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Alibertia edulis (L.C. Rich.) A.C. Rich – A potent diuretic arising from Brazilian indigenous species

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ABSTRACT

Ethnopharmacological relevance: Although *Alibertia edulis* (L.C. Rich.) A.C. Rich decoction is used in Brazilian folk medicine due to its possible antihypertensive effect, this species has never been critically investigated as a hypotensive drug. So, the aim of this study was to evaluate the possible hypotensive and antihypertensive effects of the oral administration of *Alibertia edulis* aqueous extract (AEAE) in normotensive and hypertensive rats, and evaluate its inter-relation with a possible diuretic activity.

Material and methods: Different doses of AEAE (20, 65 and 200 mg/kg) were tested on the mean arterial pressure (MAP) of normotensive Wistar rats and after induction of renovascular hypertension (two-kidney, one-clip Goldblatt model). In addition, the diuretic effects of AEAE were compared with hydrochlorothiazide (HCTZ) in an acute and repeated-dose treatment for 7 days. Volume, sodium, potassium, chloride, calcium contents, pH and density were estimated in urine samples collected after 8 or 24 h. Plasma sodium, potassium, total protein, urea, creatinine, AST and ALT concentrations were measured in samples collected at the end of the experimental period (seventh day). Finally, the antioxidant activity of the AEAE was assessed using the DPPH radical scavenging and ferric ions reducing power assay.

Results: The intraduodenal administration of the HCTZ and AEAE significantly reduced, in a dose-dependent manner, the MAP in both normotensive and hypertensive rats. Otherwise, the heart rate was not affected by any treatment. Acute and prolonged oral administration of AEAE (200 mg/kg) and HCTZ caused a significant increase in volume and urinary concentrations of sodium, potassium and chloride. Moreover, urinary calcium concentration was significantly increased after administration of AEAE (200 mg/kg). Finally, AEAE was able to present important in vitro antioxidant properties.

Conclusion: The results obtained have shown that AEAE presents potent diuretic activity and significant hypotensive and antihypertensive effect. In addition, this study may confirm part of the pharmacological activity popularly attributed to this species and opens perspective for the future use in various renal and cardiovascular diseases.

Abbreviations: 2K1C, Two-kidney-one-clip; AEAE, Alibertia edulis aqueous extract; AF, Acetate fraction; ALT, Alanine transaminase; ANOVA, Analysis of variance; AqF, Aqueous fraction; AST, Aspartate transaminase; BF, n-buthanol fraction; BW, Body weight; Ca^{+2}, Calcium; Cl^{-}, Chloride; DPPH, 2,2-diphenyl-1-picryl-hydrazil; EL, Excretion load; Fe, Ferro; FRAP, Ferric reducing antioxidant power; GAE, Gallic acid equivalentes; g/ml, grams/milliliters; h, hours; H_2O, Water; H_2SO_4, Sulfuric acid; HCTZ, Hydrochlorothiazide; HOAc, Acetic acid; HPLC-PDA, High-Performance Liquid Chromatography Photo-Diode Array; i.v., intravenous; IC_{50}, Half maximal inhibitory concentration; IU, International units; K^{+}:, Potassium; MAP, Mean arterial pressure; MeOH, Methanol; mEq/l, miliequivalent/liters; mEq/min/100 g, miliequivalent/minutes/100 g of body weight (BW); mg/kg, milligrams/kilograms; mg, milligrams; min, minutes; ml/min, milliliters/minutes; ml, milliliters; mm Hg, millimeters of mercury; Na^{+}, Sodium; NaCl, Sodium chloride; nm, nanometers; NMR, Nuclear magnetic resonance; °C, Degree Celsius; pH, Hydrogen potential; RE, Rutin equivalentes; rpm, rotation per minute; SBP, Systolic blood pressure; SEM, Standard error of the mean; TAA, Total antioxidant activity; TLC, Thin layer chromatography; TPTZ, 2,4,6-Tris(2-piridil)-s-triazina; Ux, Electrolytes concentration; µl/100 g, microliters/100 g of body weight (BW); µM, micrometer; v/v/v, volume/volume/volume; V, Urinary flow; w/v, weight/volume

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1. Introduction

In Brazil, the state of Mato Grosso do Sul has one of the richest biodiversity in Latin America (Cordeiro et al., 2014). The state has at least two major biomes, Cerrado and south Pantanal (Myers et al., 2000; Ribas and Schoereder, 2006). In addition to the rich and still preserved vegetation, the state has different indigenous communities that keep important popular knowledge about the therapeutic activities of various natural products (Bueno et al., 2005). In the region known as Grande Dourados, located in the southern state, there are more than 20 thousand Indians of different ethnic groups, including Guarani-Kaiowá, Kadiwéu, and Terena (Sacchi et al., 2013). It is in this place of intense cultural effervescence that much ethnopharmacological knowledge has been transmitted from generation to generation for hundreds of years, showing great potential for ethnopharmacological validation of the popular culture of these people (Leonti, 2011).

In this region, where the predominant native vegetation is Cerrado, the use of several species of the family Rubiaceae, including *Alibertia, Psychotria*, *Palicourea* and *Tocoyena* is observed, with the largest number of species with several pharmaceutical and economic interests (Martins and Nunez, 2015). Among species widely used in the region, *Alibertia edulis* (L.C. Rich.) A.C. Rich., popularly known as "marmelada-bola" and "marmelo-do-cerrado" (Brochini et al., 1994; Persson, 2000) stands out. For many years, this species has been popularly used for the treatment of hypertension throughout the state of Mato Grosso do Sul, especially in the region of Grande Dourados (Sangalli et al., 2002; Bueno et al., 2005).

Despite its widespread use, the different preparations obtained from *Alibertia edulis* lacks detailed ethnopharmacological studies that validate its popular use. It is worth mentioning that the popular widespread use does not guarantee efficacy and safety of a natural product, and only detailed studies can point out possible risks of their pharmacological activity, such as electrolyte disturbances, arrhythmias, and other indicators of toxicity.

