

In vivo hypotensive effect and *in vitro* inhibitory activity of some *Cyperaceae* species

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In 1820, French naturalist August Saint Hillaire, during a visit in Espírito Santo (ES), a state in southeastern Brazil, reported a popular use of *Cyperaceae* species as antidote to snake bites. The plant may even have a hypotensive effect, though it was never properly researched. The *in vitro* inhibitory of the angiotensin converting enzyme (ACE) activity of eight ethanolic extracts of *Cyperaceae* was evaluated by colorimetric assay. Total phenolic and flavonoids were determined using colorimetric assay. The hypotensive effect of the active specie (*Rhychonospora exaltata*, ERE) and the *in vivo* ACE assay was measured *in vivo* using male Wistar Kyoto (ERE, 0.01-100mg/kg), with acetylcholine (ACh) as positive control (5 µg/kg, i.v.). The evaluation of ACE *in vivo* inhibitory effect was performed comparing the mean arterial pressure before and after ERE (10 mg/kg) in animals which received injection of angiotensin I (ANG I; 0,03, 03 and 300 µg/kg, i.v.). Captopril (30 mg/kg) was used as positive control. *Bulbostylis capillaris* (86.89 ± 15.20%) and ERE (74.89 ± 11.95%, ERE) were considered active in the *in vitro* ACE inhibition assay, at 100 µg/mL concentration. ACh lead to a hypotensive effect before and after ERE's curve (-40±5% and -41±3%). ERE showed a dose-dependent hypotensive effect and a *in vivo* ACE inhibitory effect. *Cyperaceae* species showed an inhibitory activity of ACE, *in vitro*, as well as high content of total phenolic and flavonoids. ERE exhibited an inhibitory effect on both *in vitro* and *in vivo* ACE. The selection of species used in popular medicine as antidotes, along with the *in vitro* assay of ACE inhibition, might be a biomonitoring method for the screening of new medicinal plants with hypotensive properties.

Uniterms: *Cyperaceae*/species/pharmacognosy. *Cyperaceae*/species/*in vitro* inhibitory activity. *Cyperaceae*/species/*in vivo* hypotensive effect. Angiotensin converting enzyme/*in vitro* inhibitory. Flavonoids/determination.

Em 1820, o naturalista francês August Saint Hillaire, durante uma visita ao Espírito Santo (ES), Estado do sudeste do Brasil, relatou o uso popular de espécies de *Cyperaceae* como antídoto para picadas de cobra. As espécies podem possuir efeito hipotensor, embora nunca tenham sido devidamente pesquisadas. A inibição *in vitro* da atividade da enzima conversora da angiotensina (ECA) de oito extratos etanólicos de *Cyperaceae* foi avaliada por ensaio colorimétrico. Totais de fenólicos e flavonóides foram determinados utilizando ensaio colorimétrico. O efeito hipotensor da espécie ativa (*Rhychonospora exaltata*, ERE) no ensaio de ECA *in vitro* foi avaliada *in vivo* utilizando-se machos Wistar Kyoto (ERE, 0.01-100 mg/kg), com a acetilcolina (ACh), como controle positivo (5 µg/kg, iv). A avaliação do efeito inibidor da ECA *in vivo* foi realizado comparando-se a pressão arterial média, antes e após ERE (10 mg/kg) nos animais que receberam injeção de angiotensina I (Ang I, 0,03, 03 e 300 µg/kg, iv). Captopril (30 mg/kg) foi utilizado como controle positivo. *Bulbostylis capillaris* (86,89±15,20%) e ERE (74,89±11,95%, ERE) foram considerados ativas no ensaio de inibição da ECA *in vitro*, na concentração de 100 µg/mL. A ACh gerou um efeito hipotensor, antes e depois da curva de ERE (-40 ± 5% -41 ± 3%). ERE apresentou efeito hipotensivo dependente da dose e um efeito inibidor da ECA *in vivo*. As espécies de *Cyperaceae* mostraram uma atividade inibidora de ACE *in vitro*, bem como elevado conteúdo de substâncias fenólicas

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e flavonóides. ERE exibiu um efeito inibidor da ECA tanto *in vitro* como *in vivo*. A seleção das espécies utilizadas na medicina popular como antiofídicos, juntamente com o ensaio *in vitro* de inibição da ECA, pode ser um método de biomonitoramento para a seleção de novas plantas medicinais com propriedades hipotensores.

