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Research Paper

Hancornia speciosa Gomes (Apocynaceae) as a potential anti-diabetic drug

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ABSTRACT

Ethnopharmacological relevance: The leaves of *Hancornia speciosa* Gomes are traditionally used to treat diabetes in Brazil. The aim of the study is to evaluate the potential anti-diabetic effect of *Hancornia speciosa* extract and derived fractions.
Materials and Methods: The ethanolic extract from *Hancornia speciosa* leaves and chromatographic fractions thereof were evaluated on α-glucosidase assay, on hyperglycemic effect and glucose uptake. The chemical composition of the extract and its most active fraction was investigated by ESI–LC–MS.
Results: The ethanolic extract and derived fractions inhibited α-glucosidase in vitro. However, only the crude extract and the dichloromethane fraction inhibited the hyperglycemic effect induced by starch or glucose. Both the extract and dichloromethane fraction were also able to increase glucose uptake in adipocytes. Bornesitol, quinic acid, and chorogenic acid were identified in the extract, along with flavonoid glycosides, whereas the dichloromethane fraction is majorly composed by esters of lupeol and/or α/β-amirin.
Conclusions: Hancornia speciosa has a potential anti-diabetic effect through a mechanism dependent on inhibition of α-glucosidase and increase on glucose uptake. These results give support to the use on traditional medicine of this medicinal plant.

1. Introduction

Diabetes is defined as a metabolic disorder characterized by chronic hyperglycemia with disturbances of protein and fat metabolism resulting from defects in insulin secretion, insulin action, or both (WHO, 2013). Diabetes type 2 is the most frequent and has been evolved into a global epidemic (WHO, 2013).

Despite the great number of hypoglycemic drugs available, most of them exhibit undesirable side effects, high cost and ultimately cannot efficiently control alone the glycemia (Hung et al., 2012), unless if associated with the adoption of lifestyle measures, such as physical activity, control of a healthy body weight, avoid smoking and maintenance of a healthy diet (WHO, 2013).

Several plant species are traditionally used as anti-diabetic around the world and some of them have had their efficacy corroborated by pre-clinical and clinical assays (Hung et al., 2012; Chang et al., 2013; Mata et al., 2013). *Hancornia speciosa* Gomes (Apocynaceae), popularly known as mangabeira or mangaba, is a tree widely distributed in the *Cerrado* biome from Brazil. It is traditionally used as anti-diabetic, as anti-hypertensive and to treat obesity (Hirschmann and de Arias, 1990; Rodrigues and Carvalho, 2001; Macedo and Ferreira, 2004). The potential anti-hypertensive, chemopreventive, antioxidant and anti-inflammatory effects of *Hancornia speciosa* have been consistently demonstrated by our research group (Ferreira et al., 2007a; Ferreira et al., 2007b; Endringer et al., 2009; Endringer et al., 2010; Silva et al., 2011).

The lack of a scientific report supporting the large use of this plant for the treatment of diabetes led us to investigate the potential anti-diabetic effect of *Hancornia speciosa* using in vitro and in vivo methods.

2. Material and methods

2.1. Preparation of the extract and fractions

The leaves of *Hancornia speciosa* were furnished by Empresa Estadual de Pesquisa Agropecuária da Paraíba (EMEPA), João Pessoa,

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Brazil. The vegetal material was collected in April 2009 from the Active Germplasm Bank of Mangaba (BAG-Mangaba) in João Pessoa (geographic coordinates: 6° 33' 13" S and 34° 48' 31" W; altitude of 30 m). After drying at 40 °C for 72 h, the plant material was powdered (100 g) and percolated with 96% ethanol to afford a green dark residue (EEH, 23.45 g) after solvent elimination under reduced pressure. A portion of EEH (20 g) was submitted to chromatography on a silica gel 60 (0.2–5 mm) column (330 × 60 mm^2 i.d.) eluted with solvents of increasing polarity. After solvent elimination in a rotatory evaporator, the n-hexane (0.031 g), DCM (1.198 g), DCM/EtOAc; (1:1) (1.252 g), EtOAc (0.108 g), EtOAc/MeOH; (1:1) (16.0 g) and MeOH (0.827 g) fractions were obtained.