Few pharmacological studies have been conducted for this species, most of them focused on its antitumor activities (Gupta et al., 1996). A phytochemical investigation with the stem from *Alibertia edulis* led to the isolation and identification of an iridoid ester and a saponin, which showed moderate inhibitory activity against *Candida albicans* and *Candida krusei* (Da Silva et al., 2008). Moreover, it was also possible to identify the presence of oleanane (Brochini, 1994), tannins and some alkaloids (Soto-Sobenis et al., 2001).

Thus, considering the available ethnobotanical information and extensive popular use of this species in the treatment of hypertension, the aim of this study was to evaluate the possible hypotensive and antihypertensive activities of the oral administration of *Alibertia edulis* aqueous extract in normotensive and hypertensive rats and evaluate its inter-relation with a possible diuretic activity.

2. Materials and methods

2.1. Drugs and spectral measurements

Hydrochlorothiazide (HCTZ), Gallic Acid, Rutin, 2,2-diphenyl-1-picrylhydrazil (DPPH), TPTZ (2,4,6-Tripyridyl-s-Triazine) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteu was obtained from Merck (Darmstadt, Germany); spectroscopy- grade acetonitrile and methanol were purchased from Merck (Darmstadt. Germany); water was purified using a Milli-Q system (Millipore). Antioxidant activity assays were recorded in MeOH using 700S Femto UV Spectrophotometer at 517 nm wavelength. Preparative Thin Layer Chromatography (TLC) was carried out on silica 60 F254 TLC plates (Merck). Sephadex LH-20 (Sigma-Aldrich) was used for column chromatography.

2.2. Phytochemical study

2.2.1. Plant material

Alibertia edulis leaves were collected in November/December 2013 from the local vegetation of the Federal University of Grande Dourados (UFGD) (Dourados, Brazil) at 458 m above sea level (S22°11′43.7 - W54°56′08.5). A voucher specimen was authenticated by Dr. Zefa Valdevina Pereira under number 4649 and deposited in the herbarium of UFGD. *Alibertia edulis* leaves were air-dried in an oven at 40 °C for 10 days (58.1% humidity) and then the dry plant was cut and ground into a powder using mechanical milling.

2.2.2. Alibertia edulis aqueous extract (AEAE) preparation and fractionation

Dry leaves were extracted by decoction (1:10 w/v at temperature 97 °C by 15 min) in a similar manner to that used popularly in Brazil (Bueno et al., 2005). The extract was filtered and lyophilized, yielding 36.9%. Further liquid/liquid extractions on the AEAE were carried out using ethyl acetate (AF) and n-buthanol (BF). Fractions were obtained after decantation and evaporated under vacuum at approximately 40 °C (AF and BF) or lyophilized (aqueous fraction - AqF) yielding 12.4% of AF, 15.8% of BF and 65.7% of AqF. The AEAE and the three fractions were analyzed by TLC using n-buthanol/acetic acid/water (65:25:15 v/v/v) and BF was selected in order to be fractioned. A BF (103.0 mg) sample was chromatographed on a Sephadex LH-20 column (50.0 cm x 2.0 cm) with MeOH as eluent in order to give seven fractions: AE-1 (25.2 mg), AE-2 (5.5 mg), AE-3 (22.4 mg), AE-4 (12.5 mg), AE-5 (8.9 mg) AE-6 (4.5 mg), and AE-7 (5.8 mg). Compounds were visualized under UV254/366 light and by spraying with H2SO4/H2O/HOAc (4:16:80 v/v/v).

2.2.3. Phytochemical analysis

2.2.3.1. Screening. The total phenolic content of AEAE and fractions (AF, BF and AqF) were determined by using the Folin & Ciocalteu's phenol reagent. An aliquot (1 ml) of the extracts was added to 25 ml volumetric flask, containing 9 ml of distilled water. Blank using distilled water was prepared. One milliliter of Folin & Ciocalteu's reagent was added to the mixture and shaken. After 5 min, 10 ml of 7% Na2CO3 solution was added to the mixture. The solution was diluted to volume (25 ml) with distilled water and mixed. After incubation for 120 min at room temperature, the absorbance against prepared blank was determined at 750 nm with an UV–vis Spectrophotometer. Standard curve of Gallic acid solution (25, 100, 300, 400, 500, 600 and 700 μg/ml) was prepared using the similar procedure. Samples were measured in three replicates.

Aluminum chloride colorimetric method was used for determination of total flavonoids. The extract and its fractions (0.5 ml of 1:10 g/ml) in methanol was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in triplicate. The calibration curve was prepared by preparing quercetin solutions at concentrations 10–100 μg/ml in methanol (Chang et al., 2002).

2.2.3.2. Nuclear Magnetic Resonance (NMR) spectroscopy. NMR spectra was recorded on Agilent 500 DD2 spectrometer equipped with 5 mm 1 H{15N–31 P} PFG high-field inverse detection z-gradient probe. ^{1}H (499.719 MHz) and ^{13}C (125.666 MHz) NMR spectra were recorded in methanol-d_4 at 25 °C. Chemical shifts are given on the δ scale and are referenced to residual methanol (δH 3.30 and δC 49.00).