Unitermos: Cyperaceae/espécies/farmacognosia. Cyperaceae/espécies/inibição *in vitro*. Cyperaceae/espécies/efeito hipotensor. Enzima conversora de angiotensina/inibição *in vitro*. Flavonóides/determinação.

INTRODUCTION

French naturalist August Saint Hillaire, during his stay in ES, noted that locals used some *Cyperaceae*, *Aristolochiaceae* and *Fabaceae* plants to treat wounds caused by snake bites - two of which applied orally and the other one applied locally (Saint-Hillaire, 1820/2002). Regarding the study of antihypertensive plants, the selection can be ethnobotanical, based on species used as diuretics, antidotes and antihypertensives (Ribeiro, *et al.*, 1986). Several species commonly used as antivenins also present anti-inflammatory, antioxidant and hypotensive activity (Balestrin *et al.*, 2008). Species of the *Cyperus* genus, *Cyperaceae* family, are reported as anti-inflammatory (Falcão, *et al.*, 2005), antimalarial (Schwikkard, Heerden, 2002) and hepatoprotective extracts (Amani *et al.*, 2012).

Concerning the advanced ecosystem destruction together with the potentialities of the *Cyperaceae* family, the aim of this study were to identify whether any of the the ethanolic extracts of *Cyperaceae* species presents an inhibitory effect on *in vitro* ACE, and whether the most active species *in vitro* shows an acute hypotensive effect *in vivo* and ACE inhibitory effect *in vivo*.

MATERIAL AND METHODS

Plant materials and extraction

Ten specimens from *Cyperaceae* family were harvested (July, 2010) from Paulo César Vinha State Park (PEPCV), with the previous approval of local environmental authorities (IEMA, protocol 629/09). The samples gathered were identified by the botanists Oberdan José Pereira and Márcio Lacerda Lopes Martins, and a voucher specimens were deposited at the herbarium of the Espírito Santo Federal University (UFES, VIES): *Bulbostylis capillaris* (L.) C.B. Clarke (20°36'22.5"S; 40°25'02.0"W, VIES 26082 and VIES 26085) *Cyperus tenuis* Sw. (20°36'12,8"S; 40°25'27.7"W, VIES 26083), *Eleocharis intersticta* Vahl (20°36'17.6"S; 40°25'16.1"W, VIES 26380), *Remirea maritima* Aubl (20°36'33.2"S;

40°24'43.7"W, VIES 26379), *Rhynchospora exaltata* Kunth (20°36'22.8"S; 40°25'07.8"W, VIES 26079), *Rhynchospora holoschoenoides* Rich. (20°36'23.1"S; 40°25'01.09"W, VIES 26086), *Rhynchospora tenuis* Link (20°36'17.1"S; 40°25'2.66"W, VIES 26081 and VIES 26087), *Scleria secans* L. (20°36'21.5"S; 40°25'10.5"W, VIES 26080). Two of the ten collected specimens were identified as the same (*B. capillaris* and *R. tenuis*).

The collected plants were dried at 40 °C (48 h) and grounded. The ethanolic extract was prepared by successive ultrasound-assisted (15 min, 40Hz) extraction. The extract was then concentrated using a rotatory-evaporator at 40 °C, under low pressure conditions, obtaining the crude ethanolic extract of the species: *B. capillaris* (0.16 g), *C. tenuis* (0.06 g), *E. intersticta* (0.71 g), *R. exaltata* (1.4 g), *R. haloschoenoides* (0.8 g), *R. tenuis* (0.91 g), *R. maiítima* (0.3 g) and *S. secans* (2.1 g).

Determination of total phenolic

The total phenolic content was determined using Folin-Ciocalteu method, along with some modifications (Singleton, Rossi, 1965). Solutions at a concentration of 1.5 mg/mL of the ethanolic extracts were prepared. The calibration curve was plotted with a pyrogallol solution (from 10 to 350 µg/mL), and the equation was $y = 0.001244x + 0.002889$, $r^2 = 0.9977$. The results were expressed as micrograms of pyrogallol equivalents (PE) per milligram of dry sample. The analyses were performed in triplicates. The limit of quantification was 13.7 µg/mL and the limit of detection was 4.1 µg/mL.

