Revista Brasileira de Farmacognosia 26 (2016) 634–639

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original article

Hypoglycemic effect of formulation containing hydroethanolic extract of *Calophyllum brasiliense* in diabetic rats induced by streptozotocin

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ARTICLE INFO

Article history:
Received 9 February 2016
Accepted 24 April 2016

Keywords:
Calophyllum
Hypoglycemic
Granulated
Diabetes mellitus
Polyphenols

ABSTRACT

Diabetes mellitus is a chronic and severe metabolic dysfunction, it's slow and progressive evolution interferes directly in the metabolism of carbohydrates, fats and proteins, causing hyperglycemia, glycosuria, polydipsia, hyperlipidaemia, among others. The aim of this study was to evaluate the antidiabetic effect of hydroethanolic extract and granulated of *Calophyllum brasiliense* Cambess., *Clusiaceae*, species in diabetic rats as well as it's biochemical parameters. The results demonstrated that both the pharmaceutical forms, hydroethanolic extract and granulated, were able to reduce significantly ($p < 0.001$) hyperglycemia and glycosuria, in addition to improve polydipsia, polyuria, and weight loss. Treatments using hydroethanolic extract and granulated were also able to reduce significantly levels of triacylglycerides, cholesterol and low-density lipoprotein, as well as the transaminases, urea and creatinine levels. Therefore, it is concluded that these pharmaceutical forms have anti-diabetic effect and act improving the biochemical parameters, this effect is probably due to the high content of polyphenolic compounds found in the formulations.

Introduction

Calophyllum brasiliense Cambess. (Cb) species belongs to Clusiaceae family; it can be found spontaneously throughout Latin America, with predominance of the species in regions such as Amazon and Atlantic Forest. Pharmacologically, is used for several diseases such as diabetes, bronchitis, liver disorders, gastrointestinal diseases, pain, inflammation, hypertension and rheumatism (Silva et al., 2001).

Diabetes mellitus (DM) is a chronic and severe metabolic dysfunction, it's slow and progressive evolution interferes directly in the metabolism of carbohydrates, fats and proteins and is characterized by absent or decreased production of insulin and/or its failure to properly exercise their effects on cells, leading mainly to hyperglycemia, glycosuria, polyuria, polydipsia, among others. DM is not a single disease but a group of several metabolic disorders that have in common hyperglycemia and dyslipidemia, and lead to serious complications causing damage to many organs, especially the eyes, kidneys, nerves, heart and blood ducts, making DM the seventh cause of death in developed countries (Ada, 2011; Sacks et al., 2002).

There is a growing demand by the world population for medicinal plants, where about 65–80% of the people use plants because of poverty, precarious health system or the easy access to these natural products that are sold in street markets and popular markets (Calixto, 2000). Therefore, studies of new anti-DM drugs have been conducted with special focus on medicinal plants; a good example is the plant *Galega officinalis* that led to the development of Metformin, an oral hypoglycemic drug (Noel et al., 1997).

Phytotherapics are developed by technological processes from vegetable raw materials, and among the main pharmaceutical forms produced, lyophilized dry extracts and granulated, which can be intermediate or final formulations for obtaining tablets and capsules, are found (Carvalho et al., 2013).

The aim of this study was to evaluate the antidiabetic effect of hydroethanolic extract and granulated of Cb species and

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http://dx.doi.org/10.1016/j.bjp.2016.04.004

biochemical parameters of normal and diabetic rats induced by streptozotocin.

Materials and methods

Plant material

Stem barks of *Calophyllum brasiliense* Cambess., Clusiaceae, was collected in the municipality of Ferreira Gomes, Amapá State, Brazil (0.859831 N and −51.158938 W). The fertile material was identified in the Herbarium of the Instituto de Estudos e Pesquisas do Estado do Amapá, with 0598AP as number of voucher specimen.

Obtaining the C. brasiliense *hydroethanolic extract*

To obtain the *C.* brasiliense *hydroethanolic extract* (ECb), 2 kg of crushed and milled hulls were subjected to maceration in 70% hydroethanol solution at 45 °C for 4 days in a ratio of 1:8 (w/v). The extractive solution was filtered through filter paper and concentrated on rotaevaporator Model Q.218.2 (Quimus Ltda, São Paulo, Brazil) at a temperature of 40 °C until complete evaporation of the solvent, yielding 32.80%. Later it was lyophilized to complete elimination of water, with final yield of 7%.