Ixoxide (AE-3): ^{1}H NMR: 7.46 (d, 1.0 Hz, H-3), 6.79 (td, 2.6, 1.5 Hz, H-7), 5.65 (d, 5.0 Hz, H-1), 4.61 (d, 7.9 Hz, H-1′), 3.84 (dd,12.2, 2.0 Hz, H-6a'), 3.67 (dd, 12.2, 5.8, H-6b'), 3.38 (dd, 9.1, 9.1 Hz), H-3′), 3.28 (m, H-5, H-4′, H-5′), 3.19 (dd, 9.1, 7.9 Hz, H-2′), 3.18 (m, H-9), 2.89 (dddd, 18.4, 8.1, 2.3, 2.3 Hz, H-6a), 2.41 (dddd, 18.4, 4.7, 2.2, 2.2 Hz, H-6b). ^{13}C NMR: 170.9 (C-11), 169.4 (C-10), 153.3 (C-3), 145.62 (C-7), 137.8 (C-8), 112.9 (C-4), 100.3 (C-1′), 96.4 (C-1), 78.2 (C-5′), 77.8 (C-3′), 74.6 (C-2′), 71.4 (C-4′), 62.6 (C-6′), 47.6 (C-9), 40.1 (C-6), 35.1 (C-5).

Caffeic acid (AE-5): ^{1}H NMR: 7.56 (d, 15.9 Hz, H-7), 7.04 (d, 2.1 Hz, H-2), 6.93 (dd, 8.2, 2.1 Hz, H-6), 6.77 (d, 8.2 Hz, H-5), 6.29 (d,15.9 Hz, H-8).

Quercetin 3-rhamnosyl-(1→6)-galactoside (AE-7): ^{1}H NMR: 7.85 (d, 2.2 Hz, H-2′), 7.59 (dd, 8.5, 2.2 Hz, H-6′), 6.88 (d, 8.5 Hz, H-5′), 6.42 (d, 1.9 Hz, H-8), 6.21 (brs, H-6), 5.04 (d, 7.8 Hz, H-1″), 4.52 (brs, H-1‴), 3.83 (dd, 8.8, 7.8 Hz, H-2″), 3.80 (d, 3.4 Hz, H-4″), 3.73 (dd, 10.4, 5.6, Hz, H-6a″), 3.64 (dd, 6.8, 5.6 Hz, H-5″), 3.58 (dd, 3.4, 1.7 Hz, H-2‴), 3.56 (dd, 9.7, 3.4 Hz, H-3″), 3.52 (m, H-5‴), 3.50 (dd, 9.2, 3.4 Hz, H-3‴) 3.41 (dd, 10.4, 6.8, H-6b″), 3.29 (dd, 9.4, 9.4 Hz, H-4‴), 1.18 (d, 6.0 Hz, H-6‴).

2.3. Pharmacological studies

2.3.1. Animals

Male Wistar rats (180–200 g), supplied by the Federal University of Grande Dourados (UFGD), were housed in a temperature- and light-controlled room (22 ± 2 °C; 12-h light/dark cycle) and acclimatized in the laboratory for a period of at least 10 days before any experimental procedures, with free access to water and food. All experimental procedures adopted in this study were previously approved by the Institutional Ethics Committee of the Federal University of Grande Dourados (UFGD, Brazil; authorization number 04/2015).

2.3.2. Systemic blood pressure studies

2.3.2.1. Induction of renovascular hypertension (two-kidney, one-clip model). The Goldblatt 2K1C hypertension model was induced according to procedure described by Umar et al. (2010). Briefly, rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), intraperitoneally administered. The left renal artery was exposed by retroperitoneal flank incision and dissected free of the renal vein and connective tissue. A silver clip with 0.22 mm of lumen was placed around the artery for partial occlusion; in sham operations, the artery was not clipped. After 6 weeks, systolic blood pressure (SBP) was measured using the tail-cuff method in conscious rats. Only hypertensive rats (2K1C; SBP above 150 mm Hg) were used in experiments.

2.3.2.2. Direct blood pressure measurement in anesthetized rats. Normotensive and 2K1C male rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), intramuscularly administered and supplemented at 45–60 min intervals. A polyethylene catheter was inserted into the right femoral vein for drug administration. Immediately after venous cannulation, a bolus injection of heparin (30 IU. *i.v.*) was administered. Animals were allowed to breath spontaneously through a tracheotomy. The left carotid artery was cannulated and connected to a pressure transducer coupled to a PowerLab® recording system, and an application program (Chart, v 4 .1; all from ADI Instruments; Castle Hill, Australia), which recorded the mean arterial pressure (MAP). For intraduodenal administration, a small incision was made below the xiphoid process and the duodenum was isolated with the aid of tweezers. For blood pressure stabilization after the surgical process, an interval of 15 min was given before recordings. At the end of experiments, animals were euthanized with an overdose of thiopental (over 40 mg/kg, i.v).

2.3.2.3. Hypotensive and antihypertensive AEAE dose response. Groups of normotensive and 2K1C rats, prepared for direct blood pressure measurements as previously described, received intraduodenal AEAE at 20, 65 and 200 mg/kg, respectively. The positive and negative controls groups received, respectively, HCTZ (25 mg/kg) and vehicle at constant volume of 100-µl/100 g-body weight (BW). Changes in MAP were recorded for 45 min after treatments.

2.3.3. Renal function studies

2.3.3.1. Diuretic activity assessment: experimental design. The diuretic activity was determined according to method previously described (Kau et al., 1984) with minor modifications (Gasparotto Junior et al., 2009). Animals were divided into five groups (n =5) for the acute study (single dose), and other five groups (n =5) for the continuous study (7-day repeated-dose). Rats fasted overnight with free access to water and were submitted to treatment as described below. Each animal was placed in an individual metabolic cage 24 h before the beginning of treatments for environmental adaptation. AEAE was dissolved in 5 ml of distilled water per kilogram of body weight (BW) just before administration, and orally administered to individual rats.

