

Protective Cardiorenal Effects Of *Tropaeolum majus* L. In Rats With Renovascular Hypertension

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ABSTRACT

Background: *Tropaeolum majus* L. (Tropaeolaceae) is an important medicinal plant belonging to the Tropaeolaceae family, which has 80 species distributed in South and Central America. In Brazil, it is distributed practically throughout the entire territory and it is popularly known as “capuchinha.” In folk medicine its leaves are widely used for treatment of cardiovascular disorders. Despite consistent data showing some acute effects on renal and cardiovascular system, no study has investigated whether *Tropaeolum majus* extracts is able to exert cardiorenal protective effects after 30 days of treatment, i.e. in a sustained manner. **Objective:** Investigate the prolonged cardiorenal protective effects of ethanolic extract obtained from *Tropaeolum majus* (EETM) in rats with renovascular hypertension. **Material and Methods:** First, EETM was obtained and chemically characterized. Then, we investigated the possible antihypertensive and diuretics effects, including effects on renal haemodynamics, after prolonged treatment (30 days) with EETM (3, 30 and 300 mg/kg). Finally, we examined whether treatment with EETM may affect the angiotensin-converting enzyme (ACE) and oxidative stress, preventing heart and kidney damage in two-kidney, one-clip (2K1C) Goldblatt hypertensive rats. **Results:** Prolonged treatment with EETM was able to prevent the evolution of renovascular

hypertension in 2K1C rats, inducing important renoprotective effects and reducing systemic blood pressure and cardiac hypertrophy. Moreover, it was also identified that these effects may be directly related to significant ACE inhibitory activity and reduction of oxidative stress. **Conclusion:** This study has brought new scientific evidence of preclinical efficacy of EETM as a cardiorenal protective agent in rats with renovascular hypertension.

Key words: Antihypertensive, Cardioprotective, Diuretic, Renoprotective, *Tropaeolum majus* L.

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INTRODUCTION

Hypertension, a worldwide public health problem, is responsible for millions of deaths per year.¹ The disorder, characterized by multiple factors that involves complex interactions between the mechanisms of homeostatic control and environmental factors, is an important risk factor for cardiovascular complications.² Since cardiovascular diseases are progressive and chronic disorders, the treatment has to be performed for a prolonged period resulting in tachyphylaxis, toxicity, and elevate financial cost, which culminates in low adhesion of patients. Thus, drugs based on natural products can be a good therapeutic strategy.³ However, most of studies evaluated only acute cardioprotector effects of natural products.

One of these plants is *Tropaeolum majus* L. (Tropaeolaceae), an important medicinal plant belonging to the Tropaeolaceae family, which has 80 species distributed in South and Central America. In Brazil, it is distributed practically throughout the entire territory and it is popularly known as “capuchinha”.⁴ In folk medicine its leaves are widely used for urinary infections, asthma, constipation, and cardiovascular disorders.⁴⁻⁶ Various compounds belonging to different classes of secondary metabolites have been isolated and identified in *T. majus* preparations. Glucosinolates,⁷ terpenoids,⁸ and flavonoids, mainly isoquercitrin (chemical marker for the quality control),⁹ are the three major groups of secondary

metabolites in the plant, which have been considered responsible for the medicinal properties.

The pharmacological properties attributed to this plant in preclinical studies include antithrombin,¹⁰ antibacterial,¹¹ diuretic,^{12,13} and acute antihypertensive activity.⁹ Toxicological parameters of this species were deeply investigated and showed important safety information. No toxic effects were observed after acute treatment or within 28 days of *T. majus* extract administration.^{12,14} In addition, *T. majus* extract was unable to produce (anti) estrogenic or (anti) androgenic activities in the short-term in *in vivo* screening assays.¹⁵

Despite consistent data showing some acute effects on renal and cardiovascular system, no study has investigated whether *T. majus* extract is able to exert cardiorenal protective effects after 30 days of treatment, i.e. in a sustained manner. So, the present study investigated the prolonged cardiorenal protective effects of ethanolic extract obtained from *T. majus* (EETM) in rats with renovascular hypertension. First, EETM was obtained and chemically characterized. Then, we investigated the possible antihypertensive and diuretics effects, including actions on renal haemodynamics, after prolonged treatment (30 days). Finally, we examined whether treatment with EETM can produce some response on the angiotensin-converting enzyme (ACE) and oxidative stress, preventing tissue damage in two-kidney, one-clip (2K1C) Goldblatt hypertensive rats.

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MATERIAL AND METHODS

Drugs

Furosemide, isoquercitrin (ISQ), N-hippuryl-L-histidyl-L-leucine hydrate, o-phthalaldehyde, and captopril, were obtained from Sigma-Aldrich[®] (St. Louis, MO, USA). All other drugs and reagents used were purchased from Merck[®] (Darmstadt, Germany).