Obtaining granulated of C. brasiliense *extract (GCb)*

The granule was obtained by manual mixing for granulating using the following combination of excipients and extract: 21.80% Avicel® cellulose (Sigma–Aldrich Co., St. Louis, USA), 3.87% magnesium stearate Riedelde Haën® (Sigma–Aldrich Co., St. Louis, USA), 33.35% lactose monohydrate D-Vetec® (Vetec Fine Chemicals Ltd., Rio de Janeiro, Brazil) 6.9% corn starch Duryea® (Unilever Brazil Industrial Ltda, Pernambuco, Brazil), 28.5% water and 26.64% dry ECb (Carvalho et al., 2013).

Quantitative analysis of polyphenols and total tannins in ECb and GCb

In analysis of polyphenols and total tannins 0.750 g of lyophilized ECb and 2.81 g of GCb equivalent to 0.750 g of ECB were used. Subsequently we used the reduction of phosphomolybdotungstic acid technique in an alkaline medium (20% sodium carbonate). Later the absorbance was measured at 760 nm in UV-VIS model UVmini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) (Sá et al., 2015).

Levels of polyphenols and tannins were calculated from the absorbance submitted to the equation of the straight obtained by the standard curve of the pyrogallic acid in concentrations of 0.02–0.10 mg ml^{-1} in reaction with the phosphomolybdotungstic acid in an alkaline medium, lyophilized bovine serum albumin was used to complexation with tannin. The percentage of polyphenols and tannins was obtained applying the formula described by Carvalho et al. (2013).

$$\%Pf = \frac{x\,(\mathrm{mg/ml}) \cdot FD \cdot 100}{m\,(mg)}$$

where *%Pf* = polyphenols percentage; *x* = sample concentration obtained in straight equation; DF = dilution factor of the solution; *m* = mass of the sample.

Animals used in the study

Wistar male rats were used, weighing around 210 ± 30 g, during the animals activities were placed in individual metabolic cages stainless steel, measuring 60 cm × 50 cm × 22 cm, kept in air-conditioned environment with temperature around 25 ± 3 °C and humidity of 50 ± 10%, photoperiod of 12 h light and dark, fed with standard feed for rodents and water *ad libitum*. This study was approved by the Ethics Committee of the Federal University of Amapá under the Protocol 002A/2012 August 6, 2012.

Oral test for glucose tolerance (OTGT)

To determine glucose tolerance, hyperglycemia was induced in normoglycemic and diabetic rats with 16 h of fasting by administering by mouth of a glucose solution of 4 g/kg of body weight 30 min after treatment. Blood glucose levels were assessed at 0, 30, 60, 90, 120 and 180 min. The animals were divided into five groups ($n = 5$), a diabetic group treated with distilled water 0.5 ml/animal (DTA), the group treated with glibenclamide by mouth 3 mg/kg (GBC), diabetic group treated with GCb by mouth 500 mg/kg, diabetic group treated with ECb by mouth 500 mg/kg, non-diabetic group of animals treated with distilled water 0.5 ml/animal (NDC).

Induction of Diabetes mellitus

The induction of diabetes was performed in animals after 16 h fasting period, by intraperitoneal injection of streptozotocin (STZ) (SIGMA-Aldrich Inc., St. Louis, MO, USA) dissolved in 0.01 M sodium citrate buffer (pH 4.5), with a dose of 55 mg/kg in a volume of 1 ml/kg body weight. Four days after STZ injection, animals were considered diabetic with blood sugar >300 mg/dl urine glucose >100 mg/dl, polydipsia and polyuria. The animals were divided into five groups ($n = 5$), a diabetic group treated with distilled water 0.5 ml/animal (DTA), the group treated with glibenclamide by mouth 3 mg/kg (GBC), diabetic group treated with GCb by mouth 500 mg/kg, diabetic group treated with ECb by mouth 500 mg/kg, animals and non-diabetic group treated with distilled water 0.5 ml/animal (NDC).

