the State of Amapá - IEPA, Brazil, with 0598AP as voucher specimen number.

Hydroethanolic extract obtaintment (CBE)

To obtain the CBE, 2 kg of crushed and milled barks were subjected to a dilution in a percolator (LM20) with 70% hydroethanolic solution at a temperature of 45°C during 4 days in ratio of 1:8 (w/v). The extracted solution was filtered through filter paper and concentrated on rotaevaporator model Q.218.2 (Quimis Ltda, São Paulo, Brazil) at a temperature of 40°C until complete evaporation of the solvent, obtaining a yield of 32%. Finally, it was lyophilized to complete elimination of water, with final yield of 6.95%.

Quantitative analysis of total polyphenols by spectrophotometry and of gallic acid by HPLC

Total polyphenols analysis was performed using 0.750g of lyophilized extract diluted in 250 mL of distilled water and heated for 30 min at 60°C in a water bath. 5 mL of this solution were transferred to a 50 mL volumetric flask, and then 2 mL of acid phosphomolybdotungstic reagent (Sigma Co., São Paulo, Brazil) were added and the volume was completed with sodium carbonate solution of 15% (Sigma Co., São Paulo, Brazil). Later, the absorbance was measured in a 760 nm UV-VIS spectrophotometer (Shimadzu, UVmini-1240 model), according to the methodology described by Carvalho et al. (2013).

For the HPLC chromatographic analysis, 10 mg of CBE were weighed and added to 10 mL of methanol:water solution (2:8 v/v); then, filtered through membrane filter with a pore size of 0.45 micrometers (Millepore®) and analyzed by high-performance-HPLC (Shimadzu Corporation) equipped with auto injector, diode array detector scanned from 190 to 500 nm. Chromatographic conditions were: Chromatograms obtained at 350 nm, oven with temperature kept at 30°C, reverse phase column (C18), Shim-pack VP-ODS (150 x 4.6 mm; 5 μm), injection volume of 10 μL using methanol as phase A: water acidified with 0.05% of acetic acid (70:30) and acetonitrile as phase B, in proportions isocratic system of 70% phase A with flow rate of 1 mL/min. The quantification of gallic acid (Sigma Co., São Paulo, Brazil) was accomplished by constructing standard curve with concentrations from 2 to 16 mg/mL.

*Corresponding author. Email: farmacos@unifap.br.

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Animals

Male Wistar rats were used, with an average weight of 210 ± 50 g. During the experiment, animals were placed into individual metabolic cages of stainless steel (60×50×22 cm), in an air-conditioned environment with constant temperature of 25 ± 3°C and humidity of 50 ± 10%. They were subjected to a light regime of 12-hour light / dark, water was offered ad libitum and they were fed with standard rodent chow. The project was approved by the Ethics Commission of the Federal University of Amapá, Amapá, Brazil, under Protocol Number 02A/2014.

Oral glucose tolerance test (OGTT)

To determine glucose tolerance, hyperglycemia was induced in normoglycemic and diabetic rats mice after 16 h of fasting, by the administration (VO) of a glucose solution at a dose of 4 g/kg of body weight. Glucose levels were measured after 0, 30, 60, 90, 120 and 180 min. The animals were randomly divided into 5 groups (n = 5/group). Non-treated group (NTD), group treated (VO) with 3 mg/kg of glibenclamide (GBC), group treated (VO) with 250 mg/kg of CBE (CBE250), group treated (VO) with 500 mg/kg of CBE (CBE500) and the group treated (subcutaneous injection) with 14 IU/kg of NPH insulin (INS).

Diabetes mellitus induction

The DM induction in animals was performed after a 16 h fasting period by peritoneal injection of streptozotocin (Sigma-Aldrich Inc., St. Louis, MO, USA), dissolved in sodium citrate buffer 0.01M (pH 4.5), with a dose of 55 mg/kg of body weight. 4 days after the injection of streptozotocin, animals showed blood glucose levels greater than 300 mg/dL urine glucose concentration higher than 250 mg/dL, polydipsia and polyuria were considered as diabetic animals (Carvalho et al., 2016).