Chemical characterization and standardization of the isoquercitrin content

Plant material and preparation of EETM

T. majus leaves were collected on February 2013 from the botanical garden of the Paranaense University (UNIPAR; Umuarama-PR, Brazil; S23°47'55"-W53°18'48"). A voucher specimen is cataloged at the Herbarium of the University under number 2230. The plant material was air-dried and pulverized. The resulting product was macerated with 70% ethanol for seven days. The solvent was removed using a spray-drying equipment yielding 12.55%.

Sample solution

Accurately weighed amounts of the EETM were dissolved in methanol/water (1:1) to prepare a 5.00 µg/mL solution and the ISQ was identified by preparative High-Performance Liquid Chromatography (HPLC) analysis and quantified using the same chromatographic conditions as used for ISQ standard.

Standard solution

In order to prepare standard solutions, ISQ standard was dissolved in methanol/water (1:1, v/v) in nine different concentrations, from 1.00 to 20.00 µg/mL. Preparative and quantitative HPLC analyses were conducted using the conditions described.⁹

High-Performance Liquid Chromatography (HPLC) measurements

HPLC analyzes were performed on a Shimadzu LC-2010C system coupled to a UV detector. For analysis, we used a RP-18 column (5 µM, 225 × 4.6 mm) (LiChrospher[®], Merck KGaA, Darmstadt, Germany) protected by a guard Merck RP-18 column (5 µM, 4.0 × 3.0 mm). Peak areas were calculated by an integrator Shimadzu LASS-VP7.2.1.

The analyses were carried out in triplicate, at a flow rate of 0.6 mL/min, with the UV detector set at 330 nm and an injection volume of 10 µL. Calibration graphs were plotted showing a linear relationship between concentrations versus peak areas for the reference compound. The attribution of the chromatographic peak was based on the retention times of the single compound and confirmed by comparison with the isolated standard. Under our working conditions, the mean of retention time for ISQ was 41.98 min. The concentration of the ISQ was calculated from the experimental peak area by analytical interpolation on a standard calibration line. The limit of quantification (LOQ) of 1.25 g/mL for EETM was determined using signal-to-noise ratio.

Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (ESI(+)-FT-ICR MS)

The ESI(+)-FT-ICR MS analysis was performed using a micrOTOF hybrid quadrupole time-of-flight esquire 3000 plus mass spectrometer (Bruker Daltonics, USA) equipped with an Apollo II electrospray ion source in positive ion mode. For acquisition of mass spectra, ions were selected using an isolation width of ±4 Da and fragmented using nitrogen as the collision gas with collision energies in the range of 10–30 eV. The eluted compounds in methanol/water 1:1 (v/v) were analyzed in positive ion mode with the following instrument settings: nebulizer gas, 1.6 bar; dry gas, nitrogen, 4L/min, 180°C; capillary, -5500 V; end plate offset, -500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; insource CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas,

argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 µs; prepulse storage, 5 µs; pulser frequency, 10 kHz. The calibration was performed using a Cole Palmer syringe pump connected to the interface, passing a solution of sodium acetate cluster containing 5 mM sodium hydroxide in a sheath liquid of 0.2% acetic acid in water/isopropanol 1:1 (v/v).

Nuclear magnetic resonance (NMR) measurements

¹H NMR spectra were recorded at room temperature on a Bruker 300 spectrometer (Institute of Chemistry, UFMS, Brazil), 10% in Acetone-D₆ solution at 298K operating at 300.132. Data processing was carried out on a Solaris workstation. The ¹H NMR parameters were as follows: spectral width, 4789.27 Hz; data points, 64 k, zero-filled to 64 k; acquisition time, 6.84 s and digital resolution, 0.07 Hz. Spectral widths of 4789.27 Hz were used for ¹H. The ¹H chemical shifts are given on the δ scale (ppm) and were referenced to internal Acetone; coupling constants (*J*) are reported in hertz (Hz). The abbreviations s, d, and m were used for singlet, doublet, and multiplet, respectively.

Pharmacological studies

Animals

Male Wistar rats (250–280 g) were obtained by Federal University of Paraná (UFPR, Curitiba-PR, Brazil), and were kept in light- and temperature -controlled room (12-h light/dark cycle; 22 ± 2°C) with *ad libitum* access to food and water. All experimental protocols were previously approved by the Institutional Ethics Committee of the UFPR (authorization number 240) and were performed in accordance with international standards and ethical guidelines on animal welfare.

Induction of renovascular hypertension (2K1C Goldblatt model)

The Goldblatt model of hypertension was induced according to procedure described.¹⁶ Initially, rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) by intraperitoneal route. The left renal artery was exposed by retroperitoneal incision and dissected. A silver clip (lumen of 0.22 mm) was placed around the artery for partial occlusion; in negative control animals (SHAM operated) the artery was not clipped. After 30 days, the systolic blood pressure (SBP) was measured using the tail-cuff method. Only hypertensive rats (SBP above 145 mm Hg) were used in the experiments.

