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Short Communication

ACE inhibition by astilbin isolated from *Erythroxylum gonocladum* (Mart.) O.E. Schulz

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ABSTRACT

Erythroxylum species have several traditional uses in different countries, including the treatment of hypertension. The ethanol extract from *E. gonocladum* aerial parts, a species endemic to the Brazilian *cerrado*, elicited a concentration-dependent inhibition of angiotensin converting enzyme (ACE) ($pIC_{50}=4.53\pm0.05$). Extract fractionation led to the isolation of two compounds, whose structures were assigned by spectrometric data as astilbin and β-sitosterol, along with a mixture of palmitic, stearic and linolenic acids. This is the first report on the occurrence of these compounds on *E. gonocladum*. Astilbin promoted significant ACE inhibition *in vitro* ($pIC_{50}=5.86\pm0.33$) and its activity did not differ from captopril, when both compounds were assayed at 10 μM concentration.

Introduction

Erythroxylum (Erythroxylaceae) species are distributed in tropical regions of South America, Africa, and the island of Madagascar, being Brazil considered a center of diversity and endemism of the genus. A total of 114 out of 187 *Erythroxylum* species found in the tropical America occur in the country (Plowman and Hensold 2004). Despite its wide distribution in Brazil, data on chemical composition and biological activities are scarce for *Erythroxylum* species, even though several ethnopharmacological uses have been described for them, such as anti-inflammatory, antibacterial, diuretic, tonic, stimulant, for treating liver, kidney and vesicular diseases, as well as for rheumatism, arthritis and respiratory disorders (Adsersen and Adsersen 1997; Bohm et al. 1988; Cano and Volpato 2004; González-Guevara et al. 2006).

The chemistry of *Erythroxylum* is characterized by the presence of tropane alkaloids (Chin et al. 2006; Griffin and Lin 2000) and flavonoids (Chávez et al. 1996; Johnson et al. 2003), commonly found as 3-*O*-monoglycosides of glucose, galactose, arabinose, xylose and rhamnose (Hegnauer 1981). Kampferol and quercetin, along with their corresponding 3-*O*-glycosides, are frequently found in *Erythroxylum*, being considered chemical markers for the genus (Inigo and Pomilio 1985).

Erythroxylum gonocladum (Mart.) O. E. Schulz is a shrub found in the Brazilian *cerrado*, a savannah like vegetation. As far as we know, the chemistry of the species has never been investigated and no ethnomedical use has been reported so far. On the other hand, in a preliminary screening carried out by our group directed towards the search of anti-hypertensive plants, the ethanol extract from *E. gonocladum* aerial parts promoted significant *in vitro* inhibition of angiotensin converting enzyme (ACE). Therefore, the main goal of the present study was to investigate the chemical composition of the species and to isolate the potential anti-hypertensive constituents.

Material and methods

General

^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), NOESY, HSQC, HSQC-TOCSY and HMBC spectra were obtained in CD_3OD+D_2O or $CDCl_3$ with TMS as internal standard and were recorded on a Bruker Avance DRX-400 equipment. Silica gel (70-320 mesh, Merck, Germany) was used for CC separation while silica gel 60 (Merck) was used for analytical (0.25 mm) TLC. Preparative HPLC was carried out on a Shimadzu system (Japan) composed of pump LC-8A, UV-VIS detector SPD-GAV, controller system SCL-8A and

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integrator C-R4A. A Zorbax SB-C18 column (250 × 20 mm i.d., Agilent, USA) was employed.

Plant material

The aerial parts of *E. gonocladum* were collected in Serra da Piedade, in the municipality of Caeté, Minas Gerais state, Brazil, in July 2007. The species was identified by Dr. J. R. Stehmann, Institute of Biological Sciences, UFMG, Belo Horizonte, Brazil, where a voucher specimen (BHCB 118.812) is deposited. The fresh vegetal material was washed under current water and dried in a ventilated oven at 40 °C for 72 h. The plant material (264.5 g) was ground and percolated with 96% EtOH at room temperature. The solvent was removed in a rotatory evaporator under vacuum at 40 °C, leaving a dark residue (EGE 62.5 g), which was kept in a desiccator until constant weight.