Development and experimental evaluation

Diabetic animals were maintained in metabolic cages throughout treatment (30 days), where body weight, water intake, feed intake and urine output were daily recorded. Glycemia and glycosuria were evaluated every 5 days, where the blood collection was performed by retro-orbital plexus and glucose levels were estimated by colorimetric glucose oxidase method (Glucox 500, Doles Reagentes and Equip. for Lab., Ltd., Goiânia GO, Brazil)

Histopathological evaluation

The animals were euthanized and the pancreas was removed; then, was inserted in a 10% buffered formaldehyde for 24 h and then dehydration in alcohol, clarification in xylene, impregnation and paraffin embedment at 60°C took place. The histological observations were performed in a semi-automatic rotary microtome model (CUT 5062; SLEE) in 5 μm sections after coloring with hematoxylin-eosin (H/E).

Statistical analysis

For polyphenols and tannins dosage, a linear regression test was used. Anti-diabetic activity was analysed by a variance analysis (ANOVA) followed by Tukey’s test. Results with P < 0.05 significance levels were considered as statistically significant. Statistical software used was GraphPad Instat and Prism (version 5.03).

RESULTS AND DISCUSSION

Polyphenols are chemically characterized by different classes of substances which have at least one aromatic ring with one or more hydroxyl substituents and are derived from the metabolism of shikimic acid and of phenylpropanoids. Polyphenols are widely distributed in the plant kingdom, and serve as essential secondary metabolites for the plant. They are formed during stress conditions, such as infection, injury, ultraviolet radiation with the intention to protect plant molecules (King and Young, 1999; Lee et al., 2005; Naczk and Shahidi, 2004; Swain and Hillis, 1959).

From the quantification method using reduced levels of phosphomolybdotungstic reagent, the linear equation of pyrogallic acid was obtained (y = 10.450x - 0.0118), enabling the determination of the total polyphenols content present in the hydroethanolic extract of Cb, which was 0.025 ± 0.0028 mg/mL (n = 3), corresponding to 4.77%.

As was shown by the HPLC analysis, the tannin retention time (gallic acid) in the Cb extract was 3.827 min. (Figure 1), and from the standard curve (Figure 2) the gallic acid content was equal to 8.262 ± 0.417 (n = 3) μg/mL.

In a study conducted by Carvalho et al. (2013), the Cb extract showed high levels of polyphenolic compounds, and these natural antioxidants have great potential for the treatment of DM, as several studies have proven the hypoglycemic activity attributed to these compounds (Hou et al., 2007; Jia et al., 2009; Panda and Kar, 2007).

The oral glucose tolerance test (OGTT) is characterized as a clinical test used to assess the ability of pancreas’ β-cells to secrete insulin and to evaluate the tissue sensitivity to insulin. This allows to check the carbohydrate metabolism behaviour (Bhuiyan et al., 2011), as through this test it is possible to evaluate the ability of probable anti-diabetic drugs to reduce the postprandial blood glucose and to define the dose responsible for such activity.

After the implementation of the OGTT in normal rats (Figure 3), it was observed that blood glucose levels were not significantly reduced in the ECB250 compared to the NTD group, but ECB500 and GBC groups had significantly lower values (P < 0.001). According to these findings, we selected the dose of 500 mg/kg of CBE in the present study for diabetic rats (Figure 4), and it was observed that the treatment with 500 mg/kg of CBE (CBE500) significantly reduced blood glucose levels. At the end of the analysis time, blood glucose levels in CBE500 group were still quite high, as well as in the
group treated with glibenclamide with levels of 443 ± 13.5 and 343 ± 19.6 mg/dL, respectively. These high values can be justified by the fact that there are few functional β cells due to the deleterious effect of diabetes drugs induction.

Considering the results obtained in the OGTT with normal and diabetic rats, we can conclude that the CBE at the dose of 500 mg/kg had an acute hypoglycemic activity. Similar results were found in the study of Jia et al. (2009), where doses of 100, 200 and 300 mg/kg of *C. parthenoxyylon* extract were offered and the highest dose showed the greatest hypoglycemic activity in the OGTT.

In a study by Panda and Kar (2007), the crude extract and fractions of *Gentiana olivieri* (Gentianaceae) and its isolated polyphenols reduced blood glucose in hyperglycemic, normal and diabetic rats as shown by the OGTT, findings that are similar with that obtained in this study for the Cb species.