2.2. α-glucosidase inhibition assay in vitro

The inhibitory activity against α-glucosidase was carried out spectrophotometrically in a 96-well microplate reader as previously described (Pereira et al., 2012a). A reaction mixture containing 0.04 U/mL of enzyme, sample (0.3–1000 μg/mL) and 0.1 mM of reduced glutathione was preincubated for 15 min. The reaction was started by the addition of 0.85 mM p-nitrophenyl-α-D-glucopyranoside (*p*NPG) as substrate. After 25 min incubation at 37 °C, sodium carbonate was added to stop the reaction. Absorbance readings were recorded at 405 nm (Thermoplate Reader, USA). Samples were dissolved in DMSO and other reagents in 0.1 mM phosphate buffer pH 6.8. Acarbose (0.1–100 ng/mL) was used as a reference drug. The inhibitory effect was expressed as a percentage of inhibition related to the control.

2.3. Glucose uptake assay

Adipocytes were isolated from epididymal fat pads by the method of Rodbell (1964). After isolation, adipocytes were incubated with EEH and DCM (100 μg/mL) and insulin (50 ng/mL) for 45 min. The uptake of 2-deoxy-[^{3}H]glucose was used to determine the rate of glucose transport as previously described (Green, 1987). Briefly, glucose uptake was initiated by the addition of 2-deoxy-[^{3}H]glucose (0.2 μCi/tube) for 3 min. Thereafter, cells were separated by centrifugation through silicone oil and cell-associated radioactivity was determined by scintillation counting.

2.4. Animals

Animal experimental protocols conformed international guidelines and were approved by the local ethics committee [protocol 163/2010, UFMG]. Male Swiss mice (10–12 week old) were maintained with free access to standard diet (Nuvilab, Brazil) and tap water was supplied ad libitum, at a constant temperature (23 ± 2 °C), with a 12:12 h dark/light cycle. All experiments were carried out using at least five animals per group.

2.5. Acute effect of EEH and its fractions on blood glucose in mice

The indirect evaluation of α-glucosidase inhibition in animals was performed after cornstarch overload. Swiss mice were deprived of food for 6 h before the test but allowed free access of tap water throughout the experiment. Blood samples were collected from the tail tip before EEH and fractions administration (time 0). EEH, fractions, and cornstarch were administrated orally. EEH and fractions (300 mg/kg) were dissolved in 50% labrasol. After 20 min, cornstarch (2 g/kg) was administrated, and blood samples were collected at 15, 30, 60 and 120 min after starch overload. Acarbose (10 mg/kg) was used as a reference drug.

In order to evaluate the mechanism of action of the samples, the glucose tolerance test was performed as described above, except that glucose replaced cornstarch and glibenclamide (5 mg/kg) replaced acarbose as reference drug.

2.6. Drugs

Unless otherwise mentioned, all reagents were purchased from Sigma-Aldrich. Blood glucose concentration was determined with an Accu-Chek active glucose meter (Roche Diagnostics, Brazil). Labrasol was purchased from Gatefossé SAS (France).

2.7. Statistical analysis

The percentage of α-glucosidase inhibition was evaluated using the pIC_{50} ($-\log$ of the sample concentration that inhibits activity of the enzyme by 50%), which was calculated using non-linear regression curve and expressed as mean ± S.E.M.. Two-way or one-way ANOVA analysis followed by the Bonferroni post-test were employed, and significance was accepted at $P < 0.05$.