Determination of total flavonoids

The total flavonoids content was determined by colorimetric aluminum chloride method (Marinova *et al.*, 2005). The calibration curve was prepared with rutin solutions, at the 31.25-1000 µg/mL range. The calibration regression equation was $y = 0.0033x + 0.0121$, $r^2 = 0.9991$. The results were expressed as micrograms of rutin equivalents (RE) per milligram of sample. The analyses

were done in triplicates. The limit of quantification was 5.3 µg/mL and the limit of detection was 1.6 µg/mL.

High performance liquid chromatography (HPLC) analysis

The analysis of the eight Cyperaceae extracts, especially of ERE by HPLC was performed in a Waters 1515 separation device, along with a binary pump, and an UV/VIS detector (model 2489). The data were processed using the Breeze software. A C18 XBridge reverse-phase column was used (150 x 4.6 mm i.d., 3.5 µm, Waters), along with an also C-18 XBridge precolumn (20 x 4.6 mm i.d., 3.5 µm, Waters), at room temperature. Two isocratic elution systems were used. The first one was composed by a MeOH:H₂O (9:1) solution, wavelength detection at 254 and 365 nm, and isocratic flow at 0.8 mL/min; the second was employed a MeOH:H₂O:ACN: AcOOH: CHOOH (20:10:20:1:1) eluent, same wavelengths, and 1.2 mL/min flow. The solvents were HPLC quality (Merck, Darmstadt, Germany), the water was ultrapure (ELGA, 18.2Ω) and was degassed by sonication and vacuum pump prior to use. Both the reference rutin and apigenin solutions (Sigma, St. Louis, USA) and the ERE sample were dissolved in MeOH, HPLC degree (Merck, Darmstadt, Germany). The sample concentration was 1.0 mg/mL. The final concentration of the reference solutions used on the plotting of the standard curve ranged from 0.03 to 0.6 µg/mL. Samples, as well as reference solutions, were centrifuged at 8400 g for 5 min. Each sample and standard solution (20 µL) was done manually. All analyses were done in triplicates.

ACE in vitro inhibition assay

Effects of the Cyperaceae crude ethanolic extracts on ACE were determined by measuring the accumulation of Gly-Gly (glycine-glycine), the ACE cleavage product of Hip-Gly-Gly. This assay was performed as previously described (Endringer *et al.*, 2014). In brief, the assays were initiated by an addition of 10 µL of the sample (final concentration of 100 µg/mL), 10 µL of MeOH/HEPES (1: 4), pH 8.0 (negative control), or 10 µL of a captopril solution (final concentration of 64 nM; positive control) into a 96-well plate. A rabbit lung solution (10 µL for the final concentration of 9.1 mg/mL) was used as the ACE source. The plate was then homogenized and pre-incubated at 37 °C for 10 min. The enzymatic reaction was initiated by an addition of 60 µL of the HEPES buffer (final concentration of 27.3 mM), pH 8.15 and 30 µL of a Hip-Gly-Gly solution (final concentration of 27.3 mM).

After the homogenization, the mixture was incubated for 40 min at 37 °C. The reaction was stopped by an addition of 100 µL of 0.3 M sodium tungstate and 100 µL of 0.3 M sulfuric acid. In the sequence, an aliquot (64 µL) was diluted with 100 µL of water and kept in rest for 5 min. Subsequently, the system was mixed with the color reagent 2,4,6-trinitrobenzenesulfonic acid solution, TNBS (6.92 mM) (Sigma, USA). The plate was kept in the dark at room temperature for 20 min and its absorbance was measured using a microplate reader (BioRad, Model 550) at 415 nm. The readings were compared against a blank solution, which was prepared in a similar manner with the exception of sodium tungstate and sulfuric acid solutions being added before the rabbit lung solution. Based on the measured ACE inhibitory activity, the compounds were classified as inactive (<30 %), weak (>30 % to 50 %), moderate (>50 % to 80%), and strong (>80 %) inhibitors.

