

# ***Combretum lanceolatum* flowers extract shows antidiabetic activity through activation of AMPK by quercetin**

**Carlos Roberto Porto Dechandt, Juliany Torres Siqueira, Damiana Luiza Pereira de Souza, Lais Cobianchi Junqueira Araujo, Virginia Claudia da Silva, Paulo Teixeira de Sousa Junior, Claudia Marlise Balbinotti Andrade, Nair Honda Kawashita, Amanda Martins Baviera\***

Departamento de Química, Universidade Federal de Mato Grosso, Brazil.

**Abstract:** The present study evaluated the antidiabetic activity of the *Combretum lanceolatum* Pohl ex Eichler, Combretaceae, flowers extract (ClEtOH) in diabetic rats. Streptozotocin-diabetic rats were divided into four groups: diabetic control, diabetic treated with 500 mg/kg of metformin and diabetic treated with 250 or 500 mg/kg of ClEtOH for 21 days. The treatment of diabetic rats with 500 mg/kg of ClEtOH promoted an increase in the weight of liver, white adipose tissues and skeletal muscles, improving body weight gain. Diabetic rats treated with 500 mg/kg of ClEtOH also presented reduction in glycemia, glycosuria and urinary urea levels, and increase in liver glycogen content. HPLC chromatogram showed that quercetin is the major compound in the extract. The phosphorylation levels of adenosine monophosphate-activated protein kinase were increased in liver slices incubated in vitro with 50 µg/mL of ClEtOH, similarly to the incubation with metformin (50 µg/mL) or quercetin (10 µg/mL). The antihyperglycemic effect of ClEtOH was similar to that of metformin and appears to be through inhibition of gluconeogenesis, since urinary urea was reduced and skeletal muscle mass was increased. These data indicate that the antidiabetic activity of the *Combretum lanceolatum* extract could be mediated, at least in part, through activation of adenosine monophosphate-activated protein kinase by quercetin.

## Article

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## Introduction

Diabetes mellitus, a chronic metabolic disease characterized by a deficiency in the pancreas insulin production and/or by peripheral insulin resistance, can be referred as a global epidemic disease; data from the World Health Organization (WHO) estimate that this disorder affected 285 million people worldwide in 2010 and projections rise to 439 million in 2030 (Shaw et al., 2010). In Brazil, diabetes mellitus appeared as one of the ten main causes of deaths in Brazil in 2002 (WHO, 2006). Schmidt and collaborators (2011) have shown that the prevalence of diabetes in Brazil has been rising in association to obesity and to increased western diet and physical inactivity, affecting 5.3 % of Brazilians aged 20 years or older in 2008, in comparison to 3.3 % in 1998.

The adverse effects of hypoglycemic drugs and insulin and the excessive cost of these medications can be mentioned as some disadvantages regarding the diabetes treatment, which stimulate the search for new therapeutic agents that present safety, effectiveness and

low cost. Nowadays, there is growing trend towards using herbal preparations and/or derivatives in traditional and complementary medicine to treat diabetes symptoms (Yeh et al., 2003). In this way, it has been crescent the interest of current ethnopharmacological research to investigate the plants species with antihyperglycemic effect, focusing in the evaluation of the efficacy and safety of plant preparations for diabetes treatment, as well as the mechanisms of action that explain their antidiabetic activities (Kawashita & Baviera, 2010; Prabhakar & Doble, 2011).

*Combretum lanceolatum* Pohl ex Eichler, Combretaceae, commonly known as “pombeiro-vermelho”, is distributed from northern to southeastern of Brazil and is found in several phytogeographic domains including Amazon, Pantanal, Caatinga, Cerrado and Atlantic Forest (Marquete & Valente, 2010). Ethnopharmacological studies have demonstrated that plants from *Combretum* genus presented antidiabetic activity. The six weeks treatment of streptozotocin-diabetic rats with ethanolic extract of *Combretum decandrum* Jacq. leaves decreased fasting blood glucose levels (Pannangpetch et al.,

2008). Chika & Bello (2010) showed that *Combretum micranthum* G. Don leaves aqueous extract treatment improved glucose tolerance and reduced fasting glycemia in both normal and alloxan-diabetic rats. The treatment of streptozotocin-diabetic rats with mollic acid glucoside, a hydroxycycloartenoid isolated from leaves of *Combretum molle* R. Br. ex G. Don, reduced glycemia levels in a dose-dependent manner (Ojewole & Adewole, 2009). Phenolic compounds, especially flavonoids, are widely reported in *Combretum* genus, and they are probably the responsible for its antihyperglycemic activity (Eloff et al., 2004; Lopes et al., 2010). These findings stimulate the present study, since there is no systematic study attempting to the investigation of the antidiabetic activity of *C. lanceolatum*, one of the most important species of Combretaceae in the Brazilian Pantanal (Pott et al., 2011). In this way, our laboratory initiated the evaluation of the antidiabetic properties of several parts (flowers, fruits, leaves) of this plant, attempting to its further indication in phytotherapeutic formulations. The present study was undertaken to assess the subchronic antidiabetic activity of the *C. lanceolatum* flowers ethanolic extract (CIEtOH) in streptozotocin-diabetic rats.