2.8. Chemical characterization of EEH and DCM by ESI–LC–MS

The chemical composition of EEH and DCM was analyzed using an Acquity UPLC system (Waters, Milford, USA) with a Photodiode Array (PDA) detector and interfaced to a triple quadrupole mass spectrometer (TQD) (Waters Micromass, Manchester, UK). The analyses were carried out on a reverse phase column (Acquity UPLC BEH C18, Waters, Ireland; 50 × 2.1 mm^2 i.d., 1.7 μm) in combination with a guard column (Acquity UPLC BEH C18 VanGuard pre-column, Waters, Ireland; 2.1 × 5 mm^2 i.d., 1.7 μm), using a gradient elution of water (A) and acetonitrile (B), both containing 0.1% v/v formic acid (5–95% B in 10 min; 95–5% B in 1 min, followed by 2 min of isocratic elution), at a flow rate of 0.3 mL/min, and temperature of 40 °C. Sample volume injected was 3 μL for EEH (2 mg/mL) and 5 μL for DCM (5 mg/mL), both prepared as MeOH solutions. For the ESI (negative and positive mode) source, the following inlet conditions were applied: capillary voltage 3.54 kV; cone voltage 17 V; source temperature 120 °C; desolvation temperature 300 °C. The mass range was set at *m*/*z* 100–2000.

3. Results and discussion

Inhibition of α-glucosidase has been proven to be a valid therapeutic option for the prevention of type 2 diabetes (Chiasson et al., 2002). In the present study, the effect of EEH and its derived chromatographic fractions on α-glucosidase were evaluated in vitro (Fig. 1). EEH, DCM, DCM:EtOAc, EtOAc, EtOAc:MeOH and MeOH fractions promoted a concentration-dependent inhibition of α-glucosidase activity (Fig. 1A) with similar potency, excepting EtOAc, which showed a lower pIC_{50} value (Fig. 1B). The small yielding of the hexane fraction did not allow us to perform the test with α-glucosidase. Considering the difference of polarity among the fractions and the crude extract, the obtained results suggest that the different classes of compounds may account for the α-glucosidase inhibition. The idea of a broad class of natural products as inhibitors of α-glucosidase is compatible with reports from the literature (Reddy et al., 2009; Bräunlich et al., 2013). L-bornesitol, quinic acid, and rutin were previously reported in an ethanol extract from *Hancornia speciosa* leaves (Endringer et al., 2009). In our experimental conditions, L-bornesitol did not inhibit α-glucosidase (data not shown). Quinic acid and rutin derivatives are well-known inhibitors of α-glucosidase (Iwai et al., 2006; Pereira et al., 2011), and probably contribute to the observed effect. Moreover, chemical studies with *Hancornia speciosa* leaves have also indicated the presence of terpenoids, steroids and tannins (Honda et al., 1990; Brandão et al., 2011), which could also play a role in the inhibitory effect of this plant on α-glucosidase.

The starch tolerance test demonstrated that EEH, DCM, and MeOH fractions were able to decrease the plasma concentration of