RP-HPLC analysis of EGE, fractions and compounds

RP-HPLC analyses were carried out on a Waters alliance 2695 HPLC system composed of a quaternary pump, an auto sampler, a photodiode array detector (DAD) 2996 and a Waters Empower pro data handling system (Waters Corporation, Milford, USA). The analyses were performed on a LiChrospher 100 RP-18 column (125 × 4 mm i.d., 5 μm; Merck, Darmstadt, Germany), in combination with a LiChrospher 100 RP-18 guard column (4 × 4 mm i.d., 5 μm; Merck, Darmstadt, Germany). The chromatograms were obtained employing a linear gradient of H_2O (A) and CH_3CN (B): 0 min 95% A, 5% B; 60 min 5% A, 95% B; followed by 10 min of isocratic elution, at a temperature of 40 °C and flow rate of 1.0 ml/min. Samples were dissolved in MeOH, in ultrasonic bath for 15 min, to concentration of 10 mg/ml for extract, 5 mg/ml for fractions and 1 mg/ml for the isolate substance. The chromatograms were obtained after centrifugation at 10,000 rpm (10 min) and automatic injection of 10 μl of supernatants onto the apparatus. The chromatograms were obtained at 210 nm and UV spectra from 190 to 400 nm were recorded on line.

GC-FID analysis

The composition of the fatty acids mixture (solid **3**) was analyzed by gas chromatography as methylated derivatives (FAME). The sample (1 mg) was derivatized by adding 100 μl of BF_3/MeOH (14% w/v) to the free fatty acids mixture in a microcentrifuge tube. The closed tube was placed in boiling water for 5 min. The methyl esters formed were diluted in MeOH to a final concentration of 100 ppm. A Varian gas chromatography model 3380 with flame-ionization detector was used for analysis, employing hydrogen as carrier gas at 2 ml/min. A DB-Wax capillary column (30 m × 0.25 mm i.d.; J&W Scientific, USA) was used with gradient temperature program: 100 °C (1 min) to 240 °C at 7 °C/min. The injection volume was 1 μl. The detector (FID) and injector (split 1:100) temperatures were kept at 260 °C. The FAMEs were identified by comparing retention times to a standard mixture (Supelco 37, Supelco, USA).

Fractionation and isolation

A portion of EGE (2.0 g) was suspended in H_2O-MeOH (11:1; 120 ml) and sequentially partitioned with equal volumes (3 × 40 ml) of *n*-hexane, CH_2Cl_2 and EtOAc. MeOH was removed in a rotavapor before partitioning the extract suspension with CH_2Cl_2 and EtOAc. Solvents were eliminated in a rotatory evaporator, at maximum temperature of 50 °C. The process was repeated ten times (20 g of EGE), yielding the *n*-hexane (2.08 g), CH_2Cl_2 (80 mg), EtOAc (870 mg) and aqueous fractions (8.26 g), along with a precipitate (PPT, 7.70 g) formed during partition with *n*-hexane. PPT (3.0 g) was further subjected to silica gel column chromatography eluted with *n*-hexane, CH_2Cl_2, CH_2Cl_2 -EtOAc (9:1, 8:2, 6:4, 4:6, 2:8, 1:9), EtOAc, EtOAc-MeOH (9:1, 8:2, 6:4, 4:6, 2:8), MeOH and water. The EtOAc fraction (700 mg) was additionally chromatographed over a silica gel column eluted with CH_2Cl_2-EtOAc (4:6, 3:7, 1:9), EtOAc, EtOAc-MeOH (9:1, 8:2, 6:4, 2:8) and MeOH. The eluate CH_2Cl_2-EtOAc (3:7) (225.0 mg) was further purified by HPLC on an ODS column using MeOH-H_2O (1:1) as eluent to give astilbin (**1**) (Fig. 1) (11.0 mg).