## Materials and Methods

### *Plant material collection and preparation of the CIEtOH extract*

Flowers of *Combretum lanceolatum* Pohl ex Eichler, Combretaceae, were collected in Poconé Porto Cercado road (km 10), Poconé-MT, Brazil (S 16°18'56.4"; W 056°32'21.5"; 126 m of elevation) in July 2010. The access to plant samples was authorized by Conselho de Gestão do Patrimônio Genético of Ministério do Meio Ambiente (license numbers 010457/2010-0). The plant material was identified by Dr. Germano Guarim Neto, Central Herbarium, Universidade Federal de Mato Grosso, where a voucher specimen (numbers 39,149) was deposited for future reference. The flowers of *C. lanceolatum* were dried at room temperature and grounded in electric grinder. Later, 5,960 kg of the botanical material was placed in maceration with ethanol at room temperature under occasional shaking, in seven cycles of seven days. The mixture was then filtered and concentrated on the rotary evaporator at reduced pressure and 38 °C approximately, obtaining the crude ethanol extract of *C. lanceolatum* - CIEtOH (2,350 kg; 39.43 % w/w).

### *Preparation of sample solutions*

Quercetin (Riedel-de Haën, Germany) standard stock solution and CIEtOH stock solution were prepared in methanol (1.0 mg/mL and 5.0 mg/ml respectively). For the sample fortification quercetin and CIEtOH methanol

solutions were mixed in the ratio of 2:1. All sample solutions were filtered through 0.45 µm membrane filter (Millipore, USA), and injected directly into the HPLC-UV for analysis.

### *Chromatographic analysis*

High performance liquid chromatographic analyses were carried out on a Varian ProStar liquid chromatograph, equipped with binary gradient pumping and a ProStar 325 Dual Wavelength UV-Vis Detector (Varian). A HICHROM 5C18 reversed-phase column (ø 4.6 mm×250 mm) packed with 5 µm diameter particles with a Kromasil® 100-5C18 guard column (4.6 mm×10 mm i.d., 5 µm) was employed. As the mobile phase, a gradient of methanol-water containing 0.1% trifluoroacetic acid (50-80% in 35 min) was employed. The detections were performed at 368 nm. Flow rate and injection volume were 0.5 mL/min and 10 µL, respectively. The presence of quercetin was confirmed by comparing its retention time and by fortification of the extract with standard quercetin. All chromatographic operations were carried out at ambient temperature.

Classical column chromatography on silica gel and preparative thin layer chromatography were performed in CIEtOH, affording the isolation of quercetin, among other flavonoids, as a majoritary compound. The details will be published elsewhere (Araujo, 2012).

### *NMR analyses*

NMR analyses were recorded in CD<sub>3</sub>OD, using TMS as internal standard in a Bruker spectrometer (500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C).

### *Animals*

Male Swiss-Webster mice weighing 25-30 g (acute toxicity study) and male Wistar rats weighing 180-210 g (subchronic antidiabetic activity) were housed in a room under standard laboratory conditions (12:12 h light-dark cycle, 24±1 °C) and had free access to water and commercial lab chow diet (Purina®Labina). During the experiments of the antidiabetic activity, rats were housed in individual metabolic cages. All experiments took place between 8 and 10 am. Experimental procedures were made according to the Brazilian College of Animal Experimentation and received prior institutional approval by the Committee for Ethics in Animal Experimental from UFMT (protocol number 23108.029613/09-3).

### *Acute oral toxicity study (Hippocratic test)*

Groups of male mice received orally by gavage a single administration of CIEtOH at crescent doses

(100; 250; 500; 1,000; 2,500 and 5,000 mg/kg). The control group received vehicle (water). The animals were individually observed at 0, 5, 10, 15, 30 min; 1, 2, 4 and 8 h and after four and fifteen days (once a day) following the extract administration. General behavioral observations were noted in the table described by Malone (1977).

#### *Subchronic evaluation of extract in streptozotocin-induced diabetic rats*

Streptozotocin (STZ, 40 mg/kg) dissolved in 0.01 mol/L citrate buffer (pH 4.5) was administered through a single intravenous injection in 15 h fasted rats. Five days after STZ administration, rats with glycemia levels of approximately 400 mg/dL were selected to the experiments and randomly assigned into four groups: DC, diabetic control rats treated with vehicle (water); DMet, diabetic rats treated with 500 mg/kg of metformin; DT250, diabetic rats treated with 250 mg/kg of CIEtOH; DT500, diabetic rats treated with 500 mg/kg of CIEtOH. The groups received vehicle, metformin or freshly prepared extract by oral gavage for 21 days. Body weight, food and water intake and urinary volume were daily monitored. At every five days, plasma glucose levels were determined (Bergmeyer et al., 1974), as well as the urea (Bernt & Bergmeyer, 1965) and glucose urinary levels (Summerson et al., 1947) in 24 h urine. At the end of the treatment, white adipose tissues (retroperitoneal, perirenal and epididymal), skeletal muscles (soleus and extensor digitorum longus - EDL) and liver were removed and weighted. Liver glycogen was extracted with 30% KOH and precipitated with ethanol (Sjörgren et al., 1938) and the quantity recovered was measured by colorimetric phenol-sulfuric acid method (Montgomery, 1957).