Development and experimental evaluation

Diabetic animals were kept in metabolic cages during the 30 days of treatment, where they were evaluated daily, body weight, water intake, food intake, and urine volume. Glyemia and glycosuria were evaluated every five days, the blood collection was performed by retrorbital plexus and glucose levels evaluated by photocolorimetric method glucose-oxidase (Glucox 500, Doles® Reagents and Equipment Lab. Ltda., Goiânia-GO, Brazil). On the 30th day of treatment was held blood collection for biochemical analysis of total protein, total triacylglycerides, total cholesterol, high density lipoprotein (HDL), low-density lipoprotein (LDL), urea, creatinine, transaminase glutamic oxaloacetic and glutamic pyruvic transaminase. All tests were performed using reagents Doles® Reagents and Equipment Lab industry. Ltda. (Goiânia, GO, Brazil) and the samples analyzed in UV-VIS model UVmini-1240 (Shimadzu Corporation, Kyoto, Japan).

Statistical analysis

We used analysis of variance (ANOVA) followed by Tukey's test, results with significance level of $p < 0.05$ were considered statistically significant. GraphPad Instat® and Prism® (version 5.03) softwares were used for analyzes.

Results

Quantification of polyphenols and total tanins

With the equation of the straight $y = 10.64x - 0020$ (Fig. 1) it was possible to realize the quantification of polyphenols and total tannins equivalents to pyrogallic acid, where the levels present in

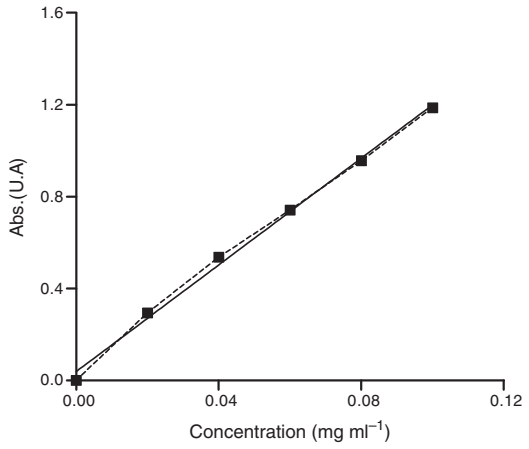

Fig. 1. Standard curve pyrogallic acid by spectrophotometry (λ = 760 nm) at concentrations from 0.02 to 0.10 mg ml^{-1}, straight line equation $y = 10.64x - 0020$, the correlation coefficient $r^2 = 0.9964$.

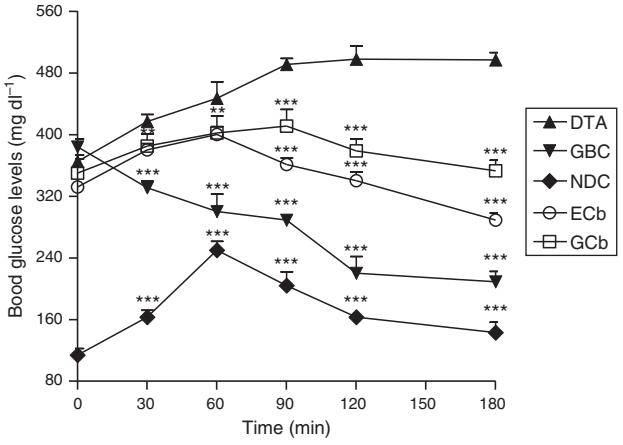

Fig. 2. Effect of treatments on the blood glucose levels of diabetic rats and non-diabetic in the TOTG. Significance: $^{**}p < 0.01$ and $^{***}p < 0.001$, Compared with DTA group. Values express the mean ± SD ($n = 5$/group).

the ECb were 0.025 ± 0.0019 mg ml^{-1} representing 8.36% of total polyphenols and 0.016 ± 0.0014 mg ml^{-1} in of tannins with 5.33%. In GCb polyphenols and total tannins contents were 0.021 ± 0.0023 and 0.014 ± 0.0017 mg ml^{-1} with percentages of 7% and 4.66% respectively.

Oral test for glucose tolerance (OTGT)

In the OTGT performed in normal and diabetic mice (Fig. 2), all groups presented a reduction of blood glucose levels in all analyzed times with exception of the DTA group. The groups of diabetic rats treated with GCb and ECb reduced blood sugar levels significantly ($p < 0.001$) when compared to the DTA group, reaching 28.98% (144 ± 6.9 mg dl^{-1}) and 41.86% (208 ± 13.58 mg dl^{-1}) of reduction in the end of the analysis period respectively. The group treated with the standard drug (GBC) also presented statistically significant ($p < 0.001$), with glucose reduction values of 288 ± 13.46 mg dl^{-1}, equivalent to 57.95%. The NDC group presented the characteristic profile of non-diabetic animals, with peak glucose levels at 60 min and then decrease in subsequent times.