The *n*-hexane fraction resulting from the extract partition between immiscible solvents was fractionated by silica gel column chromatography using gradient elution of *n*-hexane and CH_2Cl_2 resulting in the isolation of β-sitosterol (**2**, 10.0 mg) and a mixture of fatty acids (**3**, 132.7 mg) composed of palmitic, stearic and linolenic acids.

*Astilbin (dihydroquercetin 3-a-L-rhamnopyranoside) (**1**)*

^{1}H NMR (400 MHz, CD_3OD+D_2O): 1.18 (d, *J* 6.2 Hz, CH_3-6″), 3.31 (m, H-4″), 3.54 (dd, *J* 3.3 and 1.5 Hz, H-2″), 3.67 (dd, *J* 9.6 and 3.3 Hz, H-3″), 4.06 (d, *J* 1.5 Hz, H-1″), 4.25 (m, H-5″), 4.58 (d, *J* 10.7 Hz, H-3), 5.08 (d, *J* 10.7 Hz, H-2), 5.90 (d, *J* 2.2 Hz, H-8), 5.92 (d, *J* 2.2 Hz, H-6), 6.81 (d, *J* 8.3 Hz, H-5′), 6.84 (dd, *J* 8.3 and 1.9 Hz, H-6′), 6.95 (d, *J* 1.9 Hz, H-2′). ^{13}C NMR (100 MHz, CD_3OD+D_2O): 17.99 (C-6″), 70.66 (C-5″), 71.93 (C-2″), 72.33 (C-3″), 73.97 (C-4″), 78.72 (C-3), 84.10 (C-2), 96.43 (C-8), 97.55 (C-6), 102.29 (C-1″), 102.64 (C-4a), 115.64 (C-2′), 116.48 (C-5′), 120.63 (C-6′), 129.35 (C-1′), 146.69 (C-3′), 147.52 (C-4′), 164.25 (C-8a), 165.66 (C-5), 168.82 (C-7), 196.11 (C-4).

Determination of the absolute configuration of 1

Absolute configuration was determined by circular dichroism spectra (Fig. 2) recorded on a JASCO J-715 spectropolarimeter (Jasco Inc., Easton, USA), equipped with a Peltier type temperature controller. The instrument was calibrated using (+)-10-camphorsulfonic acid (Sigma-Aldrich, St. Louis, USA) (Chen and Yang 1977). Spectra were obtained from 220 to 300 nm at 25 °C using 0.1 cm path length cells. Compound **1** was dissolved in methanol (Mallinckrodt Baker Inc., Phillipsburg, USA) to a final concentration of 0.125 mg/ml. The solution was stirred and incubated at room temperature for 10 min before each spectrum was recorded. The spectra were an average of eight scans recorded at a speed of 10 nm/min with a bandwidth of 1.0 at 0.5 nm step size and a 2 s time constant. After background subtraction and smoothing, CD data was expressed in molar circular dichroism

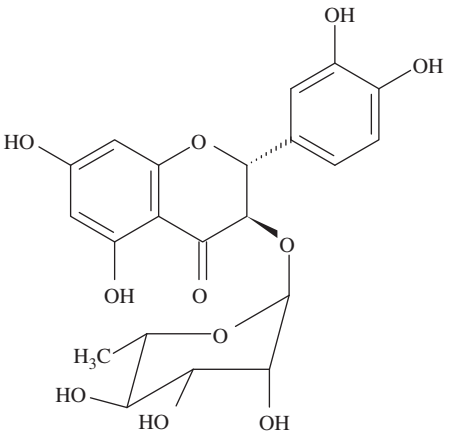

Fig. 1. Structure of Astilbin.