#### *Incubation procedure and Western blotting analysis*

Measurement of the adenosine monophosphate-activated protein kinase (AMPK) activation was performed in liver slices incubated *in vitro* in the presence of CIEtOH, quercetin or metformin. Rats were killed, liver was rapidly dissected and uniform-shaped liver slices (1 mm thick) were obtained from the right lobe with a tissue chopper (Dogterom, 1993; Bach et al., 1996). Liver slices were incubated during 1 h at 37 °C in Krebs-Ringer bicarbonate buffer (pH 7.4) equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub> and containing glucose (5 mM) in the absence (basal sample) or in the presence of CIEtOH (50 and 100 µg/mL), quercetin (10 and 50 µg/mL) or metformin (10 and 50 µg/mL). After incubation, tissues were immediately frozen in liquid nitrogen before Western blotting analysis. Following, tissues were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10

mM sodium orthovanadate, 5 µg/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride; supernatants samples were used for protein levels determination (Bradford, 1976) and for electrophoresis separation. Samples (100 µg of protein) were subjected to SDS-PAGE analysis on 10% acrylamide gels (Laemmli, 1970). Gels were electroblotted onto nitrocellulose membranes (Towbin et al., 1979) and blotted with anti-AMPK $\alpha$  (1:1,000, Cell Signaling Technology, USA) and anti-phospho-[Thr172]-AMPK $\alpha$  (1:1,000, Cell Signaling Technology, USA). Primary antibody was detected by peroxidase-conjugated secondary antibody (1:7,500, Santa Cruz Biotechnology, USA) and visualized with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, USA). Band intensities were quantified using the ImageJ Program (Version 1.38, National Institutes of Health, USA, 2004).

#### *Statistical analysis*

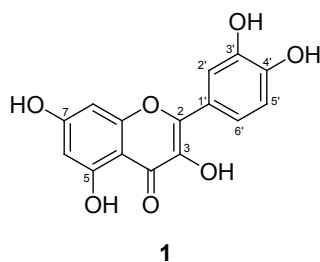
Data were expressed as mean $\pm$ SEM. One-way analysis of variance followed by Tukey's multiple comparison tests was used to analyze the differences between treated and control groups. Unpaired t test was used to compare the means of AMPK phosphorylation values from liver slices incubated under different conditions with basal sample. Differences were considered significant at  $p < 0.05$  and  $p < 0.01$ .

## Results

#### *Isolation and identification of quercetin in CIEtOH*

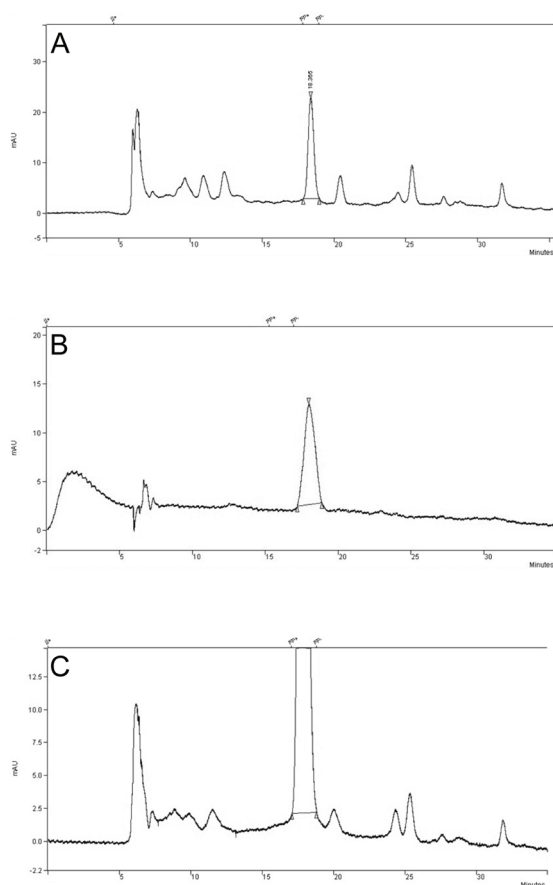
The HPLC chromatogram (Figure 1A-C) has shown that the flavonoid quercetin seems to be the major compound in CIEtOH, and its presence was evidenced by fortification with standard quercetin solution, through the increase in peak area at 18.365 min. Furthermore, quercetin (**1**) has been isolated from CIEtOH through classical column chromatographic fractionation (Araujo, 2012) and identified by <sup>1</sup>H and <sup>13</sup>C NMR experiments and comparison with literature data (Adeyemi et al., 2010) and an authentic sample.

*Quercetin (1)*: (256 mg; 0.039 %) yellow crystals, mp. 318-319 °C (315°C; [29]), <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): 7.75 (d,  $J_{2',6'}$ , 2.05 Hz, H-2'), 7.65 (dd,  $J_{6',2'}$ , 2.05 Hz and  $J_{6',5'}$ , 8.5 Hz, H-6'), 6.90 (d,  $J_{5',6'}$ , 8.5 Hz, H-5'), 6.40 (d,  $J_{8,6}$ , 1.95 Hz, H-8), 6.20 (d,  $J_{6,8}$ , 1.95 Hz, H-6). <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD): 147.4 (C-2), 135.8 (C-3), 175.9 (C-4), 161.1 (C-5), 97.8 (C-6), 164.2 (C-7), 93.0 (C-8), 156.8 (C-4a), 103.1 (C-8a), 122.7 (C-1'), 114.6 (C-2'), 144.8 (C-3'), 146.6 (C-4'), 114.8 (C-5'), 120.3 (C-6').