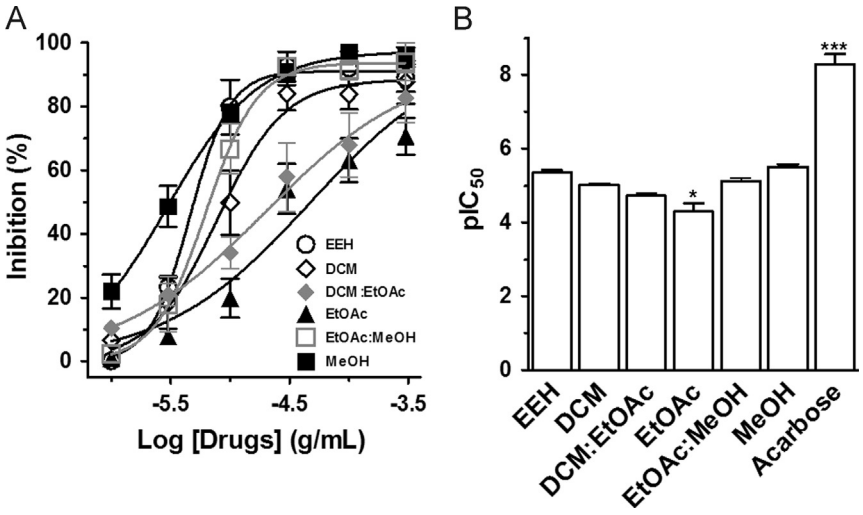

Fig. 1. Inhibitory effect of *Hancornia speciosa* crude ethanolic extract (EEH) and its dichloromethane (DCM), ethyl acetate (EtOAc), DCM:EtOAc, methanolic (MeOH) and EtOAc:MeOH fractions on α-glucosidase. (A) Concentration–response curves for EEH, DCM, EtOAc and MeOH (0.3–1000 μg/mL). (B) pIC_{50} values for EEH, DCM, DCM:EtOAc, EtOAc, EtOAc:MeOH, MeOH and acarbose. Data represent mean ± S.E.M. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus EEH data.