Experimental design

Three weeks after hypertension induction, animals were divided into 12 groups (*n* = 5; six groups to studies on renal function and six groups to hemodynamic study) and submitted to the following treatments, once a day (by gavage), during 30 days. Two SHAM operated groups were orally treated with filtered water (vehicle) and defined as negative control. Two hypertensive groups were orally treated with filtered water (vehicle) and defined as positive control. Another six groups of 2K1C rats received EETM (3, 30 and 300 mg/kg). Finally, two additional groups of rats received furosemide (FURO; 25 mg/kg) or captopril (CAP; 60 mg/kg).

Studies on renal function

Diuretic activity

The diuretic activity was assessed according to methods previously described with some modifications.¹⁷ Rats were food-deprived overnight (12 h). On the thirtieth day of treatment all animals received an oral load of isotonic saline (0.9% NaCl, 5 mL/100 g) to impose a controlled water and salt balance. Immediately after treatment, rats were placed in metabolic cages. Urine was collected and its volume was recorded for 8 h (expressed as mL/100 g of body weight). At the end of the experimental period, animals were euthanized (by decapitation) and blood was collected for biochemical analysis.

Samples collection and storage

Plasma and serum were obtained by centrifugation (1500 g, 10 min, 4°C), and stored at -80°C until analyses. Kidneys and hearts were excised, cleaned and weighed. Atria and right ventricle were then removed and the remaining left ventricle was weighed. The renal, heart, and the left ventricle indexes were calculated by dividing the absolute weight of these organs by the body weight of animals on the day of the euthanasia.

Plasma and urine analytical procedures

The urinary and plasmatic Na⁺ and K⁺ levels were quantified by flame photometry (Quimis model Q398112). Urinary Cl⁻ and HCO₃⁻ concentrations were quantified by titration. pH and conductivity were directly determined on fresh urine samples using Q400MT pH meter and Q795M2 conductivity meter (Quimis Instruments, Brazil), respectively. Density was estimated by weighing using Mettler AE163 (± 0.1 mg) analytical scale on urine volume measured using Nichiryo micropipette. Total plasma protein, urea and creatinine levels were determined by enzymatic method for automated analyzer BM/Hitachi 912 (Cobas Mira, Roche, Indianapolis, USA).

Hemodynamic study

Direct blood pressure, heart rate, and renal cortical blood flow measurement

At the end of experimental period, 2K1C rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg), intramuscularly administered and supplemented at 45 to 60 min intervals. Animals were allowed to spontaneously breathe through a tracheotomy. The left carotid artery was cannulated and connected to a pressure transducer coupled to a PowerLab⁷ recording system (Chart, v 4.1; all from ADI Instruments; Castle Hill, Australia) to record both mean arterial pressure (MAP) and heart rate (HR). For measuring renal cortical blood flow (RCBF) a standard plastic tube (DP2b, Moor Instruments) was placed on the ventral surface of the contralateral not clipped kidney (renal capsule below 2 mm) according to previous methods.¹⁸ After 15 min for stabilization of the surgical procedure, changes in MAP, HR, and RCBF were recorded for 30 min. At the end of the experiments, animals were euthanized with an overdose of thiopental (over 40 mg/kg, i.v.).

Evaluation of mechanisms involved in the renal and cardioprotective effects of EETM

Effects on the renin-angiotensin system

ACE activity was determined by indirect fluorimetry as previously described.¹⁹ For this, serum samples (10 µL) were incubated for 15 min at 37°C with 490 µL of assay solution (composition: NaCl 0.9 M and Hip-His-Leu at 5 mM in 0.4 M sodium borate buffer, pH 8.3) The reaction was stopped by addition of 1.2 mL of NaOH (0.34 N). The production of His-Leu was measured fluorometrically (365 nm excitation and 495 emission, Aminco Model J4-7461 fluorometer, American Instrument Co., Silver Springs, MD) after the addition of 100 µL of o-phthalaldehyde (20 mg/mL in methanol), and 200 µL of HCl (3 N), followed by centrifugation (800 g, 5 min) at room temperature. To correct intrinsic fluorescence of plasma, time-zero blank samples were prepared by adding plasma after NaOH treatment. In addition, aldosterone levels were measured by Enzyme Linked Immunosorbent Assay (ELISA, Immuno-Biological Laboratories, Inc). All measurements were made in triplicate.

Evaluation of serum lipid peroxidation (TBARS)

Thiobarbituric acid (TBARS) levels were measured using commercial assay kits (Nanjing Jiancheng Institute, Nanjing, China) on a spectrophotometer (DU7400, Beckman Co., Fullerton, CA, USA), according to manufacturer's instruction. Absorbance was read at 535 nm in a microplate reader, and the concentration of TBARS was expressed as mmol/L.

Determination of nitrate/nitrite serum (NOx)

The plasma nitrite concentration was determined by reducing nitrate enzymatically using the enzyme nitrate reductase, as previously described.²⁰ Plasma samples were deproteinized with zinc sulfate (30 mmol) and diluted 1:1 with Milli-Q water. For the conversion of nitrate to nitrite, samples were incubated at 37°C for 2 hours in the presence of nitrate reductase expressed in *Escherichia coli*. After the incubation period, samples were centrifuged (800 g, 10 minutes) to remove the bacteria. Then, 100 µL of supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 10% phosphoric acid/0.1% alpha-naphthyl-ethylenediamine in Milli-Q water) in a 96-well plate and read at 540 nm. Standard curves of nitrite and nitrate (0-150 mM) were performed simultaneously.