### Hippocratic test

In the acute toxicity assay, it was demonstrated that the treatment with CIeOH in doses ranging from 100 to 5,000 mg/kg did not promote any behavioral changes or mortality in animals. The doses of 250 and 500 mg/kg were selected for the evaluation of the antidiabetic activity of CIeOH in the subchronic experiment.



**Figure 1.** A. Chromatographic profile of *Combretum lanceolatum* flowers ethanolic extract (CIeOH); B. quercetin standard; and C. CIeOH with quercetin standard. Analysis in HPLC-UV with detection at 368 nm.

### Improvement in body and tissues weights of diabetic rats treated with CIeOH

#### Body weight

At the beginning of the experiment, rats from different groups showed similar body weight values. The treatment of diabetic rats with 500 mg/kg of CIeOH promoted a 2-fold increase in the diary body weight gain when compared to DC group (Table 1), leading to an increase (21%) in the final body weight (Table 1, Figure 2A). The body weight in DT500 group was higher in comparison to the beginning since the 12<sup>th</sup> day of treatment until the end of the experiment. Although the animals treated with metformin or with 250 mg/kg of CIeOH presented a daily body weight gain significantly higher than controls, the final body weight of animals from these groups was not statistically different of values from untreated animals (Table 1, Figure 2A).

#### Weight of peripheral tissues

It was observed an increase of 20% in the weight of liver from diabetic rats treated with CIeOH, in both doses, as well as in metformin-treated rats, when compared to DC. The treatment of diabetic rats with 500 mg/kg of CIeOH also promoted increase in the weight of epididymal (92%) and retroperitoneal (4-fold) adipose tissues and of soleus (28%) and EDL (29%) muscles (Table 2), which corroborated with the increased body weight gain of animals from DT500 group.

#### Reduction on food intake, liquid intake and urinary volume of diabetic rats treated with CIeOH

There were no changes in the daily values of food and water intake or urinary volume between DT250 and DC groups (Table 1). The treatment with 500 mg/kg of CIeOH promoted decrease of 14, 33 and 39%, respectively, in daily food intake, water intake and urinary volume in comparison to DC group (Table 1, Figure 2B, 2C and 2D). As expected, the metformin-treated rats also presented a decrease in daily values of food and water ingestion and of urinary volume in comparison to controls. The food and water ingestion and the urinary volume of diabetic rats treated with 500 mg/kg of CIeOH were not different to values of metformin-treated rats, showing that plant extract improved these parameters likewise metformin.

#### Treatment with CIeOH ameliorates biochemical parameters of diabetic rats

#### Plasma glucose

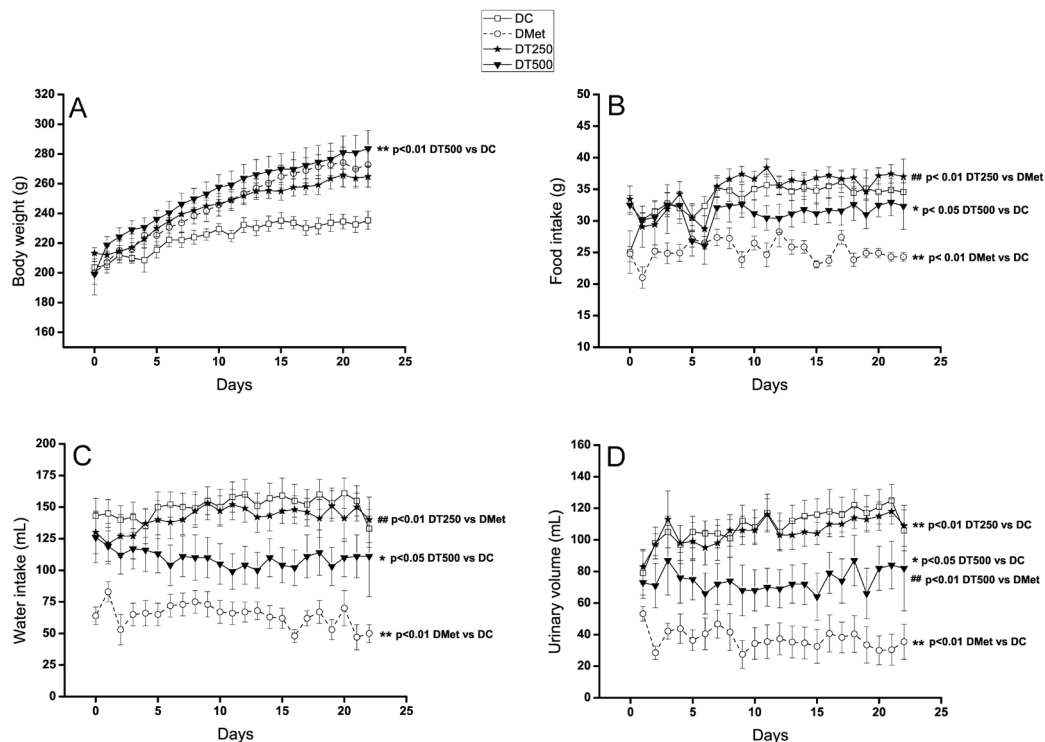
Prior to treatment beginning, all the groups presented blood glucose levels at very similar values (DC =  $493.0 \pm 17.6$ ; DMet =  $505.4 \pm 56.36$ ; DT250 =  $473.3 \pm 17.22$ ; DT500 =  $484.9 \pm 27.77$ , mg/dL, n=8-9 animals). At the end of the treatment, diabetic animals that received CIEtOH presented a reduction of 16% (250 mg/kg) and 22 % (500 mg/kg) in the glycemia levels when compared to non-treated rats. As expected, the glycemia levels were