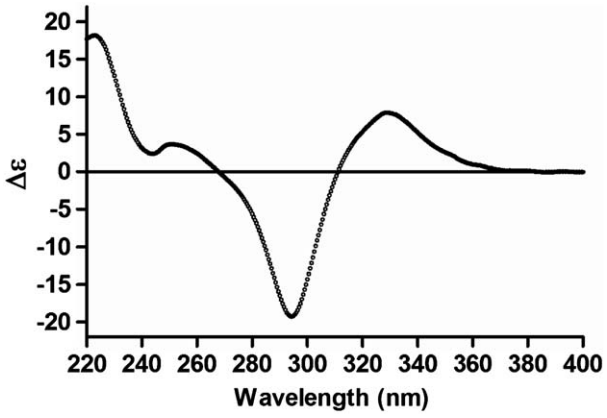

Fig. 2. CD curve of (−)(2*R*,3*R*)-astilbin (**1**) in MeOH.

units. The software Spectra Manager (Jasco) was used for data collection and analysis.

ACE inhibition assay

ACE inhibitory activity was determined using a method described by Serra et al. (2005), modified to employ rat plasma as enzyme source. Briefly, an aliquot (25 μl) of rat plasma was added to a microtitre plate containing 5 μl of the sample solution to be tested in different concentrations. Phosphate buffer (pH 8.3), ethanol and DMSO were employed as negative controls. Captopril (10 μM) was employed as positive control. The enzymatic reaction was started by adding the assay buffer and the substrate solution Hip-Gly-Gly (100 μM) (Sigma, USA). After homogenization, the mixture was incubated for 35 min, at 37 °C. The reaction was stopped by the addition of sodium tungstate (100 g/l) and sulfuric acid (0.33 mM). The system was mixed with the color reagent TNBS (Sigma, USA). After 20 min in dark, the plate absorbance was read in a microtitre plate reader (BioRad, Model 550) at 415 nm against a blank solution similarly prepared, except by adding the sodium tungstate and the sulfuric acid solutions before enzyme solution.

Statistical analysis

The experimental data are expressed as mean ± standard error mean (S.E.M.) of at least six experiments. Statistical analyses were performed using one-way ANOVA plus Tukey's multiple comparison post-test. The value of pIC_{50} represents the −log of concentration values for the ethanol extract from *E. gonocladum* aerial parts (g/ml) and astilbin (M) that induce 50% inhibition of ACE activity.

Results and discussion

The RP-HPLC fingerprint recorded for *E. gonocladum* aerial parts (EEG) indicates the predominance of a major peak with retention time of 13.5 min (compound **1**), whose UV spectra showed a λ_{max} at 289.9 nm and a shoulder around 340 nm, being these values compatible with a dihydroflavonol (Justesen et al. 1998; Bohm 1975). RP-HPLC analysis of PPT, a precipitate formed during the partition of the hydromethanolic suspension of EGE with *n*-hexane, indicated that compound **1** has been concentrated in this precipitate. Its further purification by silica gel column chromatography and preparative RP-HPLC afforded the yellowish solid **1**. Analysis of 1D and 2D NMR data, along with acid microhydrolysis on TLC and comparison with literature records for dihydroflavonoids suggested that compound **1** is dihydroquercetin-3-*O*-α-rhamnoside (Lu and Foo 1999). However, the molecule possesses two chiral centers at C-2 and C-3 resulting in four possible diastereoisomers (astilbin, neoastilbin, isoastilbin and neoisoastilbin). The ^{1}H NMR spectrum showed that **1** has *trans* configuration since it shows coupling constant $J_{2,3}$ of 10.7 Hz, thus limiting the possible isomers to astilbin and neoastilbin. In order to identify compound **1** unequivocally, its absolute configuration was investigated by circular dichroism. The chiroptical properties of dihydroflavonoids has been thoroughly investigated and the sign of the Cotton effect of $n\rightarrow\pi^*$ origin can be used for establishing the absolute configuration at C-2. Therefore, the CD measurement of **1** was carried out in the UV absorption region in methanol (Fig. 2). The absolute configuration at C-2 was concluded to be *R* based on a positive Cotton effect observed in the CD spectrum of **1**, ascribed to the absorption band at 329.0 nm ($\Delta\varepsilon = +7.88$) (Slade et al. 2005; Gaffield et al. 1975). Therefore, compound **1** was identified to be the 3-*O*-α-L-rhamnoside of (−)(2*R*,3*R*)-5,7,3′,4′-tetrahydroxydihydroflavonol or (−)(2*R*,3*R*)-astilbin. Despite its presence as a major peak in the extract from *E. gonocladum* aerial parts, only a small mass of pure astilbin (**1**) was obtained. The interconversion of isomeric astilbins is well documented (Gaffield et al. 1975) and we experienced it during the chromatographic procedures carried out for the isolation and purification of **1**.