Acute hypotensive assay

The *in vivo* experiments were performed according to the ethical principles determined by Brazilian School of Animal Experimentation (COBEA, 1991), and were approved by the Ethical, Bioethical and Animal Welfare Committee (CEUA - UVV, 120/2010). Male Wistar Kyoto (WKY; n=6) and Spontaneously Hypertensive Rats (SHR; n=6) were used. The animals were about three months old, with a body weight from 200 to 250 g. The rats were kept at temperatures of 22 ± 3 °C, in a 12 h light/12 h dark cycle, and had free access to standard pellet diet (Probiotério stockfeed, Moinho Primor SA) and water.

Under sodium pentobarbital anesthetic (50 mg/kg, intraperitoneal Hypnol, Crystal, Brasil) (Andrade *et al.*, 2008), both femoral arteries and veins were catheterized (polyethylene catheter, PE 50, Clay Adans, USA). The venous catheter was used for the injection of the raw ethanolic extract from *R. exaltata* (ERE - 267 mg/mL in saline solution) and/or saline, the arterial catheter was connected to pressure pipes (Spectramed, Statham, P23XL, USA) by a flexible catheter, and the register values of the mean arterial pressure (MAP) were collected by a computer (Pentium MMX 233 MHz) and by a biological data acquisition software (Biopac, Biopac Systems, USA, mod. 100A/94111065 MP series).

The acute hypotensive effect was evaluated by the plotting of a dose-effect curve of the ERE solution. The WKY and SHR animals received saline 1 mL/kg (Soncini *et al.*, 2011) and, afterwards, increasing doses, *in bolus*, of ERE (0.01 – 100 mg/kg). The subsequent dose was administrated only after MAP returned to the levels observed prior to the first application. ACh was utilized as

positive control (5 µg/kg, i.v.) both before and after the ERE curve (Lahlou *et al.*, 2003).

ACE in vivo inhibition assay

ACE *in vivo* inhibitory evaluation was performed according to Mangiapane *et al.* (1994), with slight modifications. Briefly, the effect of angiotensin I (ANGI; 0.03, 0.3 and 300 µg/kg i.v.) was analyzed before and after the injection of a single dose of ERE ethanolic extract (10 mg/kg, i.v.). As positive control, ANG I was administered before and after captopril (30 mg/kg).

Statistical analysis

The values of the biological evaluation were expressed as the mean ± standard error of the mean (S.E.M.). Basic values of MAP and FC, and MAP after ERE's injection were submitted to analysis of variance (ANOVA), followed by a post-hoc Fisher test, $p < 0.05$. Correlation coefficient of the standard curves was obtained by linear regression by the least squares method and the data were then analyzed by ANOVA, on a 95% LC and degree of freedom of $n-1$. The limits of detection (LOD) and quantification (LOQ) were computed by the division of the standard deviation of the slope of the curves on the calibration assay.

RESULTS AND DISCUSSION

There are just a few taxonomical studies about the *Cyperaceae* family, which makes it hard to identify and/or differentiate the species (Martins *et al.*, 1999). From the gathered *Cyperaceae* specimens, two of them were of

the same species (*B. capillaris* and *R. tenuis*), and one of them (*B. capillaris*) is on August de Saint-Hillaire Virtual Herbarium species list (Virtual Herbarium, 2012). *B. capillaris* as well as *R. exaltata* were considered active on the ACE inhibition assay (Table I).

The 50% inhibitory *in vitro* ACE concentration (CI_{50}) of the active species could not be calculated, as the observed inhibitory effect was dose-independent. The presence of total polyphenols, including tannins, on the extracts might be related to this not specific *in vitro* inhibition. Nyman *et al.* (1998), observed that only four species, from the thirteen considered active in the ACE assay, remained presenting dose-dependent inhibitory ACE activity after tannins were removed. Polyphenols, distinctly from flavonoids, might be related to the inhibitory ACE activity of *B. capillaris* species.