also reduced in diabetic rats treated with metformin, in comparison to DC and DT250 groups (DC =  $505.6 \pm 17.50$ ; DMet =  $338.4 \pm 13.53$ ; DT250 =  $426.8 \pm 6.62$ ; DT500 =  $391.6 \pm 21.13$ , mg/dL, n=8-9 animals). It is important to highlight that the glycemia values from DT500 and DMet groups were similar at the end of the experiment. In the same way, the area under the curve (AUC) of glycemia from DMet, DT250 and DT500 groups were also lower than controls (Figure 3).

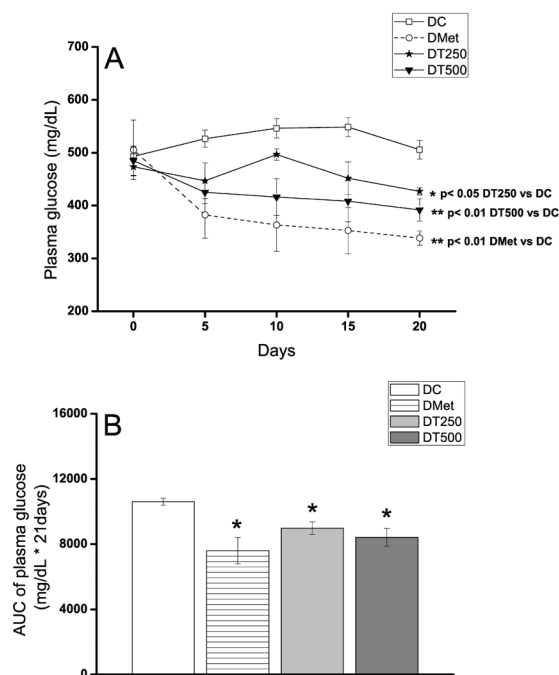
**Table 1.** Values of body weight, food intake, water intake and urinary volume from diabetic rats treated during 21 days with 500 mg/kg of metformin (DMet) and with 250 (DT250) or 500 mg/kg (DT500) of *Combretum lanceolatum* flowers ethanolic extract.

	DC (n= 8)	DMet (n= 9)	DT250 (n=9)	DT500 (n=8)
Initial body weight (g)	203.7 $\pm$ 3.91	200.0 $\pm$ 7.64	213.2 $\pm$ 3.77	199.2 $\pm$ 14.03
Final body weight (g)	235.4 $\pm$ 6.03	269.9 $\pm$ 11.66	264.8 $\pm$ 7.08	283.7 $\pm$ 12.19**
Daily weight gain (g)	1.45 $\pm$ 0.30	3.33 $\pm$ 0.41**	2.60 $\pm$ 0.32*	3.44 $\pm$ 0.35**
Daily food intake (g)	35.1 $\pm$ 1.12	25.3 $\pm$ 0.84**	34.9 $\pm$ 1.40###	30.1 $\pm$ 1.64*
Daily water intake (mL)	151.3 $\pm$ 10.70	64.1 $\pm$ 6.60**	142.3 $\pm$ 10.79###	100.7 $\pm$ 13.03*
Daily urinary volume (mL)	109.2 $\pm$ 9.61	36.8 $\pm$ 8.31**	105.1 $\pm$ 8.15###	66.1 $\pm$ 12.11*.,###

Values represent mean $\pm$ SEM; n: number of animals; \* $p < 0.05$  and \*\* $p < 0.01$  vs DC; ### $p < 0.01$ . vs DMet.



**Figure 2.** Physiological parameters of diabetic rats non-treated (DC) and treated during 21 days with 500 mg/kg of metformin (DMet) and with 250 (DT250) or 500 mg/kg (DT500) of *Combretum lanceolatum* flowers ethanolic extract. A. Body weight, g; B. food intake, g; C. water intake, mL; D. urinary volume, mL. Each point represents mean $\pm$ SEM of 8-9 animals. \* $p < 0.05$  and \*\* $p < 0.01$  vs DC; ### $p < 0.01$  vs DMet.