2.3.3.1.1. Acute diuretic activity. Before treatment, all animals received an oral dose of 5 ml/100 g of physiological saline solution (0.9% NaCl) to impose a uniform water and salt load (Benjumea et al., 2005). The first group received oral administration of vehicle (deionized water) and was used as control. Different groups of rats received, by oral route, 20, 65 and 200 mg/kg AEAE or 10 mg/kg HCTZ. Urine was collected in a graduated cylinder and the volume was recorded at 2 h intervals for 8 h. Cumulative urine excretion was calculated in relation to body weight and expressed in ml/100 g. Electrolyte concentrations (Na^{+}, K^{+}, Ca^{+2} and Cl^{-}), pH and density were estimated from a pooled urine sample of each pair of rats at the end of the experiment (8 h).

2.3.3.1.2. Prolonged diuretic activity. AEAE (20, 65 and 20 mg/kg) or HCTZ (10 mg/kg) was daily administered by oral gavage to different groups of rats for 7 days; control animals received vehicle daily. The total amount of urine was collected every 24 h and volume, Na^{+}, K^{+}, Ca^{+2} and Cl^{-} concentrations, pH and density were measured. At the end of the experimental period serum Na^{+}, K^{+}, total protein, aspartate transaminase (AST), alanine transaminase (ALT), urea and creatinine concentrations were determined.

2.3.3.1.3. Analytical procedures. For serum analysis, blood samples were collected in conical tubes after decapitation. Plasma and serum were obtained by centrifugation (2000 rpm, 10 min, 4 °C), and stored at −20 °C until analysis. Urinary and plasma Na^{+}, K^{+}, Cl^{-}, and Ca^{+2} levels were quantified by ion-selective electrodes (Cobas Integra Plus, Roche, Indianapolis, USA). pH was directly determined on fresh urine samples using HIP 3221 (Hanna Instruments) pH-meter. Density was estimated by weighing with a Mettler AE163 (± 0.1 mg) analytical scale of urine volume measured with a Nichiryo micropipette. Plasma total protein, aspartate transaminase (AST), alanine transaminase (ALT), urea and creatinine concentrations were determined by enzymatic method using BM/Hitachi 912 automated analyzer (Cobas Integra Plus, Roche, Indianapolis, USA). Additionally, excretion load (El) of Na^{+}, K^{+}, Cl^{-} and Ca^{+2} was obtained according to the equation below **(1)**:

$$\mathbf{El = U_x x V}$$

Ux: Electrolyte concentration (mEq/l).
V: Urinary flow (ml/min).
Results were expressed as mEq/min/100 g.

2.3.4. Evaluation of the in vitro antioxidant activity

2.3.4.1. DPPH radical scavenging activity. The ability of AEAE to scavenger the DPPH free radical was assessed according to Gupta and Gupta (2011) with some modifications. Briefly, a 0.004% DPPH solution in ethanol was prepared. To 900 µl of this solution, 100 µl of extract or rutin (positive control) were added at different concentrations. The mixture was vigorously shaken and incubated for 30 min in the dark at room temperature. The DPPH reduction was measured by the decrease in absorption at 517 nm. Distilled water plus extract solutions were used as blank, while DPPH solution plus distilled water were used as control. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH radical scavenging activity was calculated by the following equation:

Scavenging effect (%) =(Acontrol 517 – Aextract 517/ Acontrol 517) x 100.

The EC50 value is the extract concentration required to scavenge 50% of the DPPH free radical.

2.3.4.2. Ferric reducing antioxidant power (FRAP) assay. Ferric ion reducing power was measured according to the method of Benzie and Strain (1996). Different concentrations of AEAE were mixed with 2.5 ml of 20 mM phosphate buffer and 2.5 ml 1%, w/v potassium ferricyanide, and then the mixture was incubated at 50 °C for 30 min. Afterwards, 2.5 ml of 10%, w/v trichloroacetic acid and 0.5 ml 0.1%, w/v ferric chloride were added to the mixture, which was kept aside for 10 min. Finally, the absorbance was measured at 700 nm. Rutin was used as positive reference standard. All assays were run in triplicate way and averaged.

2.4. Statistical analysis

The results are expressed as mean ± standard error of mean (S.E.M) of 5 animals per group. Statistical analyses were performed using one-way and two-way analysis of variance (ANOVA) followed by Bonferroni's test. A p value less than 0.05 was considered statistically significant. Graphs were drawn and statistical analyses were performed using GraphPad Prism software version 5.0 for Mac OS X (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Phytochemical analyses

Aqueous extract obtained from *Alibertia edulis* leaves showed that AEAE contains large amounts of flavonoids and phenols, which correspond to 87.09 ± 6.10 mg rutin equivalents (RE)/g of extract, and 348.87 ± 2.88 mg gallic acid equivalents (GAE)/g of extract, respectively. The fractions AF, BF and AqF showed contains of phenols which correspond to 204.73 ± 6.82, 248.31 ± 5.58, 125.93 ± 5.64 mg gallic acid equivalents (GAE)/g of extract, respectively and the contents of the flavonoids corresponds to 13.63 ± 0.11 (AF), 20.35 ± 0.18 (BF) and 6.50 ± 0.11 (AqF) mg rutin equivalents (RE)/g of extract.

In an attempt to map major phenols present in AEAE, we choose to perform a detailed phytochemical characterization in the fraction (BF) in which we find the greatest amount of phenolic compounds.

The complete assignation of the ^{1}H and ^{13}C NMR spectra of AE-3 fraction using two-dimensional techniques as COSY and HSQC was done. The comparison of the data with those reported in the literature for iridoids identified from *Albertia edulis* led us to conclude that the main compound in AE-3 fraction was ixoside (El-Naggar and Beal, 1980).

The ^{1}H NMR of fraction AE-5 showed characteristic signals for phenolic acids. The spin system for aromatic compound was in agreement with a 1, 3, 4-trisubstitution and two doublets with 15.9 Hz as coupling constant suggest a *trans*-double bond moiety. So, we proposed caffeic acid as structure. The comparison of the ^{1}H NMR data of AE-5 with previously published for caffeic acid (Jeong et al., 2011) confirmed the hypothesis.