Statistical analysis

The results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. *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).

RESULTS

Sample characterization

The HPLC-UV fingerprint of the EETM (Figure 1) showed characteristic distributions of the flavonoids, including the ISQ with maxima absorption at 257 and 352 nm and identified by comparison with data found in the literature.²¹ These results were confirmed by ¹H NMR analysis using isolated standard of ISQ and the structures were unequivocally confirmed by co-injection of authentic standards and identified by retention values. The HPLC quantitative method was validated for ISQ.²² The content of this compound in the EETM was 2.10%, equation of measurements was $Y = 9 \times 10^{-5} X - 0.6655$ and $R^2 = 0.9973$.

¹H NMR (300 MHz, Acetone-D₆): (ppm) 3.347 (*m*, 3H), 3.443 (*m*, 4H), 4.184 (*s*, 1H), 4.276 (*s*, 1H), 5.133 (*s*, 1H), 5.234 (*d*, *J* = 7.20 Hz, 2H), 6.276 (*d*, *J* = 1.80, 1H), 6.506 (*d*, *J* = 2.1, 1H), 6.925 (*s*, 1H), 6.953 (*s*, 1H), 7.574 (*m*, 2H), 8.014 (*d*, *J* = 1.80 Hz, 1H), 12.362 (*s*, 1H).

ESI(+)-FT-ICR MS (Figure 2) provides M of C₂₁H₂₀O₁₂, where ions [M + H]⁺, [M + Na]⁺, [M + Cl]⁺ and [M + K]⁺ with *m/z* of 465.0930, 487.0178, 499.0533 and 503.0543, with mass error = -0.33, -0.24, -0.34 and -0.22 ppm, respectively, and base peak of 350.1470 were identified.

EETM induces sustained diuretic, natriuretic, and potassium-sparing effects after 30 days of oral treatment

In 2K1C animals, treatment with EETM (300 mg/kg) was able to induce a significant increase in diuresis. The final urine volume (8h) for rats treated with EETM (300 mg/kg) was 5.2 ± 0.6 vs. 2.6 ± 0.8 mL/100g/8h (*p*<0.05) for positive control animals, being similar to values obtained after treatment with furosemide (5.1 ± 0.7 mL/100g/8h) or SHAM operated animals (4.7 ± 0.5 mL/100g/8h). Moreover, a significant increase in urinary conductivity was observed in animals treated with EETM (300 mg/kg) and furosemide, while urinary density was significantly reduced in these animals (Table 1).

Animals treated with EETM (300 mg/kg) and furosemide showed high contents of Na⁺ in urine when compared to positive controls (Table 2). The contents of urinary Na⁺ in animals treated with EETM and furosemide were 137 ± 3.0 and 140 ± 4.2 mmol/L, respectively, while in positive control group were 95 ± 4.3 mmol/L (*p*<0.05).

All animals treated with EETM showed an interesting potassium-sparing effect at all doses used (Table 2), while furosemide excreted large amounts of this ion (negative control: 29 ± 7.2 mmol/L; positive control:

30 ± 5.9 mmol/L; furosemide: 59 ± 3.2; EETM 300: 24 ± 2.9). All other parameters did not show statistically significant differences when compared to the control groups.

Prolonged treatment with EETM prevents changes in serum markers of renal function in 2K1C rats

Animals treated with EETM at dose of 300 mg/kg showed significant renoprotective effect after 30 days of treatment. Untreated 2K1C rats (positive control) showed significant increase in serum urea and creatinine, associated with reductions in total protein concentration (Table 3). These changes were not observed in animals treated with EETM (300 mg/kg). Moreover, serum sodium and potassium levels were not affected by any treatment performed.

EETM treatment prevents heart hypertrophy induced by renovascular hypertension

The effects of 30-day oral administration of *T. majus* extract on cardiac and renal indices are shown in Table 4. Heart and left ventricle weight ratio were significantly reduced in rats treated with EETM (300 mg/kg) when compared with the positive control (HW/BW ratio: positive control: 2.93 ± 0.08 vs. EETM 300: 2.45 ± 0.07 p<0.05; LVW/BW ratio: control 2.13 ± 0.07 vs. EETM 300: 1.79 ± 0.08 p<0.05). All other parameters did not show any significant differences among experimental groups.