Treatment effect on clinical parameters

The DTA group presented a wide variation in the evaluated clinical parameters (Table 1), compared to the NDC group is observed

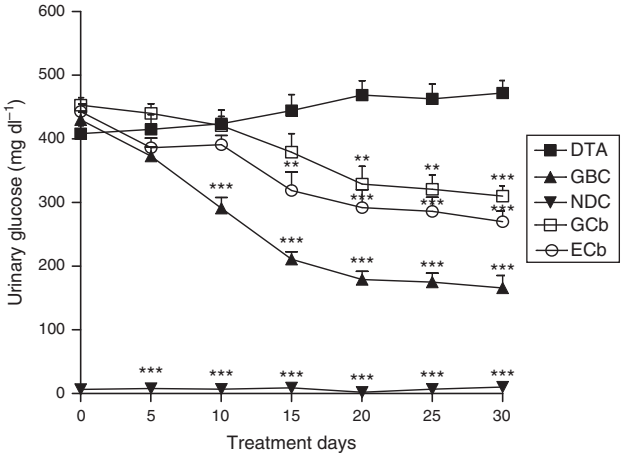

Fig. 3. Effect of treatments on glucose levels in urine of diabetic and non-diabetic mice. Significance: $^{**}p < 0.01$ and $^{***}p < 0.001$ Compared with DTA group. Values express the mean ± SD ($n = 5$/group).

that the animals in DTA group lost weight, increased feed intake (polyphagia), water intake (polydipsia) and urination (polyuria) significantly ($p < 0.05$ and $p < 0.001$), these are typical symptoms of diabetes in untreated individuals. From the treatment with standard drug (GBC) and oral formulations, extract (ECb) and granulated (GCb), it was noted that these treatments were able to improve the clinical parameters of diabetic animals significantly ($p < 0.001$) when compared with the DTA group. Regarding the weight development, an increase of body weight of 11.76% (GBC), 18.09% (GCb) and 14.02% (ECb) was observed, there were no significant findings about polyphagia because the diabetic animals had food consumption values very close. Regarding polydipsia and polyuria, significant reductions ($p < 0.001$) were observed, where the GBC group presented reductions of 41.81% and 63.10%, the GCb group presented 44.27% and 55.33% and the ECb group 47.55% and 53.39% respectively.

Effect of treatment on glycosuria and glycemia

From the determination of glucose levels in urine (Fig. 3), it was observed that the non-diabetic group (NDC) presented an average of 7.12 ± 3.51 mg dl^{-1}, but the DTA group showed high values of average, presenting 442.33 ± 44.6 mg dl^{-1}. When ECb and GCb treated groups are compared to DTA, a significant reduction ($p < 0.001$) in glucose excretion could be observed after treatment, with reductions of 42.80% and 34.33% respectively. The group treated with GBC also presented a significant reduction with $p < 0.001$, representing a decrease of 64.83%. The evaluation of blood glucose (Fig. 4) showed that the NDC group presented a glycemia mean of 160.77 ± 08.6 mg dl^{-1} whereas DTA group presented very high blood glucose levels with a mean of 455.08 ± 18.4 mg dl^{-1}. When compared to the GBC groups, GCb and ECb with the DTA group after treatment, it was observed that there was a significant reduction in blood glucose levels with $p < 0.001$, corresponding to 32.19% reductions, 18.88% and 20.89%, respectively.

Effect of treatments on biochemical parameters

Biochemical results demonstrated that DTA group presented significant increase ($p < 0.001$) in practically all biochemical parameters when compared to the NDC group, with the exception only of total proteins that presented significant reduction and HDL that did not show abnormal values (Table 2). Treatments with GCb and ECb were able to significantly reduce ($p < 0.001$) levels of triacylglycerides, cholesterol and LDL, as well as reduced transaminases

Table 1
Effect of treatments for 30 days on MD clinical parameters in animals induced with streptozotocin (weight development, polyphagia, polydipsia and polyuria).