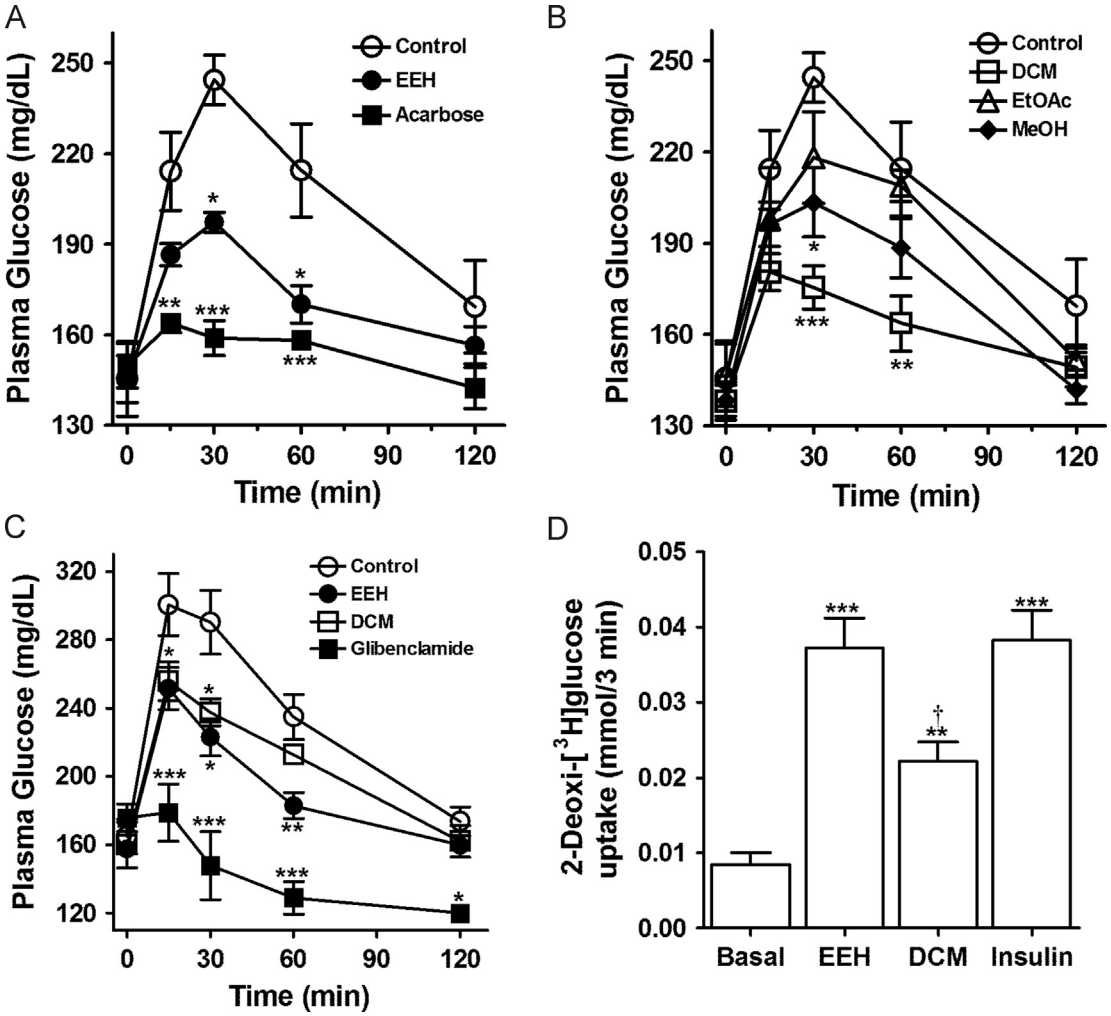

Fig. 2. Effect of *Hancornia speciosa* on the starch tolerance test, glucose tolerance test and glucose uptake. (A and B) Effect of 300 mg/kg of the ethanolic crude extract (EEH) and its dichloromethane (DCM), ethyl acetate (EtOAc) and methanolic (MeOH) fractions on the starch tolerance test and (C) on the glucose tolerance test in mice. (D) Effect of the EEH and DCM (100 μg/mL) and insulin (50 ng/mL) on glucose uptake in adipocytes. Data represent mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control or basal data. $^{\dagger}P < 0.05$ versus EEH and insulin.

glucose in mice (Fig. 2 A and B). The strongest reduction was observed with DCM, being similar to the reduction induced by acarbose in 60 min (Fig. 2B). The lowest effect was observed with MeOH, whereas EtOAc induced no significant reduction in the plasmatic glucose (Fig. 2B). Therefore, these in vivo results are compatible with the inhibitory effect of EEH, DCM and MeOH on

α-glucosidase, and the absence of effect for EtOAc might be related with its lower potency, inability to cross the intestinal mucosal barrier or low bioavailability. In order to minimize the number of animals used the fractions DCM:EtOAc and EtOAc: MeOH were not tested, considering that they had, respectively, similar effect and potency as the EtOAc and MeOH fractions.