Prolonged treatment with EETM prevents chronic blood pressure elevation in 2K1C rats

Basal MAP recorded in SHAM operated and Goldblatt hypertensive rats (positive control) after the 15-min period allowed for stabilization was 93 ± 3.81 and 138 ± 4.45 mm Hg, respectively. The oral administration of EETM caused a dose dependent antihypertensive effect (Figure 3A), reducing MAP levels to 122 ± 4.78 and 99 ± 3.93 mm Hg (100 and 300

mg/kg, respectively), with minor effects on heart rate (data not shown). In addition, an important increase in renal cortical blood flow was observed after prolonged EETM administration (100 and 300 mg/kg). Data have shown increase ranging from 15 to 30%, respectively, when compared to groups treated with vehicle alone (Figure 3B).

EETM treatment significantly reduces plasma ACE activity, serum aldosterone, and oxidative stress in 2K1C rats

The effects of 30-day oral treatment with EETM on ACE activity and serum aldosterone levels are shown in Figure 4 A-B. The plasmatic ACE activity after treatment with EETM (100 and 300 mg/kg) was reduced by 14 ± 2 and 40 ± 6%, respectively, when compared to positive control group. As expected, captopril reduced the ACE activity around 56%. Similarly, EETM (300 mg/kg) and captopril administration reduced aldosterone levels by 32 ± 6% and 45 ± 3%, respectively.

The antioxidant effect of EETM was estimated by TBARS and nitrite measurement (a marker of nitric oxide bioavailability). In 2K1C rats, EETM (300 mg/kg) was able to increase nitrite levels by approximately 78% (Figure 4C). Additionally, TBARS were reduced by approximately 52% when compared to positive control group (Figure 4D).

DISCUSSION

Currently, hypertension appears as a major public health problem worldwide, and when not properly controlled, can catalyze the emergence of various kidney and heart diseases such as stroke, renal failure and heart attack.¹ The available therapeutic arsenal for the treatment of hypertension is multivariable and highly effective, however, in various countries, the control of this disease is inappropriate, and the major reason is the poor adherence to antihypertensive medications.²³ Older age, living alone, and perception related to treatment control were significant independent factors associated with better medication adherence. Moreover, cultivating positive beliefs that hypertension is controlled by

Table 1: Effect of 30-days oral administration of *Tropaeolum majus* L. extract on urinary volume, pH, conductivity and density

Group	Urine volume (8h/100g)	pH	Conductivity (mS/cm)	Density (g/mL)
SHAM	4.7 ± 0.5	6.1 ± 0.2	14.5 ± 0.1	1014 ± 0.6
Positive control	2.6 ± 0.8 ^b	6.4 ± 0.3	15.1 ± 0.2	1016 ± 0.7
FURO (25 mg/kg)	5.1 ± 0.7 ^a	6.5 ± 0.2	17.5 ± 0.1 ^{ab}	1007 ± 0.6 ^a
EETM (3 mg/kg)	3.1 ± 0.6	6.4 ± 0.4	14.7 ± 0.2	1015 ± 0.5
EETM (30 mg/kg)	2.9 ± 0.5	6.3 ± 0.3	15.4 ± 0.1	1014 ± 0.9
EETM (300 mg/kg)	5.2 ± 0.6 ^a	6.4 ± 0.4	17.3 ± 0.2 ^{ab}	1008 ± 0.6 ^a

Values are expressed as mean ± S.E.M. in comparison to the positive control (a; p < 0.05) or SHAM operated rats (b; p < 0.05) using one-way ANOVA followed by Bonferroni's test. FURO: furosemide. EETM: ethanolic extract of *T. majus*.

Table 2: Effect of 30-days oral administration of *Tropaeolum majus* L. extract on urinary electrolyte excretion

Group	Na ⁺ (mmol/L)	K ⁺ (mmol/L)	Cl ⁻ (mmol/L)	HCO ₃ ⁻ (mmol/L)	Saluretic index ^b			
					Na ⁺	K ⁺	Cl ⁻	HCO ₃ ⁻
SHAM	110 ± 4.5	29 ± 7.2	120 ± 14	27 ± 2.0	-	-	-	-
Positive control	95 ± 4.3	30 ± 5.9	122 ± 10	25 ± 2.1	-	-	-	-
FURO (25 mg/kg)	140 ± 4.2 ^{ab}	59 ± 3.2 ^{ab}	155 ± 15	30 ± 3.1	1.49	1.96	1.27	1.20
EETM (3 mg/kg)	90 ± 6.5	33 ± 6.1	115 ± 16	24 ± 1.7	0.97	1.20	0.94	0.96
EETM (30 mg/kg)	100 ± 5.1	28 ± 4.2	112 ± 19	28 ± 2.1	1.05	0.93	0.91	1.12
EETM (300 mg/kg)	137 ± 3.0 ^{ab}	24 ± 2.9	148 ± 19	22 ± 1.6	1.44	0.80	1.21	0.88

Values are expressed as mean ± S.E.M. in comparison to the positive control (a; p < 0.05) or SHAM operated rats (b; p < 0.05) using one-way ANOVA followed by Bonferroni's test. Saluretic index = mmol/L problem group/mmol/L control group. FURO: furosemide. EETM: ethanolic extract of *T. majus*.