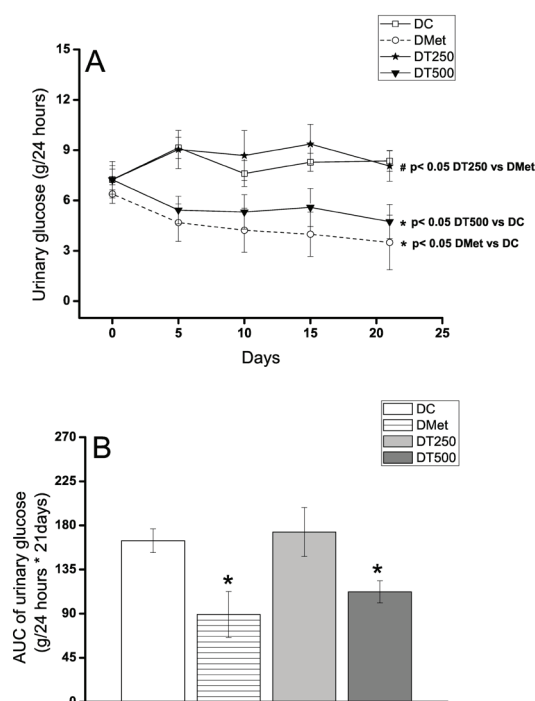


**Figure 3.** Plasma glucose levels (mg/dL) during the experiment (A) and area under the curve of glycemia (B) from diabetic rats non-treated (DC) and treated during 21 days with 500 mg/kg of metformin (DMet) and with 250 (DT250) or 500 mg/kg (DT500) of *Combretum lanceolatum* flowers ethanolic extract. Each point represents mean $\pm$ SEM of 8-9 animals. \* $p$ <0.05 and \*\* $p$ <0.01 vs DC.

#### Glycosuria and urinary urea

At the end of the experiment, the urinary glucose (Figure 4) and urea levels (Figure 5) of diabetic rats treated with 500 mg/kg of ClEtOH were, respectively, 43% and 38% lower than values from diabetic non-treated rats. The glycosuria and urinary urea levels from diabetic rats treated with 250 mg/kg of ClEtOH were not different of

values from DC group. As expected, the glycosuria values from rats treated with metformin were also lower than values from DC and DT250 groups, as well as the urinary urea levels (Figures 4 and 5). There were no differences in the urinary glucose and urea levels between DT500 and DMet groups. The AUC of glycosuria and urinary urea from diabetic rats treated with 500 mg/kg of ClEtOH were, respectively, 32% and 23% lower than values from DC group (Figures 4 and 5).

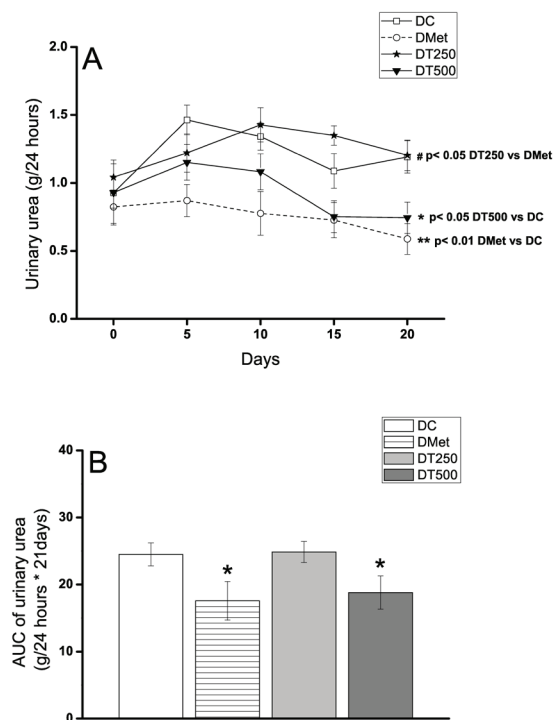


**Figure 4.** Urinary glucose levels (g/24h) during the experiment (A) and area under the curve of urinary glucose (B) from diabetic rats non-treated (DC) and treated during 21 days with 500 mg/kg of metformin (DMet) and with 250 (DT250) or 500 mg/kg (DT500) of *Combretum lanceolatum* flowers ethanolic extract. Each point represents mean $\pm$ SEM of 8-9 animals. \* $p$ <0.05 vs DC; # $p$ <0.05 vs DMet.

**Table 2.** Weight of white adipose tissues (epididymal, retroperitoneal and perirenal), skeletal muscles (EDL and soleus) and liver from diabetic rats treated during 21 days with 500 mg/kg of metformin (DMet) and with 250 (DT250) or 500 mg/kg (DT500) of *Combretum lanceolatum* flowers ethanolic extract.

	DC (n= 9)	DMet (n= 8)	DT250 (n=8)	DT500 (n=8)
Epididymal (g)	0.920 $\pm$ 0.116	1.871 $\pm$ 0.244**	1.334 $\pm$ 0.091	1.765 $\pm$ 0.228**
Retroperitoneal (g)	0.278 $\pm$ 0.077	1.210 $\pm$ 0.274*	0.733 $\pm$ 0.111	1.116 $\pm$ 0.260*
Perirenal (g)	0.146 $\pm$ 0.074	0.240 $\pm$ 0.054	0.132 $\pm$ 0.017	0.254 $\pm$ 0.064
EDL (g)	0.209 $\pm$ 0.008	0.242 $\pm$ 0.021	0.244 $\pm$ 0.008	0.270 $\pm$ 0.026*
Soleus (g)	0.252 $\pm$ 0.012	0.269 $\pm$ 0.012	0.279 $\pm$ 0.018	0.322 $\pm$ 0.019*
Liver (g)	10.78 $\pm$ 0.293	12.83 $\pm$ 0.782*	12.90 $\pm$ 0.406*	12.67 $\pm$ 0.687*

Values represent mean $\pm$ SEM; n: number of animals; \* $p$ <0.05 and \*\* $p$ <0.01 vs DC.