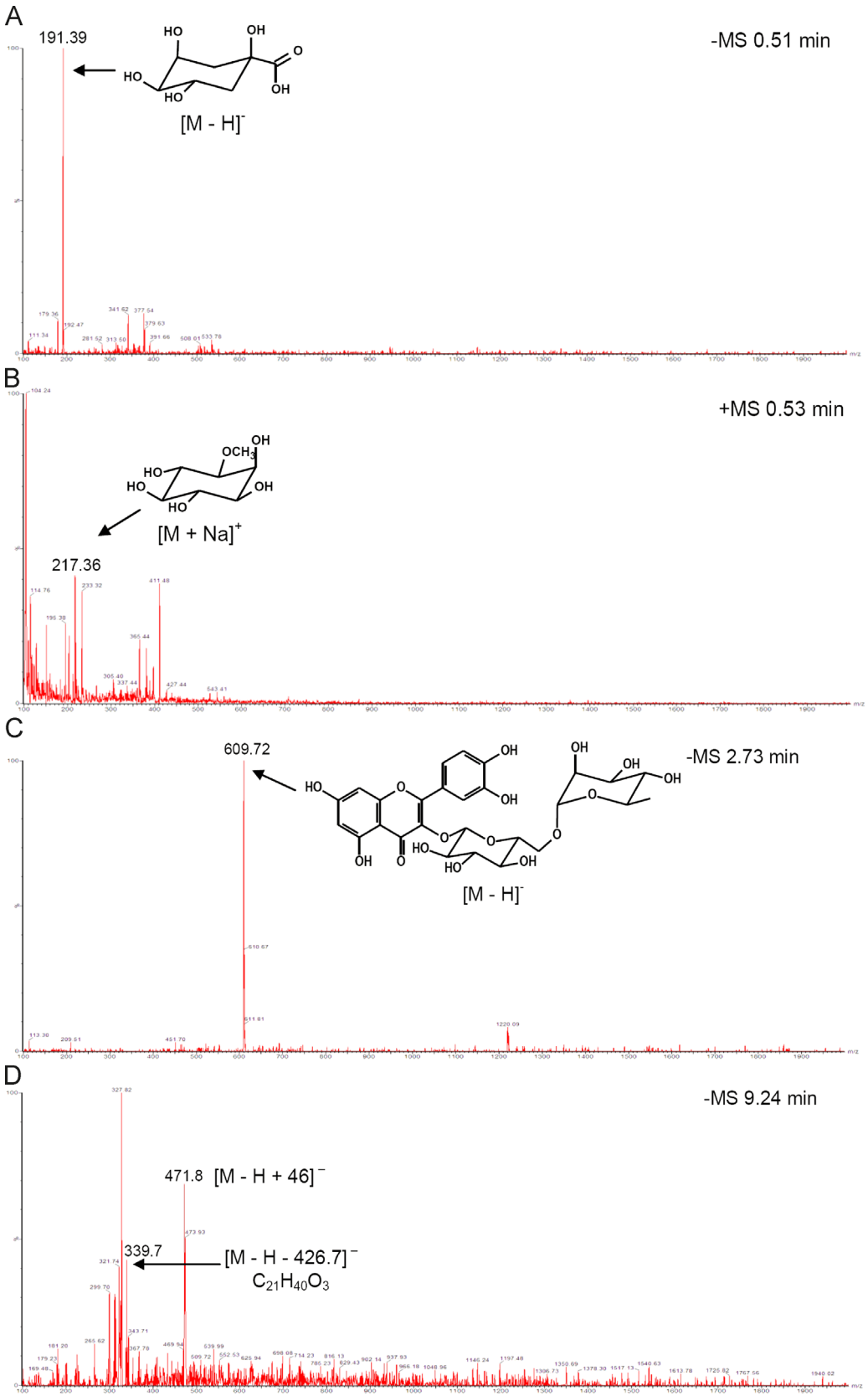

Fig. 3. ESI/LC–MS of selected peaks in the ethanolic extract from *Hancornia speciosa* leaves (A–C) and dichloromethane fraction thereof (D). Peaks: (A), quinic acid; (B) bornesitol; (C) rutin; (D) ester of lupeol.

Considering that cyclitols and flavonoids, previously described in EEH (Endringer, 2007; Pereira et al., 2012b) are able to reduce the blood glucose by increasing the glucose uptake (Shen et al., 2012; Babu et al., 2013). The effect of the crude extract (EEH) and its most active fraction (DCM) were evaluated in the oral glucose tolerance test. As illustrated in Fig. 2C, both EEH and DCM were significantly able to reduce the plasma concentration of glucose, suggesting an additional effect of EEH and DCM on glucose homeostasis. This additional effect was confirmed when EEH and DCM were tested against the glucose uptake in freshly dissociated adipocytes, where both induced a significant increase on glucose uptake (Fig. 2D). It is noteworthy to observe that the effect induced by EEH was similar to insulin while the effect of DCM was significantly smaller than EEH and insulin (Fig. 2D). Despite the reduced effect of DCM on glucose uptake, its excellent reduction on plasma glucose in the starch tolerance test suggest that the effect of this fraction is more selective for the inhibition of α-glucosidase.

Previous phytochemical studies of *Hancornia speciosa* leaves resulted in the identification of different classes of compounds, including cyclitols [L-(+)-bornesitol, quinic, 5-O-caffeoil-quinic and chlorogenic acids], cynamic acids (*cis*- and *trans*-4-hydroxy-cynamic acids), flavonoids (kaempferol-3-O-rutinoside, rutin and a quercetin triglycoside), steroids and triterpenes (β-sitosterol, obtusalin, erithrodiol and 3β-O-esters of lupeol, α- and β-amirin) (Endringer, 2007; Barros, 2008; Endringer et al., 2007; Endringer et al., 2009; Pereira et al., 2012b).