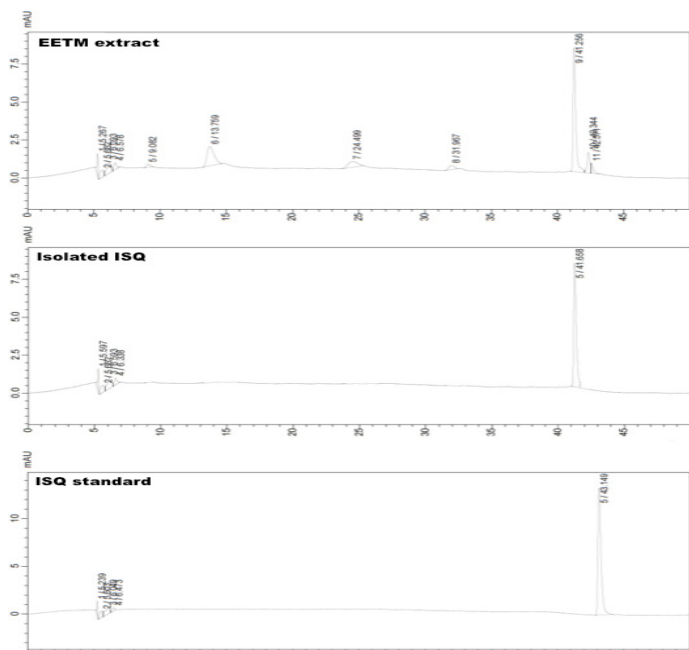


Figure 1: HPLC chromatograms of EETM extract, isolated ISQ and ISQ standard.

Table 3: Effect of prolonged oral administration of *Tropaeolum majus* L. extract on serum total protein, urea, creatinine, sodium and potassium of 2K1C rats

Group	Total protein (g/L)	Urea (mg/dL)	Creatinine (mg/dL)	Sodium (mmol/L)	Potassium (mmol/L)
SHAM	6.0 ± 0.5	28 ± 3.9	0.7 ± 0.2	144 ± 4.4	5.8 ± 1.5
Positive control	3.4 ± 0.2 ^b	59 ± 3.8 ^b	1.6 ± 0.2 ^b	141 ± 3.8	5.6 ± 2.0
FURO (25 mg/kg)	3.6 ± 0.2 ^b	47 ± 2.3 ^b	1.4 ± 0.3 ^b	143 ± 2.3	6.2 ± 1.9
EETM (3 mg/kg)	3.1 ± 0.3 ^b	50 ± 2.7 ^b	1.5 ± 0.2 ^b	141 ± 5.7	5.9 ± 1.8
EETM (30 mg/kg)	4.0 ± 0.2 ^b	42 ± 4.3 ^b	1.3 ± 0.3 ^b	144 ± 4.3	6.1 ± 1.5
EETM (300 mg/kg)	5.6 ± 0.3 ^a	31 ± 2.3 ^a	0.5 ± 0.1 ^a	149 ± 6.3	6.0 ± 2.0

Values are expressed as mean ± S.E.M. in comparison to the positive control (a; p < 0.05) or SHAM operated rats (b; p < 0.05) using one-way ANOVA followed by Bonferroni test. FURO: Furosemide. EETM: ethanolic extract of *T. majus*

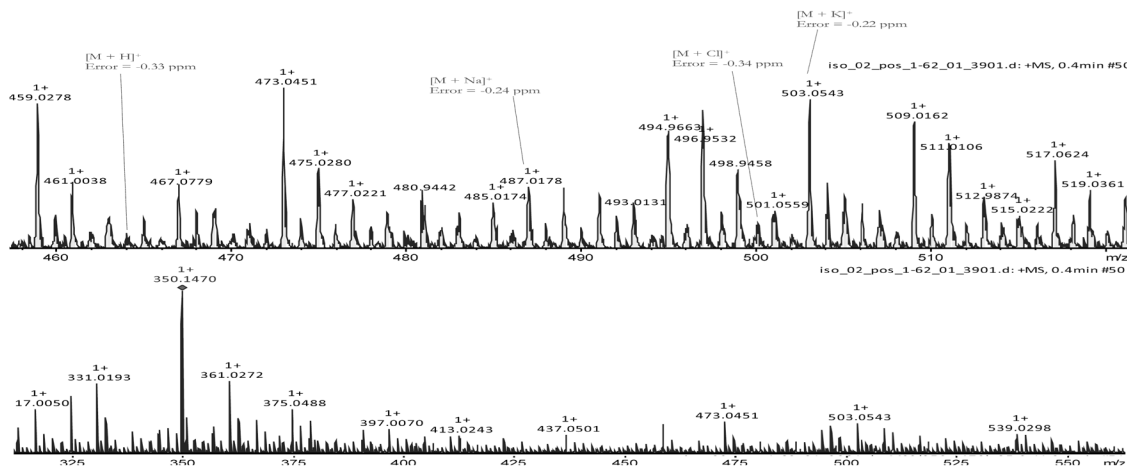


Figure 2: High-resolution mass spectrum data for isolated ISQ.