Two solids (**2** and **3**) were obtained from the *n*-hexane fraction of *E. gonocladum*. Analysis of ^{13}C and ^{1}H NMR data obtained for **2** and comparison with literature records for triterpenes and steroids allowed identifying it as β-sitosterol (Forgo and Kövér 2004). Solid **3** was submitted to GC-FID analysis and comparison with reference compounds revealed a mixture of fatty acids composed of palmitic (30.0%), stearic (21.3%) and linolenic (4.6%) acids, along with a non-identified compound. It is worth mentioning that astilbin **1**, β-sitosterol and the fatty acids were obtained for the first time from *E. gonocladum*. On the other hand, the occurrence of the aglycone in the genus seems to be common, since several glycosides of dihydroquercetin have been described for *Erythroxylum rufum*, *E. ulei* (Bohm et al. 1981), *E. coca* var. *ipadu* and *E. novogranatense* var. *truxillense* (Johnson et al. 1998).

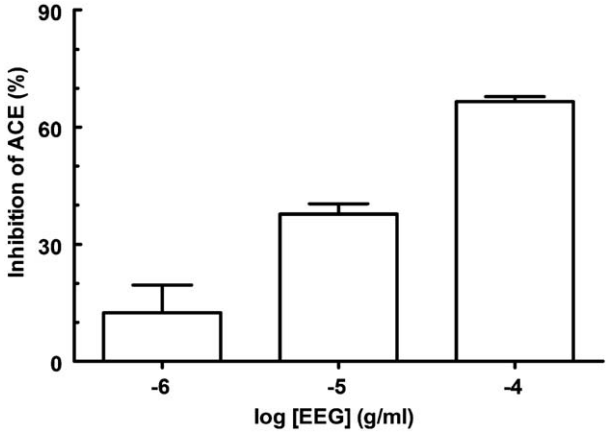

Fig. 3. Effect of the ethanol extract from *E. gonocladum* aerial parts (EEG) on the ACE *in vitro* inhibition assay. Each bar represents the mean ± SEM of at least 6 replicates. EEG was incubated with the serum 60 min before substrate addition. The results for each concentration of EEG are significantly different between each other ($p < 0.01$). The vehicle used for dilution of EEG (ethanol) did not inhibit ACE activity. The statistical analyzes was performed by One-Way ANOVA with Tukey's multiple comparison post-test.