As it is shown in Table 1, NTD group displayed the clinical characteristic of diabetes, such as increased water intake (polydipsia), increased excreted urine volume (polyuria), increased feed intake (polyphagia) and body weight reduction.

The increase in feed intake is due to the lack or the reduction of insulin release, since insulin stimulates the release of leptin from fat cells entering the central
nervous system, which may decrease food intake by affecting the actions of neuropeptide Y (NPY) of neurons in the arched nucleus of the hypothalamus (Cambraia, 2004; Halpern, 2002). With the reduction of insulin levels in diabetic as well as in the fasted rats, there was a reduction of leptin and therefore an increased feed intake. This data can be confirmed by results obtained from GBC and INS groups, where feed intake was reduced when compared with NTD group ($P < 0.05$).

Moreover, it was observed that the CBE500 and GBC groups had similar values for body weight; however, these values were significantly higher compared to the NTD group ($P < 0.001$). Finally, INS and NDC groups had the highest values for body weight ($P < 0.001$).

Weight reduction that occurs in individuals with DM is due to lack of insulin and absence of glucose uptake by cells; as a result, body seeks for new sources of energy, causing intense process of structural proteins catabolism and β-oxidation of fatty acids, which, therefore, reduce the body mass of the individual with untreated DM (Molina et al., 1989; Queiroz et al., 2009).

Water intake and urine excretion were higher in the NTD compared to the other groups ($P < 0.001$). GBC and CBE500 appeared to have decreased values and INS and NDC groups had the lowest levels of water intake and urine ($P < 0.001$).

Polydipsia present in diabetic animals is due to the blood hyperosmolarity, which makes the water to pass from intracellular to extracellular space in order to maintain the osmotic balance. The intracellular dehydration is recognized by osmoreceptors in brain that generate a response triggering intense thirst, characteristic of DM. Increased water consumption causes an osmotic imbalance in different cellular compartments, and to maintain the balance, the diabetic people excrete large amounts of urine (Jacobson, 1996; Lerco et al., 2003).

Glucose and amino acids are the most important...
substances which are absorbed specifically due a secondary active transport in the kidney’s proximal tubules, as happens for almost all substances that undergo an active reabsorption. There is a maximum level of reabsorption and substances that are not absorbed are excreted in the urine. In normal individuals the level of glucose in urine is very close to 0. Glucose levels exceeding the level of 220 mg/minute in urine, and 180 mg/dL in blood are indicative of a diabetic patient (Mather and Pollock, 2011).

According to the results obtained from the evaluation of glucose excretion levels in urine (Figure 5), the NTD group presented a large amount of glucose in the urine during the treatment, with a mean of 547.91 ± 23.9 mg/dL. The CBE500 group compared to the NTD group, showed a significant decrease in glucose excretion levels from the 15th day of treatment (p < 0.01) and at the end of treatment it was reduced by 32.40% (177.55 ± 17.8 mg/dL). The GBC group had decreased values from the 10th day of treatment (p < 0.001) and at the end of the treatment showed a reduction in urine glucose of 49.36% (270.48 mg/dL). The INS group showed significantly lower levels of urine glucose (p < 0.001) compared to all treatment groups and this reduction in excretion of glucose levels was approximately 94.32% (516.79 ± 28.6 mg/dL) in comparison to the NTD group.

The blood glucose levels evaluation (Figure 6) showed that the NTD group had high levels throughout the analysis period, with an average of 555.08 ± 16.90 mg/dL, and when this value is compared to the NDC group, there was an increase of 387.13 mg/dL. The CBE500 group showed statistical significance from the 10th day of treatment (P < 0.05), with progressive reductions in blood glucose levels, and at the end of the treatment, it was reduced by 24.09% (143.36 ± 19.6 mg/dL) when compared to NTD (P < 0.001). The GBC group also showed statistical significance (P < 0.001) from the 10th day of treatment when compared to NTD and at the end of the treatment, it showed a reduction of 36.04% (200.08 ± 14.9 mg/dL) in glucose level. INS group had reduced levels of blood glucose since the beginning of treatment (P < 0.001). In due course, glucose levels approached quite to that of the NDC group, with an average of 180.28 ± 17.870 mg/dL, a reduction equivalent to 67.52% (374.80 mg/dL).