The ^{1}H NMR of fraction AE-7 showed signals in agreement with a quercetin unit and signals for two sugar moieties. The study of TOCSY-1D of signals for anomeric protons 5.04 (d, 7.8 Hz), 4.52 (brs) and the methyl group of a 6-deoxyhexopyranose, 1.18 (d, 6.0 Hz), led us the structure of both sugars, galactopyranose and ramnopyranose. On the other hand, the ROESY-1D of rhamnopyranose anomeric signal, 4.52 (brs) showed a correlation with H-6″ signal of galactopyranose. The proposed structure for main compound of AE-7, quercetin 3-rhamnosyl-(1→6)-galactoside, was confirmed after comparison with NMR data of literature (Rayyan et al., 2005).

The iridoid ixoside was previously identified from the stems of the *Alibertia edulis* (Da Silva et al., 2008) and the caffeic acid from leaves of *Alibertia macrophylla* (Bolzani et al., 1997). The glycosylflavonol, quercetin 3-rhamnosyl-(1→6)-galactoside has not been described previously in the genus *Alibertia*.

3.2. AEAE induces hypotensive and antihypertensive effects on normotensive and 2K1C hypertensive rats

The basal MAP values recorded in normotensive rats after the 15-min period allowed for stabilization and before the administration of any drug were 98.7 ± 5.0 mm Hg. Intraduodenal administration of AEAE (20, 65 and 200 mg/kg) led to a significant dose dependent hypotension (4.6 ± 1.6, 8.1 ± 2.0, and 11.2 ± 2.3 mm Hg, respectively) (Fig. 1A), with minor effects on heart rate (data not shown).

The average values of MAP recorded in 2K1C hypertensive rats after the stabilization period and before the administration of any drug were 155.6 ± 4.8 mm Hg. In these animals, the administration of AEAE at doses of 20, 65 and 200 mg/kg reduced MAP levels in 2.7 ± 0.7, 9.3 ± 2.5 and 18.1 ± 3.1 mm Hg, respectively (Fig. 1B). Furthermore, the acute administration of HCTZ (25 mg/kg) on normotensive (MAP reduction: 9.8 ± 1.6 mm Hg) and hypertensive rats (MAP reduction: 16.3 ± 3.8 mm Hg) reduced MAP values similarly to AEAE at its highest dose (200 mg/kg) (Fig. 1A and B).

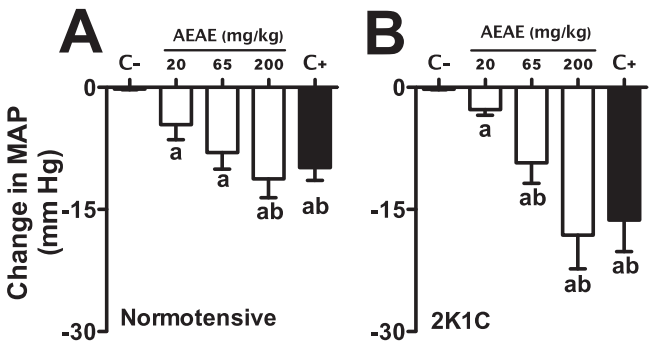

Fig. 1. Dose-dependent hypotension induced by AEAE in normotensive (A) and hypertensive rats (B). The AEAE and HCTZ (positive control; C+) were administered per intraperitoneal route in anesthetized rats. The negative control (C-) indicates the effect measured after administration of vehicle (distilled water) only. Each bar represents the mean of five animals, and the vertical lines show the S.E.M. [a] denote the significance levels in comparison with the control group. [b] denote the significance levels in comparison with the AEAE 20 mg/kg (one-way ANOVA followed by Bonferroni test) ([a and b] $p < 0.05$).

Table 1
Effect of acute oral administration of aqueous extract obtained from *Alibertia edulis* (AEAE) on urinary volume, pH, and density.

Group	Urine volume (8 h/ 100 g)	pH	Density (g/ml)
Control	3.49 ± 0.40	6.6 ± 0.07	1.079 ± 0.01
AEAE (20 mg/kg)	3.84 ± 0.38	6.6 ± 0.06	1.068 ± 0.01
AEAE (65 mg/kg)	4.28 ± 0.35	6.7 ± 0.04	1.056 ± 0.01
AEAE (200 mg/ kg)	5.55 ± 0.66[a]	6.7 ± 0.09	1.077 ± 0.01
HCTZ (25 mg/kg)	5.76 ± 0.42[a]	6.5 ± 0.01	1.076 ± 0.01

Values are expressed as mean ± S.E.M. of five rats in each group in comparison with the control using one-way ANOVA followed by Bonferroni's test ([a]p < 0.05).

3.3. AEAE induces acute diuresis and urinary electrolyte excretion in normotensive Wistar rats

Treatment with a single AEAE dose (200 mg/kg) significantly increased diuresis after 8 h (Table 1). The total urinary volume measured at 8 h in AEAE-treated animals was 5.55 ± 0.66 ml/100 g; while the urinary volume observed in the control group at the same time was 3.49 ± 0.40 ml/100 g ($p < 0.05$). The cumulative urinary volume found in AEAE-treated animals (200 mg/kg) was not different from that obtained in animals treated with HCTZ.

The effects of acute treatment with AEAE (20, 65 and 200 mg/kg) on electrolyte excretion in urine collected 8 h after treatments are presented in Table 2. Excreted amounts of Na^+, K^+, and Cl^- were significantly increased in groups treated with AEAE (200 mg/kg) when compared to controls treated with vehicle alone. Moreover, HCTZ presented an interesting sparing effect on Ca^{+2} at 25 mg/kg, while group treated with AEAE at 200 mg/kg showed high amounts of this electrolyte in urine (Control: 0.007 ± 0.0006; AEAE 200: 0.010 ± 0.004*; HCTZ: 0.008 ± 0.001 mEq/min/100 g; *$p < 0.05$). All other urinary parameters (pH and density; Table 1) showed no significant differences when compared to the control group.