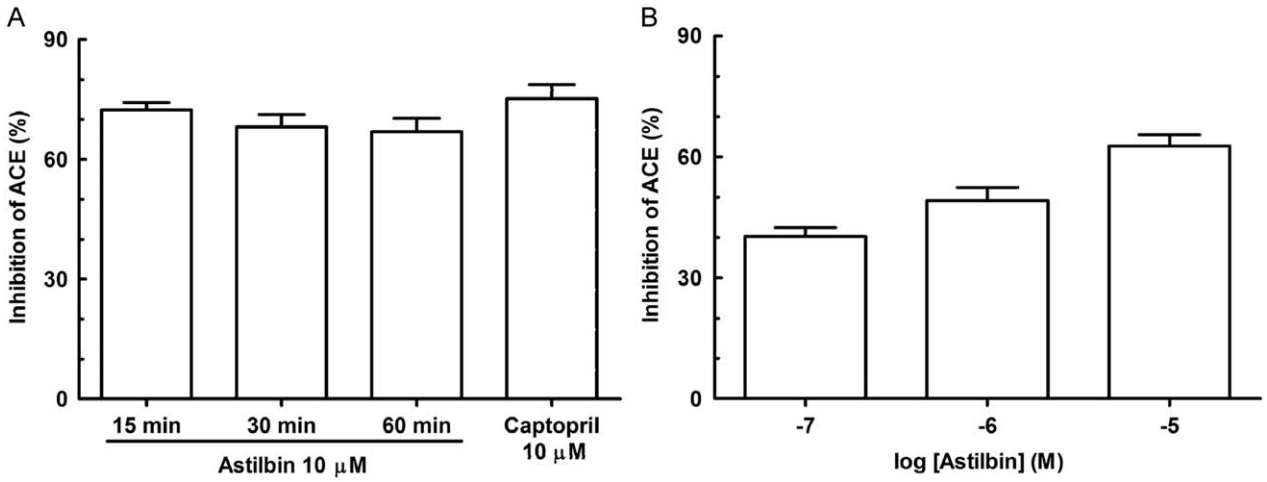

Fig. 4. Effect of astilbin on the ACE *in vitro* inhibition assay (iACE). (A) Incubation intervals for iACE with astilbin. (B) Concentration-dependent effect of astilbin on iACE. Each bar represents the mean ± SEM of at least 6 replicates. Captopril was incubated with the serum 60 min before substrate addition. Astilbin was incubated with serum 15 min before substrate addition for concentration-dependent iACE. The results illustrated in (A) are not significantly different when incubation intervals or the effect of captopril was compared with those of astilbin. The results on (B) are significantly different between each other ($p < 0.05$). The vehicles used for dilution of captopril (saline) and astilbin (DMSO) did not inhibit ACE activity. The statistical analyzes was performed by One-Way ANOVA with Tukey's multiple comparison post-test.

EEG elicited concentration-dependent ACE inhibition (pIC_{50} 4.53 ± 0.05; Fig. 3), employing a colorimetric *in vitro* assay previously described by our group (Serra et al. 2005). Keeping in mind that flavonoids have been identified as ACE inhibiting constituents of different plant species (Lacaille-Dubois et al. 2001; Oh et al. 2004; Loizzo et al. 2007), the activity of compound **1** was investigated in the present study. Astilbin was evaluated at different time intervals (15, 30 and 60 min) before substrate addition, in order to determine the optimal time of reaction (Fig. 4A). It promoted ACE inhibition over 65% at each time interval assayed, statistically different to the negative control ($p < 0.001$; not shown). Besides, the ACE inhibition showed by compound **1** (10 μM) at 15, 30 and 60 min before substrate addition did not differ from captopril (10 μM; Fig. 4A).

The ACE inhibitory activity observed for astilbin (pIC_{50}=5.86 ± 0.33; Fig. 4B) is considerably higher than that reported for other flavonoids, such as quercetin -3-*O*-α-6-caffeoyl-glycosyl-β-1,2-rhamnoside (pIC_{50}=3.9), quercetin-3-*O*-α-6-*p*-coumaryl-glycosyl-β-1,2-rhamnoside (pIC_{50}=3.4), quercetin-3-*O*-β-glucopyranoside (pIC_{50}=3.1) (Oh et al. 2004) and quercetin-3-*O*-α-arabinopyranoside (pIC_{50}=3.5) (Loizzo et al. 2007). Astilbin shows superior ACE inhibition activity even in comparison to proanthocyanidins, a class of natural products regarded as the most potent ACE inhibitors from plants, including epicatechin dimer (pIC_{50}=3.6), trimer (pIC_{50}=3.9), tetramer (pIC_{50}=4.9), pentamer (pIC_{50}=4.6) and hexamer (pIC_{50}=5.0) (Actis-Goretta et al. 2003).

In conclusion, the results described in here point out astilbin as one of the constituents responsible for the ACE inhibitory activity observed for the ethanol extract of *E. gonocladum* aerial parts and disclose **1** as a promising molecule with potential anti-hypertensive activity, via ACE inhibition.

Acknowledgments

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