Clinical parameters	Groups				
	NDC	DTA	GBC	GCb	ECb
Body weight (g/day)	275 ± 6.4[a]	221 ± 5.8	247 ± 5.1[a]	261 ± 7.3[a]	252 ± 6.4[a]
Consumption Ration (g/day)	30 ± 5.2[b]	40 ± 4.1	35 ± 3.3	36 ± 3.7	37 ± 5.5
Water intake (ml/day)	46 ± 5.3[a]	122 ± 15.2	71 ± 13.4[a]	68 ± 8.1[a]	64 ± 9.7[a]
Urine volume (ml/day)	12 ± 5.7[a]	103 ± 8.9	38 ± 6.2[a]	46 ± 5.8[a]	48 ± 6.5[a]

The data represent mean ± SD (n = 5/group).
[a] $p < 0.001$ represents a statistically significant results compared with the DTA group.
[b] $p < 0.05$ represents a statistically significant results compared with the DTA group.

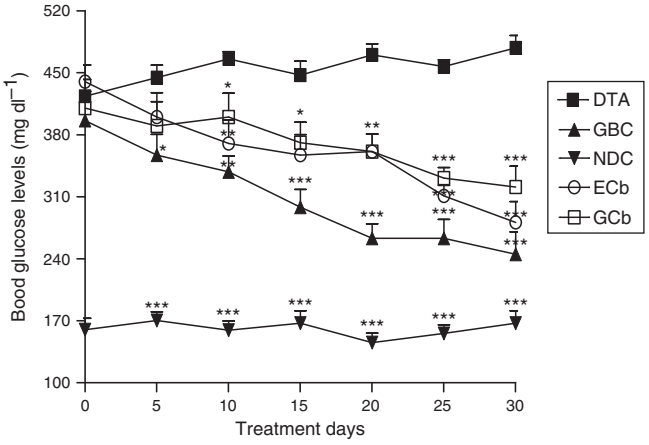

Fig. 4. Effect of treatments on the blood glucose levels of diabetic rats and non-diabetic. Significance: $^{*}p < 0.05$ and $^{**}p < 0.01$, $^{***}p < 0.001$. Compared with DTA group. Values express the mean ± SD (n = 5/group).

levels, creatinine and urea, acted increasing serum levels of total protein. In the group treated with GBC was also possible to observe significant improvement ($p < 0.001$) in the biochemical parameters, with the exception of transaminase AST.

Discussion

Several studies have been developing and standardizing formulations based on plant extracts in order to make such extracts a more technological pharmaceutical product and optimize its pharmacological effects (Linden et al., 2000; Sartori et al., 2003; Carvalho et al., 2013).

In this study, the ECb and GCb formulations were standardized as the determination of polyphenols and total tannins, where showed high content of these substances collaborating with the results obtained by Carvalho et al. (2013), but the granule production technique (GCb) from ECb demonstrated a slight decrease of 1.36% in this content, this decrease was possibly due to the great oxidative ease of these phenolic substances (Robards et al., 1999).

The great antioxidant potential of medicinal plants especially those with large content of polyphenolic compounds has been proven, these antioxidant plants has gained an important role as a source of treatment for diseases that present high production of free radicals (FR), especially metabolic and genetic disorders related diseases as diabetes, dyslipidemia and cancer. Among the antioxidant phenolic compounds we can highlight, phenolic acids, flavonoids, tannins, coumarins and carotenoids (Marles and Farnsworth, 1995; Perez et al., 1998; Ojewole, 2002; Aslan et al., 2010).

The OTGT is a test that evaluates the ability of antidiabetic drugs to reduce sharply the postprandial glycemia, examples of drugs that reduce blood glucose in this test are those who work directly in the pancreas β-cells secreting insulin such as sulfonylureas or drugs that inhibit glucose absorption by the gastrointestinal tract and increase the sensitivity to insulin in peripheral tissues such as the biguanides (Souza et al., 2009).

According to Souza et al. (2009) in normoglycemic rats, the elevation of postprandial glycemia after glucose overload, and the consequent normalization to basal levels after about 120 min, featuring a normal function in glucose metabolism.

In OTGT it was possible to observe that both the ECb and the GCb presented effect on glucose metabolism, with a significant reduction of blood glucose when compared with DTA, standard GBC drug also significantly reduced, but this reduction did not lead to basal levels as observed in normoglycemic group (NDC), this reduced effect is possibly due to destruction of pancreatic β cells by the action of STZ, so there a is little amount insulin to be secreted by GBC effect.

