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Involvement of bradykinin B_2 and muscarinic receptors in the prolonged diuretic and antihypertensive properties of *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli

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ABSTRACT

Background: Although *Echinodorus grandiflorus* (Cham. & Schltr.) Michel are used in Brazilian folk medicine as a diuretic drug, to date, no study has evaluated the mechanisms involved in this activity after prolonged administration in rats.
Aim of the study: Evaluate the possible mechanisms involved in the prolonged diuretic activity of ethanol soluble fraction obtained from Echinodorus grandiflorus (ES-EG) and to assess its relationship with hypotensive and antihypertensive activity using normotensive rats and those with renovascular hypertension (2K1C).
Methods: The diuretic effects of ES-EG (30-300 mg/kg; p.o.) were compared with hydrochlorothiazide in a repeated-dose treatment for 7 days. The urinary volume and sodium, potassium, chloride, bicarbonate contents, conductivity, pH and density were estimated in sample collected in 24 h for 7 days. Plasma sodium, potassium, total protein, urea, creatinine, aldosterone, vasopressin, nitrite, acetylcholinesterase concentration and angiotensin converting enzyme (ACE) activity were measured in samples collected at the end of the experimental period (seventh day). Using pharmacological antagonists or inhibitors, the involvement of bradykinin, prostaglandin, acetylcholine and nitric oxide (NO) in ES-EG-induced diuresis was determined. In addition, activities of erythrocytary carbonic anhydrase and renal Na+/K+/ATPase were evaluated in vitro.
Results: ES-EG increased diuresis similarly to hydrochlorothiazide and also presented HCO3-sparing effects and increased serum nitrite levels. Moreover, the intraduodenal administration of ES-EG induces significant hypotensive and antihypertensive effects in 2K1C rats. Previous treatment with HOE-140, indometacin and atropine fully avoided the diuretic effect of ES-EG, and including L-NAME pre-administration, it prevented the hypotensive and hypertensive activity induced by ES-EG. In addition, the association between HOE-140 and atropine or indometacin and L-NAME fully inhibited the hypotensive and antihypertensive effects of ES-EG. The 7-day treatment with ES-EG resulted in increased plasma nitrite levels. All other parameters were not affected by treatment with ES-EG.
Conclusions: Our results suggest that the mechanisms through which *Echinodorus grandiflorus* extracts induce prolonged diuresis and reduce blood pressure in normotensive and 2K1C rats are mainly related to activation of muscarinic and bradykinin receptors with direct effects on prostaglandins and nitric oxide pathways.

Abbreviations: ACE:, angiotensin-converting enzyme; ACTZ:, acetazolamide; ANOVA:, analysis of variance; BW:, body weight; °C:, degree Celsius; CID:, collision-induced dissociation; CG-MS:, chromatography-mass spectrometry; EDTA:, ethylenediaminetetraacetic acid; EI:, excretion load; ELISA:, enzyme linked immunosorbent assay; eV:, electron-volts; ES-EG:, ethanol soluble fraction obtained from *Echinodorus grandiflorus*; ETOH:, ethanol; FWHM:, full width at half maximum; HOE-140:, icatibant acetate; HR:, heart rate; HCTZ:, hydrochlorothiazide; HUEM:, herbarium of the Universidade Estadual de Maringá; INDO:, indometacin; LC-UV-MS:, chromatography-ultra-violet-mass spectrometry; L-NAME:, N-ω-nitro-L-arginine methyl ester; MAP:, mean arterial pressure; NO:, nitric oxide; NOx:, nitrate/nitrite serum; PGI_2:, prostacyclin; RBC:, red blood cells; ROS:, reactive oxygen species; SBP:, systolic blood pressure; SEM:, standard error of the mean; UFPR:, Federal University of Paraná; UNIPAR:, Universidade Paranaense; UPLC:, ultra performance liquid chromatography; Ux:, electrolytes concentration; V:, volts; 2K1C:, two-kidney, one-clip Goldblatt hypertensive rats.

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Introduction

In recent decades, several studies have been conducted worldwide in order to evaluate the possible diuretic properties of different natural products. Most of these studies were only qualitative and not aimed at investigating the molecular mechanisms involved in these effects (Wright et al. 2007). Only in recent years, data emphasizing the mode of action of some diuretic plants and the relationship of these effects to their secondary metabolites have been published (de Souza et al. 2013; Gasparotto Junior et al. 2009, 2012; Leme et al. 2013).

In Brazil, several medicinal species are used as diuretic drugs, but most of them lack pharmacological studies showing the molecular pathways that might be contributing to these effects. Among them, *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli, a native species that occurs from southern Mexico to Brazil stands out. This plant belongs to family Alismataceae and sporadically grows as a plant cover on flooded or wet soils. In Brazil, it is popularly known as "chapéu de couro", "chá-de-campanha" or "erva do brejo", and its leaves are habitually used as a hot water infusion orally administered as diuretic, hypotensive, hypolipidemic, anti-inflammatory and analgesic (Bolson et al. 2015; Brugiolo et al. 2009; Lorenzi and Matos 2008). Recently, this species had its monograph described in the 5th edition of the Brazilian Pharmacopoeia (Brazil, 2011, 2010) and the infusion obtained from its leaves has been indicated as mild diuretic and anti-inflammatory according to herbal form of the Brazilian Pharmacopoeia in its 1st edition (Brazil 2011).