3.4. Potent diuretic effects induced by Alibertia edulis remain during 7 days of treatment

Daily AEAE administration (200 mg/kg) for 7 days significantly increased diuresis after the second day of treatment (Fig. 2), remaining until the seventh day. At this point, the cumulative urinary flow increased from 36.46 ± 3.84 ml in control animals to 58.77 ± 7.65 ml in rats treated with AEAE. The cumulative urinary volume found in AEAE-treated animals during all experimental period was not different than values obtained in animals treated with HCTZ.

The effects of prolonged treatment with AEAE (20, 65 and 200 mg/kg) and hydrochlorothiazide on electrolyte (Na^+, K^+, Cl^-, and Ca^{+2}) excretion are presented in Figs. 2 and 3. Urinary excretion of Na^+(after the second day), K^+(from the first day) and Cl^- (after the first day) was significantly increased in groups treated with AEAE (200 mg/kg) when compared to controls groups (Figs. 2B and 3A-B).

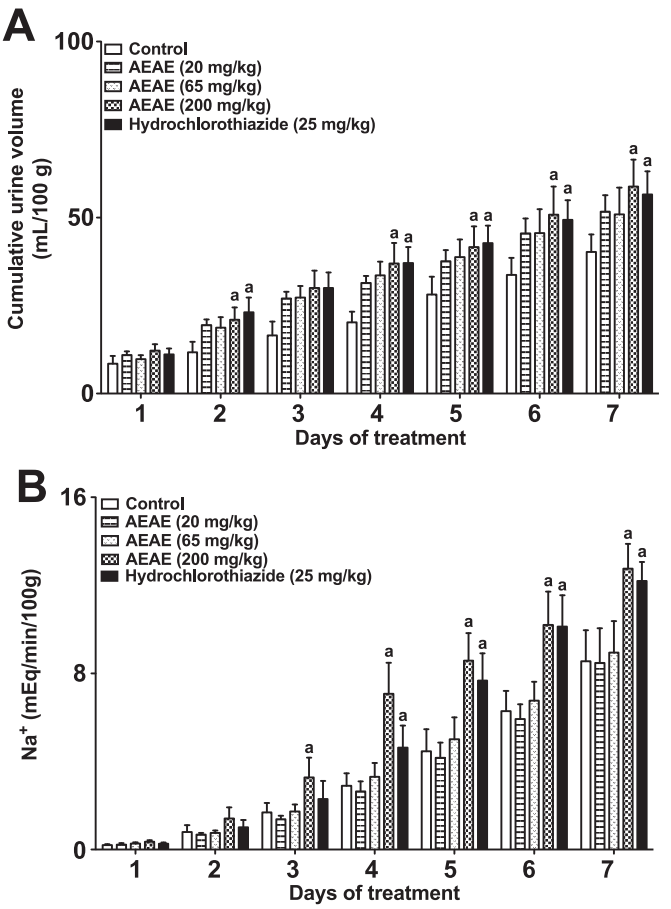

Fig. 2. Effect of prolonged oral treatment with EAEA on cumulative urinary volume (A) and Na^+ excretion (B) of rats. The urine samples were collected every day until 7 days, and cumulative urinary volume and electrolyte levels were analyzed. Each bar represents the mean of five animals and the vertical lines show the S.E.M. [a]denote the significance levels in comparison with the control group (two-way ANOVA followed by Bonferroni test) ([a]$p < 0.05$).

Although urinary Na^+, K^+, and Cl^- excretion induced by AEAE did not differ from values observed with the use of hydrochlorothiazide, group treated with only this extract showed increased amounts of Ca^{+2} in urine (Fig. 3C). Moreover, smaller AEAE doses (20 and 65 mg/kg) did not affect the urinary concentration of any of the measured electrolytes. All other urinary (pH and density) or serum parameters (Na^+, K^+, total protein, ALT, AST, urea and creatinine) showed no significant differences when compared to the control group (Table 3).

Table 2
Effect of acute oral administration of aqueous extract obtained from *Alibertia edulis* (AEAE) on urinary electrolyte excretion.

Group	El_{Na+} (mEq/min/100 g)	El_{k+} (mEq/min/100 g)	El_{Cl-} (mEq/min/100 g)	El_{Ca++} (mEq/min/100 g)	Saluretic index[b]			
					Na^+	K^+	Cl^-	Ca^{++}
Control	0.29 ± 0.02	0.18 ± 0.05	0.35 ± 0.08	0.007 ± 0.0006	–	–	–	–
AEAE (20 mg/kg)	0.20 ± 0.02	0.32 ± 0.06	0.37 ± 0.08	0.006 ± 0.0004	0.68	1.77	1.05	0.85
AEAE (65 mg/kg)	0.43 ± 0.06	0.22 ± 0.04	0.56 ± 0.08	0.008 ± 0.0003	1.48	1.22	1.60	1.14
AEAE (200 mg/kg)	0.82 ± 0.16[a,b]	0.65 ± 0.15[a,b]	1.07 ± 0.15[a,b]	0.010 ± 0.004[a]	2.82	3.61	3.05	1.42
HCTZ (25 mg/kg)	0.57 ± 0.08[a,b]	0.38 ± 0.04[a]	0.81 ± 0.11[a,b]	0.008 ± 0.001	1.96	2.11	2.31	1.14

Values are expressed as mean ± S.E.M. of five rats in each group in comparison with the control using one-way ANOVA followed by Bonferroni's test.
[a] $p < 0.05$.
[b] Saluretic index = mEq/min/100 g problem group/ mEq/min/100 g control group. **El**: Excreted load; **HCTZ**: hydrochlorothiazide.