The reduction of glycemia in diabetic rats by medicinal plants containing polyphenols and detected by OTGT has been described in several studies (Panda and Kar, 2007; Jia et al., 2009; Li et al., 2015) as well as the results obtained in this study using formulations of *C. brasiliense* species.

Table 2
Effect of treatments for 30 days on biochemical parameters of diabetes in STZ-induced animals. (Weight development, polyphagia, polyuria and polydipsia.).

Biochemical parameters	Groups				
	NDC	DTA	GBC	GCb	ECb
Total proteins (g/dl)	6.80 ± 0.12[a]	4.73 ± 0.27	6.89 ± 0.11[a]	7.03 ± 0.07[a]	6.93 ± 0.11[a]
Triacylglycerides (mg/dl)	82.4 ± 8.8[a]	241.2 ± 24.7	163.4 ± 15.4[a]	131.7 ± 10.8[a]	159.4 ± 12.3[a]
Urea (mg/dl)	45.7 ± 4.9[a]	93.2 ± 13.2	62.3 ± 9.4[a]	64.8 ± 7.5[a]	59.5 ± 9.5[a]
Creatinine (mg/dl)	0.56 ± 0.03[a]	0.94 ± 0.08	0.64 ± 0.05[a]	0.69 ± 0.03[a]	0.59 ± 0.05[a]
Total Cholesterol (mg/dl)	59.7 ± 5.5[a]	165.1 ± 16.4	115.5 ± 11.1[a]	98.3 ± 9.3[a]	96.4 ± 11.6[a]
HDL (mg/dl)	37.1 ± 4.6	34.1 ± 8.9	30.3 ± 6.8	39.8 ± 6.1	35.6 ± 7.2
LDL (mg/dl)	20.1 ± 6.1[a]	72.7 ± 7.5	52.5 ± 6.6[a]	32.2 ± 5.9[a]	28.9 ± 6.3[a]
AST (U/dl)	54.5 ± 4.4[a]	79.2 ± 6.7	75.7 ± 3.6	59.3 ± 4.8[a]	65.9 ± 5.1[b]
SGPT (U/dl)	65.4 ± 6.7[a]	94.3 ± 5.4	76.6 ± 6.1[b]	79.1 ± 7.1[b]	74.3 ± 6.3[a]

The data represent the mean ± standard deviation (n = 5/group).
[a] $p < 0.001$ represents a statistically significant results compared with the DTA group.
[b] $p < 0.05$ represents a statistically significant results compared with the DTA group.

Authors report several mechanisms of action proposed for plants containing polyphenolic compound and these can involve, protection of the pancreatic β-cells from oxidative damage, increased insulin secretion, increased sensitivity of peripheral tissues in response to insulin and reduced gastrointestinal glucose absorption (Sezik et al., 2005; Panda and Kar, 2007).

In diabetic individuals, lack of insulin leads to various clinical signs and symptoms, including chronic hyperglycemia, elevated blood glucose level in urine, constant thirst (polydipsia), increased urination (polyuria), weight loss, and severe starvation (polyphagia) (Mahendran et al., 2014).

Insulin deficiency causes hyperglycemia and when the blood glucose level is higher than the renal filtration threshold, there is the presence of glucose in urine, as well as increased excreted urine volume due to osmotic imbalance, hyperosmolarity, because of high levels of circulating glucose, causes water to pass from intracellular to extracellular medium in order to maintain this osmotic equilibrium, intracellular dehydration is recognized by brain osmoreceptors generating a response triggering intense thirst, characteristic of diabetes (Lerco et al., 2003; Mahendran et al., 2014).

Insulin is a hormone that facilitates the glucose transport into muscle cells and adipocytes, increases the synthesis and storage of cellular proteins, muscle glycogen and triacylglycerides in adipocytes, and decrease protein catabolism (May and Buse, 1989). Lack of insulin causes intense catabolism process of structural proteins and β-oxidation of fatty acids to form subproducts to gluconeogenesis, the loss or breakdown of these structural proteins directly reflect in reduced body weight (Ramesh and Pugalendi, 2006), this process can be observed in diabetic animals treated with distilled water (DTA).