Aiming to characterize the chemical composition of EEH and its DCM fraction, they were analyzed by ESI–LC–MS, being the results compared with reference compounds and/or the literature data reported for the species. Hence, negative ion mode analysis of EEH led to the detection of quinic acid (Rt = 0.51 min) by the peak at *m/z* 191.4 $[M-H]^-$ (Fig. 3) and chlorogenic acid (Rt = 1.91 min) at *m/z* 353.6 $[M-H]^-$, along with rutin (Rt = 2.73 min), identified by the peak at *m/z* 609.7 $[M-H]^-$ (Fig. 3). Bornesitol (Rt = 0.53 min) was disclosed by the peak of its sodium adduct at *m/z* 217.4 $[M+23]^+$ in the positive ion mode analysis of EEH (Fig. 3).

In addition, the compound eluted at 3.04 min gives a peak at *m/z* 593.7 $[M-H]^-$ compatible with a kaempferol diglycoside, supported by the fragment at *m/z* 447.7 $[M-H-146]^-$ resulting from the loss of one sugar residue. On its turn, the constituent eluting at 3.60 min was ascribed to a kaempferol triglycoside, disclosed by the peak at *m/z* 755.6 $[M-H]^-$. The analysis also revealed lipophilic compounds, whose MS data are compatible with esters of lupeol or α-/β-amirin. The first eluted at 6.49 min and gives a peak at *m/z* 471.8 $[M-H+46]^-$ credited to the formic acid adduct of the triterpene (lupeol or α-/β-amirin), along with a peak at *m/z* 311.7 $[M-H-426.8]^-$ assigned to the side chain fragment ($C_{19}H_{36}O_3$). The second ester eluted at 7.56 min and was suggested by the peak at *m/z* 339.7 $[M-H-426.8]^-$, attributed to the side chain residue ($C_{21}H_{40}O_3$). In addition, positive ion mode analysis of the compound eluting at 11.09 min gives a peak at *m/z* 155.2 $[M+H]^+$, compatible with the volatiles *trans*- and *cis*-linalool oxide, α-terpinol and geraniol.

HPLC and TLC analyses of DCM revealed majorly triterpenes and derivatives as constituents of the fraction (data not shown), in agreement with previous reports for the species (Endringer, 2007; Barros, 2008). ESI–LC–MS analysis of DCM disclosed different esters of lupeol or α/β-amirin as discussed above for EEH. For instance, the compound eluting at 9.24 min showed a base peak at *m/z* 471.8 $[M-H+46]^-$ ascribed to the adduct of lupeol with formic acid, along with a fragment at *m/z* 339.7 $[M-H-426.7]^-$ credited to the side chain residue ($C_{21}H_{40}O_3$) (Fig. 3). The ESI–LC data also revealed the presence of monoterpenes at *m/z* 155.2 $[M+H]^+$, similarly as found in EEH.

It is noteworthy that lupeol and α/β-amirin are well known as anti-diabetic drugs (Ali et al., 2006; Santos et al., 2012). The mechanism of action is better characterized for lupeol, as an inhibitor of α-glucosidase and protein tyrosine phosphatase-1B (Na et al., 2009; Nkobole et al., 2011). These reports from the literature suggest that lupeol and α/β-amirin may contribute to the anti-diabetic effect of *Hancornia speciosa*.

Altogether the present work demonstrates that *Hancornia speciosa* reduces the blood glucose concentration through inhibition of intestinal α-glucosidase and stimulation of glucose uptake by adipocytes. These results suggest the potential use of this plant for the treatment of diabetes mellitus.

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