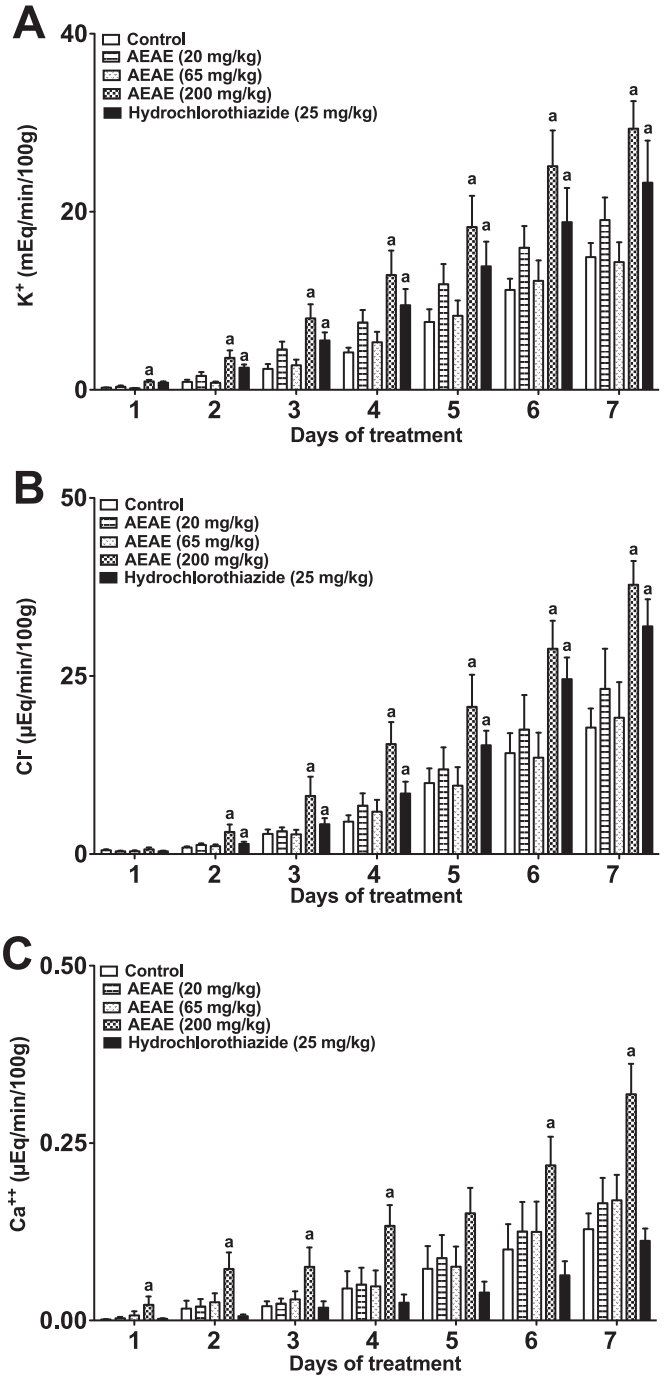

Fig. 3. Effect of prolonged oral treatment with EAEA on cumulative urinary K^+(A), Cl^- (B), and Ca^{+2} (C) excretion (B) of rats. The urine samples were collected every day until 7 days, and cumulative electrolyte levels were analyzed. Each bar represents the mean of five animals and the vertical lines show the S.E.M. [a]denote the significance levels in comparison with the control group (two-way ANOVA followed by Bonferroni test) ([a]$p < 0.05$).

3.5. AEAE induces important antioxidant properties

The Table 4 show the antioxidant activities of the AEAE and rutin (positive control) assessed using the DPPH radical scavenging and ferric ions reducing power assay. *Alibertia edulis* aqueous extract produced high DPPH scavenging activity, with estimated values of 69.91 ± 0.37% at 150 µg/ml. However, the activity of AEAE is lesser than the rutin (74.94 ± 0.12% at 10 µg/ml). Similarly, the reducing ability of AEAE (103.05 ± 0.13 µM Fe2+) was also high, but lower than the positive control (148.91 ± 0.17 µM Fe2+).

4. Discussion

In Brazil, the popular use of several natural products has increased significantly. It is likely that this behavior is related to the popular culture still highly valued in the country and the belief that substances derived from native flora are free from harmful effects (Rates, 2001). Despite the popular use is a possible indicator of medicinal species with pharmacological activity, many of these species have no studies to prove its effectiveness (Leonti, 2011).

The state of Mato Grosso do Sul, located in the mid-western region of Brazil has one of the richest biomes of the world. In this state, species from two distinct and unique biomes (Pantanal and Cerrado) are found in the same region. Many indigenous, mestizo and riverine communities with valuable knowledge about the benefits of different poorly studied species live in this rich biodiversity (Myers et al., 2000; Bueno et al., 2005; Cordeiro et al., 2014). In this work, we propose to evaluate these plants and the possible hypotensive and diuretic activity of an important medicinal species belonging to these biomes. In fact, we can prove that the aqueous extract obtained from the decoction of *Alibertia edulis* leaves is able to induce significant hypotensive and antihypertensive activity in Wistar rats; and in addition, show that part of this effect may be due to a significant acute and prolonged diuretic activity.

In general, several medicinal plants popularly known as diuretic have not been validated in controlled preclinical trials (Wright et al., 2007). In fact, some of this effect can be attributed to the increased renal hydrostatic pressure caused by the expansion of plasma volume resulting from the ingestion of large amounts of water (for review see Ivy and Bailey (2014)). Thus, it is very important not only to directly measure urinary volume, but also the increased urinary concentration of electrolytes, especially Na^+ and Cl^-. Currently, it is known that virtually all reference diuretics lead to substantial elimination of Na^+, Cl^- and other electrolytes, including K^+. In fact, one of the most important diuretic classes, inhibitors of Na+/K+/2Cl- symporter (loop-diuretics e.g. furosemide), result in substantial elimination of Na^+, K^+, Cl^-, and Ca^{+2}, and is routinely used to reduce cardiac output and, therefore, blood pressure (Oh and Han, 2015).