Treatment for 30 days with ECb and GCb formulations significantly reduced hyperglycemia (20.89% and 18.88%) and urine glucose (42.80% and 34.33%) of the diabetic rats respectively, and acted also reducing polydipsia, polyuria and loss of body mass.

The improvement of symptoms and clinical signs during 30 days of treatment with ECb and GCb, reinforce the antidiabetic activity of Cb species, as possibly this improvement is due to the high content of polyphenolic compounds, where this substances would act restoring the β-pancreatic cells from oxidative damage caused by STZ and consequently increasing the insulin production (Sezik et al., 2005), these results confirm the results obtained by several authors, who conclude that natural antioxidant substances such as polyphenolic compounds have great potential for anti-diabetic activity (Sabu et al., 2002; Hou et al., 2003; Panda and Kar, 2007; Jia et al., 2009; Alade et al., 2012).

Liver and kidneys are the main organs responsible for metabolism and excretion of endogenous substances and xenobiotics, the dysfunction of these organs leads to changes in biochemical parameters, being the markers transaminases AST and ALT who determine hepatocytes damage and elevation of creatinine and urea indicating renal dysfunction (Almdal and Vilstrup, 1988; Ohaeri, 2001).

In DTA group, a large increase in levels of AST, ALT, creatinine and urea were observed when compared to the NDC group, these results indicate that the animals in the DTA group show possible liver and kidney dysfunction. These changes are justified because the induction of diabetes by streptozotocin and the resulting chronic hyperglycemia, are factors that lead to formation of reactive oxygen species (ROS), which in turn causes lipid peroxidation and damage to cell membranes, these ROS are responsible by secondary complications of diabetes mellitus such as kidney, liver, retina, blood vessels and nerve damage (Hunt et al., 1988).

Treatment of the animals using ECb and GCb were able to significantly reduce ($p < 0.001$ and $p < 0.05$) creatinine, urea, AST and ALT levels, these results demonstrate that there was a significant reduction in liver damage and are in line with results obtained by (Ohaeri, 2001; Ramesh et al., 2010). The improvement of renal dysfunction can be justified by the increase in total protein levels, as in diabetic individuals nephropathy is the main factor for protein excretion in urine, these results are consistent with those obtained by Bakris (1993) and Tuvemo et al. (1997).

The results of renal and hepatic biochemical parameters also reinforce the lack of toxicity of the doses of the formulations used in this study, and corroborate with the results obtained by Oliveira et al. (2014), which demonstrate that the treatment for 30 days with 500 mg/kg of ECB, no showed signs of toxicity in rats.

Diabetes is a disease that has a great influence on lipid metabolism causing increases in serum triacylglycerides, cholesterol and lipoproteins, this fact can be observed in DTA group where is possible to see a very significant increase ($p < 0.001$) in triacylglyceride, cholesterol and LDL serum values. Treatment with ECb and GCb were able to reduce the increase of the triacylglycerides levels, cholesterol and LDL, but there was no significance in the results of HDL, similar results were obtained with experimental diabetic mice after treatment with plant extracts containing polyphenols, where a significant reduction in lipid levels could be observed (Ramadan et al., 2009; Islam, 2011).

The increase in lipid levels on diabetes are mainly responsible for mediating the formation of RL by peroxidation of unsaturated fatty acids, cholesterol and lipoproteins, increased lipid peroxidation leads to membrane damage and consequently organs dysfunction being this an important risk factor for atherosclerosis and coronary artery disease (Maghrani et al., 2004; Alfy et al., 2005).

Decrease on lipid levels and consequently the reduction of lipid peroxidation is improved due to the high antioxidant potential of polyphenolic compounds that act by mechanisms of reaction inhibition in the peroxidation chain and can reduce complications resulting from diabetes (Kamalakkannan and Prince, 2006; Mahendran et al., 2014).

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

HOC, BSFS, IVFS, and HK contributed to the preparation of formulations and execution of experimental tests. HOC, RLR, CPF and JCTC, contributed execution of experimental tests and to development and critical reading of the manuscript. All authors read and approved the final manuscript submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

We thank the Research Support Foundation of the State of Amapá for its financial support through project PPSUS

EFP-00007609, the CAPES for the grant bestowed, and Guyamazon Project (FAPEAP – AIRD) and CNPq Process number 407768/2013-0.

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