Table 4: Effect of chronic oral administration of *Tropaeolum majus* L. extract on cardiac and renal indices of 2K1C rats

Group	HW/BW ratio	LVW/BW ratio	CKW/BW ratio	SKW/BW ratio
SHAM	2.20 ± 0.05	1.60 ± 0.06	3.48 ± 0.21	1.61 ± 0.31
Positive control	2.93 ± 0.08 ^b	2.13 ± 0.07 ^b	3.51 ± 0.12	1.73 ± 0.30
CAP (60 mg/kg)	2.40 ± 0.06 ^{ab}	1.69 ± 0.06 ^a	3.44 ± 0.22	1.59 ± 0.33
EETM (3 mg/kg)	2.89 ± 0.07 ^b	2.15 ± 0.08 ^b	3.41 ± 0.17	1.55 ± 0.32
EETM (30 mg/kg)	2.79 ± 0.06 ^b	2.00 ± 0.07 ^b	3.54 ± 0.18	1.70 ± 0.26
EETM (300 mg/kg)	2.45 ± 0.07 ^{ab}	1.79 ± 0.08 ^a	3.40 ± 0.20	1.57 ± 0.35

Values are expressed as mean ± S.E.M. in comparison to the positive control (a; p < 0.05) or SHAM operated rats (b; p < 0.05) using one-way ANOVA followed by Bonferroni's test. BW: body weight; CAP: captopril; CKW: contralateral kidney weight; EETM: ethanolic extract of *T. majus*; HW: heart weight; LVW: left ventricle weight; SKW: stenotic kidney weight.

treatment is one of the most appropriate ways for adequate control of this pathology.²⁴

In recent years, several studies have been conducted to identify alternative therapies derived from natural products for use as an adjunct to conventional drug therapy in hypertension treatment. This initiative could encourage the improvement of adherence to pharmacological treatment, offering a product with great popular appeal and pharmacological effects complementary to conventional therapies.²⁵⁻³¹

In this study, it was shown for the first time that prolonged treatment with an ethanol extract obtained from *T. majus* is able to prevent the evolution of renovascular hypertension in 2K1C rats, showing a significant reno- and cardioprotective effect. According to previous data, the renin-angiotensin system (RAS) played a prominent role in the oxidative stress and renovascular hypertension.³² Since RAS is clearly involved in the development of this pathology, antihypertensive agents such as

ACE inhibitors are the first line treatment of these conditions.^{33,34} In fact, as the EETM showed prolonged ACE inhibitory activity similar to captopril, an important reduction in MAP and serum aldosterone were observed, leading to significant reduction of cardiac hypertrophy and improvement of renal function.

Another fact that drew attention in this work is related to the sustained diuretic effect induced by EETM. Prolonged EETM administration was able to increase the urine volume and sodium excretion in a manner very similar to furosemide, with the advantage of not excreting significant amounts of potassium. This effect can also be directly or indirectly influenced by ACE inhibition mainly due to increased bradykinin bioavailability. Recent studies have shown that classic ACE inhibitors can cause acute diuretic and natriuretic effect directly influenced by a reduction in the bradykinin metabolism, with consequent release of prostaglandins and nitric oxide in the renal arterioles, which may increase the RCBF and glomerular filtration rate, inducing diuretic effect.³⁵ In fact, we show by direct measurement of RCBF that the EETM could increase the renal blood flow, indicating a likely vasodilator effect and a significant increase in hydrostatic pressure in the renal arterioles, which directly increases glomerular filtration rate and the urine output.

Currently, it is known that ISQ, the main active metabolite of *T. majus*, has a wide range of important cardiovascular effects including, diuretic,¹³ antihypertensive,^{9,36} antiatherosclerotic³⁷ and antidiabetic.³⁸ It has been recently reported that ISQ-induced acute hypotension is an event dependent on the inhibition of angiotensin II generation by ACE.⁹ Moreover, it was also suggested that mechanisms by which ISQ exerts its diuretic effect on SHR rats are mainly related to ACE inhibition, increased bioavailability of bradykinin, prostaglandins, and nitric oxide.¹³ We also believe that ISQ can play a major role in this effect, however it is likely that the effect obtained with EETM comes from a complex interaction between different secondary metabolites acting as a powerful phytocomplex.

CONCLUSION

This study has brought new scientific evidence of preclinical efficacy of *T. majus* as a reno- and cardioprotective agent. In addition, it presents important evidence on the role of ACE inhibition in these effects, opening up the prospect that EETM can be converted into an herbal preparation in the future.