Previous pharmacological studies have shown that extracts obtained from *Echinodorus grandiflorus* leaves induce antiedematogenic (Garcia et al. 2010), antihypertensive (Lessa et al. 2008), vasodilator (Tibiriçá et al. 2007) and immunosuppressive (Pinto et al. 2007) effects, explaining, at least in part, its popular use against cardiovascular diseases. Previous phytochemical studies conducted with *Echinodorus grandiflorus* leaves have demonstrated the presence of fatty acids, diterpenoids, phenolic acids, flavonols, alkaloids, saponins and tannins, and their polyphenolic compounds have great potential to be the secondary metabolites responsible for their therapeutic properties (Brugiolo et al. 2009; Garcia et al. 2010).

Taking into account the popular use and previous evidence of effectiveness and therapeutic potential of *Echinodorus grandiflorus* against cardiovascular diseases, this study was conducted in order to investigate the molecular mechanisms involved in the possible diuretic effects induced by the aqueous extract obtained from *Echinodorus grandiflorus* and verify its relationship with a potential hypotensive and anti-hypertensive effect using normotensive rats and with renovascular hypertension (2K1C).

Material and methods

Drugs

Hydrochlorothiazide (HCTZ; thiazide diuretic), acetazolamide (ACTZ; carbonic anhydrase inhibitor), ouabain octahydrate (sodium potassium ATPase inhibitor), *N*-ω-Nitro-L-arginine methyl ester (L-NAME; NO synthase inhibitor), icatibant acetate (HOE-140; B_2 bradykinin receptor antagonist), indometacin (cyclooxygenase inhibitor), atropine (muscarinic receptor antagonist), and captopril (angiotensin converting enzyme inhibitor), were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other drugs and reagents used were purchased from Merck (Darmstadt, Germany).

Plant material and preparation of aqueous Echinodorus grandiflorus extract

Echinodorus grandiflorus leaves were collected on February 2014 from the botanical garden of the Universidade Paranaense (UNIPAR) (Umuarama, Brazil) at 430 m altitude above sea level (S23°47'55–W53°18'48). A voucher specimen of this species is cataloged in the Herbarium of the Universidade Estadual de Maringá (HUEM) under number 20810.

The plant material was air-dried in an oven at 37 °C for 5 days, and then cut and pulverized. The extracts were obtained by infusion in a similar manner to that used popularly in Brazil (Bolson et al. 2015). 1 L of boiling water was poured over 60 g of dried ground leaves; the container was closed, and the extraction was allowed to proceed until room temperature was reached (~ 6 h). The infusion was treated with 3 volumes of EtOH, which gave rise to a precipitate and an ethanol soluble fraction (ES-EG; yield 9.54%). All preparations were freeze-dried and maintained at room temperature until analyses.

Phytochemical investigation

The ethanol-soluble fraction (ES-EG) was analyzed by liquid chromatography-ultraviolet-mass spectrometry (LC-UV-MS) using an Acquity-UPLC (Waters) with LTQ-Orbitrap-XL (Thermo-Scientific). The chromatographic separation was developed on a C18 HSS T3 column (Waters) with 100 × 2.1 mm and 1.7 m of particle, at 60 °C. The solvent was composed of 0.1% formic acid (v/v) in water (A) and acetonitrile (B), with a gradient increasing solvent B: 0–30% in 13 min, –80% in 20 min, then returning to initial condition in 21 min, with flow rate of 400 μl/min. The sample was prepared at 1 mg/ml and held at room temperature (22 °C), the injection volume was 2 μl.

Detection was developed by photodiode-array at 200–400 nm and by mass spectrometry in positive and negative polarities, with mass range of 100–2000 *m/z*. The MS set up was: positive ions—spray voltage at 4.5 kV, capillary at 30 V, tube lens at 120 V, source temperature at 350 °C with nitrogen for desolvation at flow rate of 60 arbitrary units (a.u.) in sheath gas and 20 a.u. in auxiliary gas. For the negative ions, only voltages were different, being 3.5 kV on spray voltage, 10 V on capillary and 130 V on tube lens. For the structural characterization, fragmentation analyses were held by collision-induced dissociation (CID), with energy range of 20–25 eV. Mass accuracy was obtained by external calibration and the ion resolution was set at 7500 FWHM (full width at half maximum).

ES-EG sample was also analyzed by gas chromatography–mass spectrometry (GC–MS – Varian 4000) in order to characterize the composition of monosaccharides from glycosides observed by LC-UV-MS. ES-EG (1 mg) was previously hydrolyzed in 1 M trifluoroacetic acid for 12 h at 100 °C. The sample was dried at N_2 stream and the released monosaccharides were reduced with $NaBH_4$ to give their respective alditols, which were acetylated with 200 μl of acetic anhydride and pyridine (1:1, v/v), for 12 h at room temperature. The reaction was stopped by addition of 200 μl of methanol and the solvents were evaporated under N_2 stream. The resulting alditol acetates were analyzed by GC–MS with capillary column DB-225MS (30 m x 0.25 mm). The injector was held at 250 °C and column-oven heated from 50 to 220 °C, at 40 °C/min, for 20 min. Monosaccharide identification was based on the mass spectra fragmentation profile from electron ionization (70 eV), with ion trap analyzer, compared with authentic standards.

Pharmacological studies

Animals

Male Wistar rats (250–300 g) were used in experiments. The animals were provided by Universidade Federal do Paraná (UFPR, Curitiba/PR, Brazil), and were kept in a temperature- and light-controlled room (22 ± 2 °C; 12-h light/dark cycle) with free access to water and food. All experimental procedures adopted in this study were previously approved by the Institutional Ethics Research Committee of Universidade Paranaense (UNIPAR, Brazil; authorization number 25454/2014).