However, it is not possible to establish a correlation between the *in vitro* ACE inhibition percentage and the content of total phenolics, as they might be the constituents related to the ACE inhibitory effect noted for *R. exaltata* (table I). Barbosa-Filho *et al.* (2006), summarized several studies of the natural products with inhibitory ACE activity and observed that flavonoids and tannins were related to the activity of several species among them: *Erythroxylum laurifolium*, “Korean” green tea, *Allophylus edulis*. In the present study, *C. rotundus*, *Cyperaceae*, was cited, but it did not show ACE inhibition (Barbosa-Filho *et al.*, 2006).

The two species showing the most effective ACE inhibition were *B. capillaris* and *R. exaltata*. Due to the available amount of active samples, *R. exaltata* was chosen for the *in vivo* hypotensive study. The chromatographic profile obtained for ERE was simple, on the analyzed wavelengths, with a dominance of polar substances,

TABLE I - ACE inhibition percentages, total polyphenols and flavonoids amounts for the analyzed *Cyperaceae* species

	% ACEI ^a	Total polyphenols ^b	Total flavonoids ^c
Captopril	73.2 ± 20.0		
<i>Bulbostylis capillaries</i>	86.9 ± 15.2	141.0 ± 16.1	11.0 ± 3.6
<i>Cyperus tenuis</i> ^d	NA	NA	NA
<i>Eleocharis interstincta</i>	30.7 ± 7,8	30.1 ± 1.9	31.2 ± 5.0
<i>Remiria maritime</i>	NA	92.4 ± 3.4	34.3 ± 2.1
<i>Rhynchospora exaltata</i>	74.9 ± 12.0	36.7 ± 4.9	125.8 ± 2.9
<i>Rhynchospora tenuis</i>	50.4 ± 23.2	163.5 ± 18.3	149.2 ± 9.0
<i>Rhynchospora haloschoenoides</i>	NA	51.3 ± 3.6	90.8 ± 5.3
<i>Scleria secans</i>	26.5 ± 4.6	194.7 ± 19.9	93.9 ± 2.4

^aInhibition percentage of ACE. ^bResults presented as micrograms of pyrogallol equivalents per milligram of sample. ^cResults presented as micrograms of rutin equivalents per milligram of sample. ^dNA: not evaluated due to the available mass.

suggesting the presence flavonoid-like compounds. Using particular flavonoid eluents, in comparison with rutin and apigenin, peaks related to aglycones, but an overlap of peaks related to glycolized flavonoids (rutin and others).

As expected MAP of SHR (182±8 mmHg; p<0.01) animals was higher compared with WKY (99±5 mmHg). During the acute hypotensive assay, the ethanolic crude extract of *R. exaltata* determined a reduction of the pressure levels in a dose-dependent way in both normotensive and hypertensive animals (Figure 1), which were statistically different from the reduction caused by the saline injection (WKY: -7.6±3 mmHg; SHR: -8±4 mmHg). Reductions elicited in SHR animals were greater compared with WKY (Figure 1). ACh induced a hypotensive effect before and after ERE's curve (WKY: -43.2±4 mmHg and -44.3±3 mmHg; SHR: -74±5 mmHg and -78 mmHg; p<0.01 compared with WKY), suggesting vascular integrity, as the vascular relaxation induced by ACh is dependent on the integrity of endothelial cells (Batlouni, 2001). The *in vivo* ACE inhibition of ERE is reported in Table II. ANGI at all tested doses (0.03, 3.0, 300.00 µg/kg) increased the MAP (Table II, before CAP and before ERE). ERE decreased around 50% of the ANGI effect (Table II), indicating that ERE inhibits ACE *in vivo*. However, the design of the *in vivo* inhibition assay applied in the present study does not differ between the two ACE isoforms and also not between AngII and its metabolite, Ang 1-7. ACE and ACE2 isoforms are 40% similar (Watermeyer *et al.*, 2006; Sturrock *et al.*, 2004). ACE2 acts as a carboxylpeptidase, whereas ACE acts as a peptidylpeptidase (Brown, Hall, 2005). Several studies indicate that both enzymes, ACE and ACE2, have similar catalytic mechanisms. However, the ACE inhibitory agents, such as captopril, lisinopril, are inactive against ACE2. These data suggest that the proteolytic activity of ACE2 is different from the isoform ACE. Unlike ACE,