In clinical practice, there are few medicinal species that are actually able to cause a massive and sustained elimination of these electrolytes. Furthermore, when this occurs, a sustained diuretic effect is even less common (Wright et al., 2007). So, what draws attention in this work, in addition to its positive ethnopharmacological aspect, is the great potential of AEAE to vigorously excrete Na^+, K^+, Cl^-, and Ca^{+2}, and furthermore, to sustain this effect for a week with little or no tolerance to diuretic response. A common problem with several high potency diuretics refers to their reduced effectiveness after acute and sustained use. Acutely, this effect may be due to reduced renal perfusion pressure caused by the significant reduction in blood pressure, and in the long term, this effect is partly due to increased reabsorption of various electrolytes by the proximal tubule of the nephron (Kim, 2004). In fact, after the first 8 h of treatment, AEAE induced an important counter-regulatory response able to compensate the elimination of water and electrolytes to levels similar to the control group (in 24 h). However, the diuretic capacity of AEAE was properly re-established after the first or second day of treatment, showing that its diuretic effectiveness remains after sustained treatment.

As AEAE showed a potent acute diuretic activity, we chose to assess whether this effect would be able to affect blood pressure of rats with renovascular hypertension. The choice of this experimental model (2K1C) was due to the fact that renovascular hypertension is very sensitive to diuretic drugs (Sica, 2008). As expected, acute administration of AEAE induced a potent antihypertensive effect, which can also be observed in normotensive animals. We believe that this effect, which occurs before the renal elimination of salt and water, may be due to some acute vasoactive response, similarly to what occurs after the administration of other diuretics of clinical use (Chan et al., 2012).

Table 3
Effect of prolonged oral administration of aqueous extract obtained from *Alibertia edulis* (AEAE) on plasma Na^+, K^+, urea, creatinine, total protein, ALT and AST levels in rats.

Group	Na+(mmol/L)	K+(mmol/L)	Urea (mg/dL)	Creatinine (mg/dL)	Total protein (mg/dL)	ALT (mg/dL)	AST (mg/dL)
Control	153.6 ± 3.1	5.5 ± 0.3	45 ± 4.3	0.31 ± 0.02	5.8 ± 0.2	31 ± 2.9	129 ± 15.8
AEAE (20 mg/kg)	152.2 ± 4.3	5.3 ± 0.2	47 ± 2.0	0.33 ± 0.05	6.1 ± 0.4	35 ± 4.9	164 ± 24.1
AEAE (65 mg/kg)	153.7 ± 3.8	5.2 ± 0.2	45 ± 2.3	0.28 ± 0.02	5.8 ± 0.2	37 ± 2.9	165 ± 23.6
AEAE (200 mg/kg)	149.9 ± 4.1	5.0 ± 0.3	50 ± 5.1	0.27 ± 0.03	5.5 ± 0.3	31 ± 3.8	122 ± 15.0
HCTZ (25 mg/kg)	150.2 ± 3.7	5.5 ± 0.4	46 ± 4.5	0.29 ± 0.02	5.9 ± 0.2	39 ± 5.3	141 ± 22.2

Values are expressed as mean ± S.E.M. of five rats in each group in comparison with the control using one-way ANOVA followed by Bonferroni's test. ALT: alanine transaminase; AST: aspartate transaminase.

Table 4
IC_{50} and maximum activity (%) of DPPH free radical scavenging and ferric reducing antioxidant power (FRAP) of aqueous extract obtained from *Alibertia edulis* (AEAE).

	DPPH free radical scavenging			FRAP assay
	IC50 (μg/ml)	%	μg/ml	TAA (equivalent at 1.000 μM of Fe^{+2})
AEAE	0.11 ± 2.3	69.91 ± 0.37	150	103.05 ± 0.13
Rutin	6.74 ± 0.001	74.94 ± 0.12	10	148.91 ± 0.17

Values are expressed as mean ± S.E.M. of triplicate experiments. TAA: total antioxidant activity.

Further studies may clarify which pharmacological mechanisms may be involved in the cardiovascular effects of AEAE and show its correlation with acute and sustained diuretic response.

Another interesting data obtained with AEAE refers to its significant "in vitro" antioxidant activity. Phytochemical analyses have shown that the species has several phenolic compounds, including flavonoids, which classically are important antioxidants (Chanet et al., 2012). It has long been known that several hypertensive stimuli promote the production of reactive oxygen species in the brain, kidneys, and the vasculature, contributing to the establishment of the hypertension (Harrison and Gongora, 2009). Furthermore, it is known that antioxidant drugs can prevent further vascular damage due to oxidative stress, leading to a better prognosis in essential hypertension patients (Mahajan et al., 2007). It is still premature to say whether caffeic acid or flavonoid glycoside derivatives identified in BF may be directly involved with cardiovascular and renal responses observed in this study. However, the various classic recent studies (Hügel et al., 2016) allow us conjecturing that these compounds may be potential candidates for antioxidant, hypotensive and diuretic effects presented herein.

The above data show that AEAE have enough efficiency to become an herbal product endowed with important hypotensive and diuretic activity. On the other hand, one of the greatest challenges is still featuring a standard pharmaceutical preparation, which can be effective in controlled clinical trials. Future efforts should aim at outlining the pharmacokinetic profile of these drugs and form a basis for the development of safe and effective therapeutic approaches, and if possible, with low cost.

5. Conclusion

The data presented here show that the decoction obtained from *Alibertia edulis* leaves presents a potent diuretic activity, as well as a significant hypotensive and antihypertensive effect. In addition, this study may confirm part of the pharmacological activity popularly attributed to this species and opens perspective for the use of this preparation in various renal and cardiovascular diseases.

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