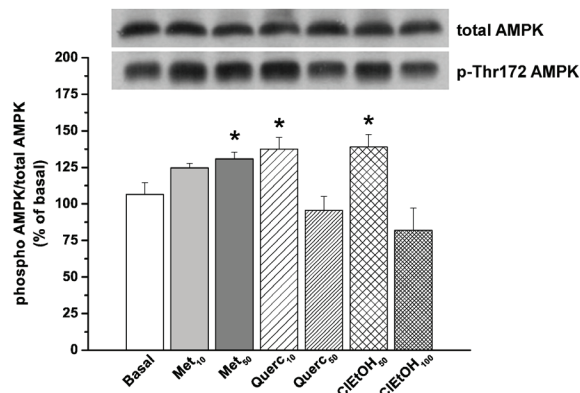
**Figure 5.** Urinary urea levels (g/24h) during the experiment (A) and area under the curve of urinary urea (B) from diabetic rats non-treated (DC) and treated during 21 days with 500 mg/kg of metformin (DMet) and with 250 (DT250) or 500 mg/kg (DT500) of *Combretum lanceolatum* flowers ethanolic extract. Each point represents mean $\pm$ SEM of 8-9 animals. \* $p$ <0.05 and \*\* $p$ <0.01 vs DC; # $p$ <0.05 vs DMet.

#### Liver glycogen content

The liver glycogen content was 90% higher in diabetic animals treated with 500 mg/kg of ClEtOH in comparison to DC group (DC = 11.36 $\pm$ 1.17; DMet = 19.00 $\pm$ 4.42; DT250 = 20.39 $\pm$ 4.62; DT500 = 21.58 $\pm$ 4.21, mg/g, n = 8-9 animals).

#### ClEtOH promotes in vitro AMPK activation in rat liver slices

As expected, the incubation of liver slices with 50  $\mu$ g/ml metformin promoted an increase (23%) in the phosphorylation levels of AMPK. There was also observed a 29% raise in the AMPK phosphorylation when liver slices were incubated with 10  $\mu$ g/mL quercetin. Incubation of liver slices with 50  $\mu$ g/mL of ClEtOH resulted in an increase in the phosphorylation levels of AMPK (31%) in a similar magnitude of that observed with metformin or quercetin. No changes were observed in the AMPK phosphorylation after incubation with metformin, quercetin and ClEtOH in the doses of 10, 50 and 100  $\mu$ g/mL, respectively (Figure 6).



**Figure 6.** Phosphorylation levels of AMPK (% of basal values) in liver slices incubated in the presence of metformin [10  $\mu$ g/mL (Met10) and 50  $\mu$ g/mL (Met50)], quercetin [10  $\mu$ g/mL (Querc10) and 50  $\mu$ g/mL (Querc50)] and *Combretum lanceolatum* flowers ethanolic extract [50  $\mu$ g/mL (ClEtOH<sub>50</sub>) and 100  $\mu$ g/mL (ClEtOH<sub>100</sub>)]. Values are means $\pm$ SEM of 3-5 liver slices. \* $p$ <0.05 vs basal.

#### Discussion

Crescent attention has been given to the investigation of the antidiabetic activity of many plant species all around the world, using different experimental models and several methodologies. However, information about the safety of plant-derived medicines is essential, considering that medicinal plants are easily acquired by the population and that many toxicological effects may be observed after the use of herbal preparations. In this way, it is common the use of mice in the experimental investigation of the acute toxic effects of plant extracts orally administered in high doses (Mu et al., 2011; Jesus et al., 2012). Data from this study showed that the *Combretum lanceolatum* Pohl ex Eichler, Combretaceae, extract did not have any acute toxicity in animals, at every tested dose. In front of the lack of toxicity, the doses of 250 and 500 mg/kg were chosen to the evaluation of the antidiabetic activity of *C. lanceolatum* flower extract in STZ-diabetic rats.

In insulin deficiency and/or resistance conditions, the hyperglycemia is a consequence of the combination of increased endogenous glucose production, related to the higher rates of glycogenolysis and gluconeogenesis processes, and reduced glucose uptake by peripheral tissues. It has been proposed that the search and/or development of new compounds that correct these glucose metabolism disturbances will be important to highlight new options for diabetes treatment (Wu et al., 2005; Loney & Tavaré, 2009).