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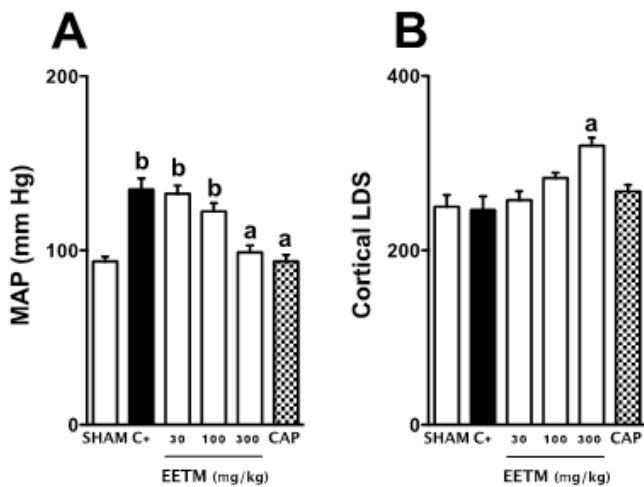


Figure 3: *Tropaeolum majus* L. extract (EETM) induced hypotension (A) and increase renal cortical blood flow (B) in Goldblatt hypertensive rats. The antihypertensive and renal cortical blood flow effects were measured for 45 minutes after 30-days of treatment. Values are expressed as mean \pm S.E.M. in comparison to the positive control (a; $p < 0.05$) or SHAM operated rats (b; $p < 0.05$) using one-way ANOVA followed by Bonferroni's test. CAP: captopril; LDS: laser doppler scale; MAP: mean arterial pressure.

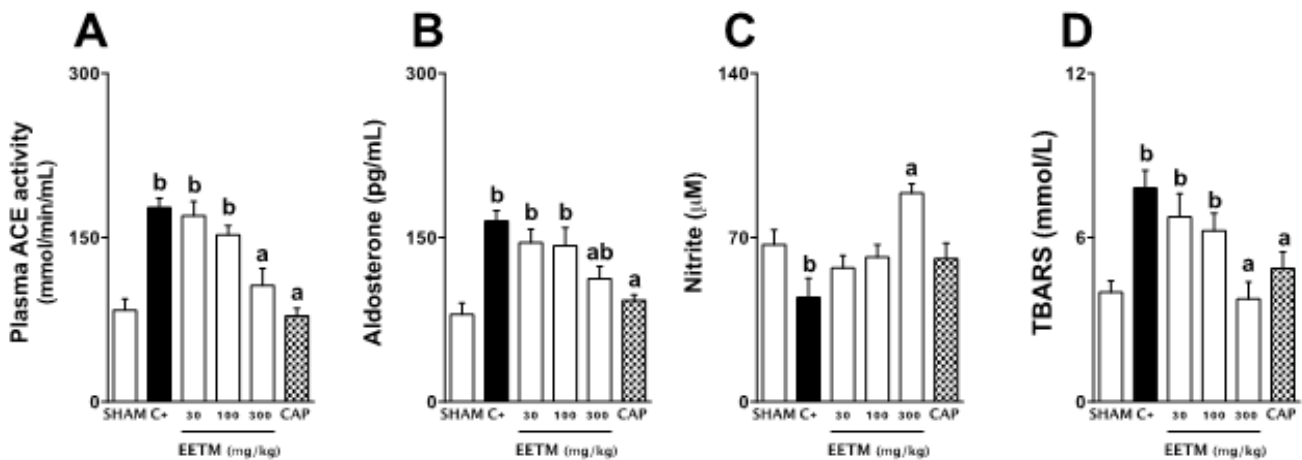


Figure 4: *Tropaeolum majus* L. extract (EETM) inhibit serum ACE activity (A), reduce levels of aldosterone (B), increases serum nitrate (C), and protect against lipid peroxidation (D) in 2K1C rats. The serum samples were obtained after 30-days of treatment. Values are expressed as mean \pm S.E.M. in comparison to the positive control (a; $p < 0.05$) or SHAM operated rats (b; $p < 0.05$) using one-way ANOVA followed by Bonferroni's test. CAP: Captopril.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures performed in animals were in accordance with the ethical standards of the institution (UFPR).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. This work was supported by grants from the Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT, Brazil, 59/300.046/2015), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil, 449464/2014-8).

ABBREVIATION USED

2K1C: two-kidney, one-clip Goldblatt hypertensive rats; **ACE:** Angiotensin-converting enzyme; **ANOVA:** Analysis of variance; **CAP:** Captopril; **EETM:** Ethanolic extract obtained from *Tropaeolum majus*; **ESI(+)-FT-ICR MS:** Electrospray ionization fourier transform ion cyclotron resonance mass spectrometry; **FURO:** Furosemide; **HPLC:** High-performance liquid chromatography; **HR:** Heart rate; **ISQ:** Isoquercitrin; **LOQ:** Limit of quantification; **MAP:** Mean arterial pressure; **NMR:** Nuclear magnetic resonance; **NOx:** Nitrite/nitrate levels; **RAS:** Renin-angiotensin system; **RCBF:** Renal cortical blood flow; **SBP:** Systolic blood pressure; **SEM:** Standard error of the mean; **SHAM:** Placebo surgery; **TBARS:** Thiobarbituric acid reactive substances; **UFMS:** Federal University of Mato Grosso do Sul; **UFPR:** Federal University of Paraná; **UNIPAR:** Paranaense University; **UV:** Ultraviolet.

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