It has been discussed in the literature that a drug is considered effective as anti-diabetic when it can lower plasma glucose levels by at least 15% (Martha et al., 2000). According to this criterion, it can be concluded that treatment with CBE in STZ-induced diabetic rats was effective because it was able to reduce blood glucose levels by 24.09% and moderate the display of clinical symptoms resulting from diabetes, such as: Weight loss, polydipsia, polyuria and glycosuria.

Histopathological findings of the pancreas (Figure 7) show that the dose of STZ caused endocrine cells destruction in the islets of Langerhans compared to the NTD group and to the NDC group, but there were remaining cells that characterize this model as type 2 diabetes. Similar results were obtained by Silva et al. (2011), who observed partial destruction of pancreatic β-cells, and the STZ cytotoxicity in β-cells was proportional to the administered dose. The cytotoxicity effects of STZ on the pancreatic β-cells are due to its similarity with
Figure 6. Effect of CBE500, GBC and INS on the blood glucose levels of diabetic rats. Where significance: *P < 0.05, **P < 0.01, ***P < 0.001, and (a) compared with NTD group, (b) compared to the group and GBC, (c) compared to all groups treated diabetics. Values express the mean ± S.D (n = 5/group).

Figure 7. Histological photomicrographs showing pancreatic islets of Langerhans, 40x magnification, stained by the method (H/E). NDC, non-diabetic; NTD, untreated diabetic; CBE500, treated with extract; INS, insulin treated; GBC, treated glibenclamide.

Glucose molecule, allowing their intracellular passage via transporters GLUT 2 (Schnedl et al., 1994). The cellular action involves the production of reactive oxygen species (ROS) that promote alkylation of DNA chain, causing irreversible damage to the metabolism of β-cells, resulting in the depletion of nicotinamide adenine dinucleotide (Asplunk et al., 1984).

The groups treated with CBE500 and INS showed a slight increase in the number of cells in the islets of Langerhans compared to the NTD group. GBC group also showed an increase, but was less than that of CBE500 and INS groups. The increase of cells observed in the INS group is due to the reduction of GLUT-2 expression in β cells, and therefore the limited damage caused by STZ (Thulesen et al., 1997). The higher number of β-pancreatic cells evidenced by CBE500 group is possibly because of its antioxidant potential, that possibly reduces reactive oxygen species (ROS) produced by STZ, restoring the β-pancreatic cells from oxidative damage (Sezik et al., 2005).

This possible mechanism was observed in several studies, which reported the hypoglycemic activity of plant extracts that have high content of polyphenolic compounds, such as: *Cinnamomum parthenoxylon* (Jia...
et al., 2009), *Lactuca indicae* (Hou et al., 2007), *Dimocarpus longan* (Li et al., 2015), *Bauhinia monandra* (Alade et al., 2012) and *Sphaeranthus indicus* (Ramachandran et al., 2011).

Despite these assumptions, the actual mechanism involved in the anti-diabetic activity of the CBE cannot be clearly described, thus, more detailed studies are needed to clarify and confirm the mechanism of CBE action.

Medicinal plants have different mechanisms for the control of carbohydrate metabolism, and these mechanisms reduce hyperglycemia by acting in the restoration of the functions of beta-pancreatic cells. Some of them involve: The increased stimulation of insulin release, the elevated glucose uptake and utilization, the reduction of the oxidative damage caused by reactive oxygen species (ROS) in the beta-pancreatic cells, the increase in the number and sensibility of insulin receptor sites, the reduction of gluconeogenesis and gastrointestinal absorption of glucose and/or the release of glucagon (Pepato et al., 2003; Rocha et al., 2006). Authors propose some mechanisms of action for polyphenolic compounds, which include: Protection of pancreatic beta-cells from oxidative damage, increased secretion of insulin, increased sensitivity of peripheral tissues in response to insulin and reduced gastrointestinal absorption of glucose.

**Conclusion**

Based on the results obtained in this study, the hydroethanolic extract of the bark of *C. brasiliense* species was able to improve clinical and laboratory parameters caused by diabetes in induced diabetic mice by streptozotocin, and this effect may possibly be related to the high content of polyphenols. Therefore, it can be concluded that the extract of this species has anti-diabetic effect and can probably develop similar effect in humans. These results confirm the information observed and described in popular medicine.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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