Specifically to gluconeogenesis, it is well known that insulin deficiency lead to a marked skeletal muscle

proteolysis, increasing the flow of amino acids into the liver and providing substrates to gluconeogenesis. Since our results showed a reduction in the urinary urea values (Figure 5) and an increase in the weight of soleus and EDL skeletal muscles (Table 2), it is reasonable to speculate that the improvement in the carbohydrate metabolism of diabetic rats treated with *C. lanceolatum* could be explained, at least in part, by a decrease in the rate of hepatic gluconeogenesis, modulating the activity and/or gene expression of the rate-limiting enzymes of gluconeogenesis, as example phosphoenolpyruvate carboxykinase (PEPCK) and/or glucose-6-phosphatase (G6Pase). If *C. lanceolatum* extract exerts an antidiabetic activity through gluconeogenesis inhibition, its action can be related to that promoted by metformin, a classical hypoglycemic drug that lead to gluconeogenesis inhibition (Hundal & Inzucchi, 2003). Recent findings have described that the hypoglycemic effect of metformin occurs through activation of adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine protein kinase that exerts a central role in the regulation of cellular metabolism and energy balance (Hardie, 2007). Indeed, it was demonstrated that metformin downregulates PEPCK and G6Pase expression through liver AMPK activation (Lochhead et al., 2000; Zhou et al., 2001). Nowadays, AMPK activation has been pointed as a potential target for the treatment of diabetes and other metabolic disorders (Towler & Hardie, 2007; Viollet et al., 2007). In addition, the search of natural compounds that activate this kinase showed that some polyphenols activate AMPK during its beneficial effects on metabolic disorders, as example quercetin (Aguirre et al., 2011), curcumin (Aggarwal, 2010), epigallocatechin-3-gallate (Collins et al., 2007) and resveratrol (Shin et al., 2009). Therefore, it can be suggested that polyphenols are potential candidates of natural origin to diabetes treatment (Hwang et al., 2009), because they stimulate a cellular target similar to that of metformin. Corroborating with this, data from this study showed that AMPK phosphorylation levels were increased in liver slices incubated in the presence of *C. lanceolatum* extract (Figure 6) in a similar way of that observed with quercetin or metformin, showing that the antihyperglycemic effect of the extract could be attributed, at least in part, to AMPK stimulation followed by inhibition of gluconeogenesis process. Confirming this hypothesis, our results also demonstrated that the *in vivo* beneficial effects of the extract on several parameters were very similar to that promoted by metformin, as example reduction on glycemia (Figure 3), glycosuria (Figure 4) and urinary urea (Figure 5). Current investigations have been carried out in our laboratory to confirm if the *in vivo* AMPK activation is involved in the inhibition of hepatic gluconeogenesis by *C. lanceolatum* extract, through the analysis of the AMPK phosphorylation in liver from diabetic rats treated with the extract, as well as the changes in the PEPCK expression and in the hepatic

glucose production (Siqueira et al., 2012). Taken together, it is believed that quercetin, identified and isolated from *C. lanceolatum* extract (Figure 1), activates AMPK *in vivo*, contributing to the treatment of diabetes symptoms. Corroborating our findings, quercetin has been reported to be the active compound that explains the antidiabetic activity of some plant species (Eid et al., 2010; Veerapur et al., 2010). In fact, both *in vivo* and *in vitro* studies performed with quercetin have also demonstrated the beneficial effects of this flavonoid on several parameters altered in diabetes (Aguirre et al., 2011). In this way, the isolation of a potential antidiabetic compound in *C. lanceolatum* extract, together with the beneficial effects observed in STZ-diabetic rats treated with the extract reinforces our purpose, focused in the continuity of the studies with *C. lanceolatum*, obtaining sufficient data to justify its future indication into phytotherapeutic formulations to treat diabetes symptoms.

Because liver glycogen content was increased in diabetic rats after *C. lanceolatum* treatment, it is possible that the extract promotes decrease in liver glycogenolysis and/or increase in glycogenesis. In addition, since the extract treatment also promoted increase in the weight of adipose tissues (Table 2) and reduction in the food intake (Figure 2B) of diabetic rats, similar to metformin effects, it can be suggested that the extract promotes a better glucose disposal by peripheral tissues. Considering that quercetin, found in the extract, stimulates AMPK, a probable beneficial effect on glucose uptake promoted by *C. lanceolatum* can be referred as a metformin-like response, since this hypoglycemic drug stimulates glucose transporter type 4 (GLUT4) translocation to plasma membranes of peripheral tissues in an AMPK-dependent manner (Lee et al., 2011). These possibilities of the extract actions are the focus of current studies.

In summary, the findings of the present study indicated that the ethanolic extract of *C. lanceolatum* flowers has antihyperglycemic activity, as well as that quercetin is the major compound in the extract. The antidiabetic effect of *C. lanceolatum* can be attributed, at least in part, to the AMPK activation in liver by quercetin, in a similar manner of metformin, inhibiting hepatic glucose production. Further investigations are ongoing in our laboratory to detail the mechanisms of action that explains the antidiabetic effect of this plant extract.

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### Authors contributions

CRPD and JTS contributed in collecting plant sample, running the laboratory work and analysis of the data. DLPS running the laboratory work. LCA contributed to chromatographic analysis. CS, PTSJ, CMBA and NHK, co-designed experiments, discussed analyses and contributed to critical reading of the manuscript. AMB designed the study, supervised the laboratory work, analysis and interpretation of the data and drafted the paper.

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#### \*Correspondence

Amanda Martins Baviera  
Universidade Federal de Mato Grosso  
Av. Fernando Correa Costa, 2367, 78060 900 Cuiab -MT,  
Brazil  
baviera@ufmt.br  
Tel.: +55 65 3615 8765  
Fax: +55 65 3615 8798/99