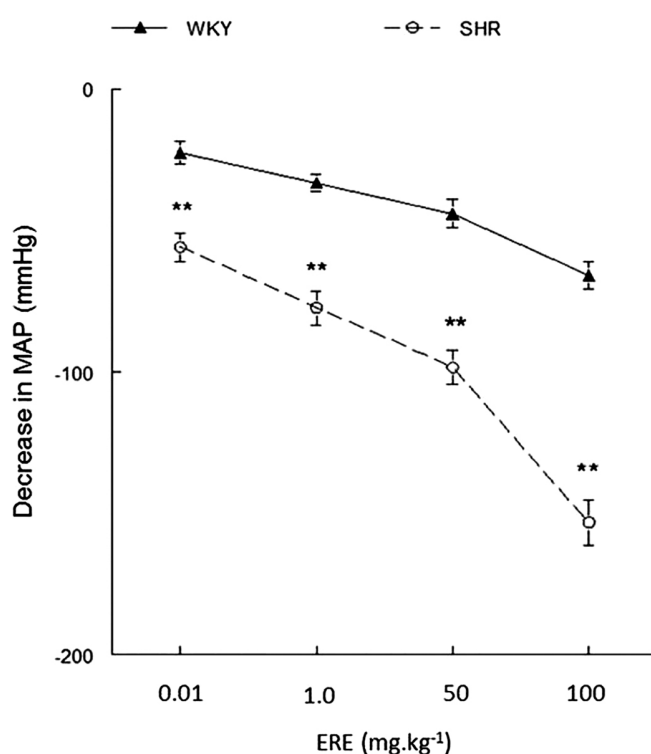


FIGURE 1 - ERE-induced dose-dependent reduction in Mean Arterial Pressure (MAP) in normotensive (WKY) and hypertensive (SHR) animals. ERE: ethanolic extract of *Rhynchospora exaltata*. The values are presented as the mean ± S.E.M. ** p<0.01 compared WKY.

ACE2 is the monopeptidylcarboxypeptidase (Guy *et al.*, 2003; Warner *et al.*, 2004; Guy *et al.*, 2005).

Some species analyzed applying a similar assay used in the present study also showed hypotensive activity (Singi *et al.*, 2005; Soncini *et al.*, 2011). Soncini *et al.* (2011) reported that aqueous extract of *Averrhoa carambola* L. (Oxalidaceae) showed a dose-dependent pressure reduction. The hypotensive activity

TABLE II - Mean arterial pressure (MAP) increase induced by the injection of ANG I (0.03, 3 and 300 µg/kg, i.v.) before and after injection of the ethanolic extract of *Rhynchospora exaltata* (ERE; 10 mg/kg) and captopril (CAP; 30 mg/kg, triangles)

Groups	ANG I (µg/kg)								
	0.03			3.0			300		
	MAP _B	MAP _A	ΔMAP	MAP _B	MAP _A	ΔMAP	MAP _B	MAP _A	ΔMAP
Before CAP	96±4	126±5	32±4	98±3	153±6	56±4	92±5	190±8	104±6
After CAP	94±3	99±4**	4±2**	93±4	101±3**	8±3**	91±6	105±5**	12±4**
Before ERE	92±4	124±6	34±5	95±3	146±5	49±6	93±4	192±7	100±8
After ERE	95±2	108±3**	14±4***	96±5	124±4***	31±5***	92±4	143±6***	53±5***

MAP_B: MAP before ANG I injection; MAP_A: MAP after ANG I injection; ΔMAP: difference between MAP_A and MAP_B. The values are presented as the mean ± S.E.M. ** p<0.01 compared with MAP values before CAP or ERE administration. *** p<0.01 compared with MAP after CAP administration.

of hydroalcoholic extracts of garlic (*Allium sativum* L.) and lemon-grass (*Cymbopogon citratus* (DC) Stapf) were reported by Singi *et al.* (2005).

The data reported in the present study indicate that some *Cyparaceae* species have ACE *in vitro* inhibitory activity, being related with the high content of total flavonoids and phenolics. ERE exhibited an inhibitory effect on both *in vitro* and *in vivo* ACE. The selection of species used in popular medicine as antidotes, along with the *in vitro* assay of ACE inhibition, might be a biomonitoring method for the screening of new medicinal plants with hypotensive properties.

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