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Alkaloids from *Croton echinocarpus* Baill.: Anti-HIV potential

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ABSTRACT

Croton belongs to the Euphorbiaceae genus, one of the major Angiosperms families, and is widely distributed throughout the world, especially in the tropics. In this study, the hydroalcoholic extract (70% EtOH) of *Croton echinocarpus* leaves afforded two alkaloids: corydine and norisoboldine, and their structures were established by spectroscopic data interpretation (UV, IR, NMR and LREIMS). Both alkaloids displayed significant in vitro anti-HIV potential, inhibiting 40% of the HIV-1 reverse transcriptase enzyme activity at a concentration of 100 μg mL^{-1} of norisoboldine and 450 μg mL^{-1} of corydine. Corydine showed IC_{50} of 356.8 μg mL^{-1}, while norisoboldine was more efficient on inhibiting the RT activity, showing IC_{50} of 153.7 μg mL^{-1}.

1. Introduction

Euphorbiaceae Juss. is one of the major Angiosperms families. It is widely distributed throughout the world, especially in the tropics, composing various types of vegetation (Secco et al., 2012) and being one of the most common families in Brazilian biomes.

Croton L. is a genus of Euphorbiaceae and it is composed by approximately 1300 species of herbaceous, shrubs, trees, and lianas forms (Riina et al., 2009). This genus is widely distributed in tropical and subtropical regions around the world and is diversely expressed in Brazil (Berry et al., 2005; Van Ee et al., 2011). Several *Croton* species have been used in traditional medicine for the treatment of various illnesses, such as infections, wounds, fever, diabetes, and obesity (Salatino et al., 2007).

Traditional knowledge has proven to be a useful tool in the search for new plant-based medicines (Cox and Balick, 1994; Cox, 2000; Lewis, 2003). Of the approximately 300,000 species of higher plants, about 10,000 have their medicinal uses documented, which is still considered a very small percentage of the plant species studied (McChesney et al., 2007). Ethnobotanic and ethnopharmacological studies have been extremely relevant to discover promising drugs for the cure and/or treatment of various diseases. Saslis-Lagoudakis et al. (2012) listed genus and plants species according to their medicinal bioactivity and included *Croton* as a high priority genus for future phytochemistry screening.

Medicinal and toxic properties of *Croton* species are derived to the presence of a wide variety of chemical compounds, such as diterpenoids, triterpenoids, steroids, volatile oils containing mono- and sesquiterpenoids, alkaloids and phenolic derivatives, mainly flavonoids, lignans and proanthocyanidins (Salatino et al., 2007).

The importance of several Brazilian *Croton* species in traditional medicine has been described. For example, *Croton celtidifolius* Baill., *Croton urucurana* Baill. and *Croton cajucara* Benth. have been used in traditional medicine for the treatment of cancer (Salatino et al., 2007). Many studies have evaluated the bioactive properties of the latex of *C. urucurana*, proving its anti-inflammatory, analgesic, antiviral and antitumoral properties (Gurgel et al., 2001). Another study of this species demonstrated the effectiveness of its latex in inhibiting the growth of some types of fungi that cause skin infections (Gurgel et al., 2005). The Brazilian shrub species *Croton betulaster* Müll. Arg. contains casticin, which has been studied as a potential drug for the treatment of neurodegenerative diseases (Spohr et al., 2010). In a recent study, it was found that the dichloromethane (CH_2Cl_2) extract *Croton macrobothrys* Baill. leaves, composed by geranyl geraniol, corydine and clerodane diterpene derivatives, showed antiproliferative activity on tumor cell

Abbreviations: NCI-H460, non-small cell lung cancer cell line; K562, leukemic cell line; RT-HIV-1, Human Immunodeficiency Virus-1 reverse transcriptase enzyme; DMSO, dimethyl sulfoxide; DEPC, diethylpyrocarbonate; anti-DIG-POD, antibody anti-digoxigenin-peroxidase; ABTS, (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)); GC-MS, gas chromatography coupled to mass spectrometry; CC, column chromatography; NC, negative control; PC, positive control; RT, reverse transcriptase; IC_{50}, concentration of each sample to achieve 50% of the inhibitory RT activity; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; LREIMS, low-resolution electron impact mass spectra.

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lines, particularly NCI-H460 (lung) and K562 (leukemia) (Motta et al., 2011).

Some *Croton* species exhibit seasonal variations in their composition. Interestingly, Pereira et al. (2001) observed that seasonal changes exerted a direct influence on the composition of terpens, steroids, alkaloids and α-tochoferol from *Croton hemiargyreus* Müll. Arg. var. *hemiargyreus* and *Croton echinocarpus* Baill.

C. echinocarpus is widely distributed in the Brazilian Atlantic Forest. It is native and endemic to Brazil, predominating in the states of Rio de Janeiro and Minas Gerais (Cordeiro et al., 2014). Moreover, species from the *Cyclostigma* section consist of large trees, which are interesting to studies of bioactive substances, since they have large amounts of both foliar and stem biomass. Another species belonging to the *Cyclostigma* Griseb. section present valuable pharmacological properties, such as *C. urucurana*.

This study aimed to investigate the antiviral potential of two alkaloids isolated from *C. echinocarpus* leaves.

2. Material and methods

2.1. General

The column chromatographic separation used silica gel (230–400 mesh, Merck-Sigma-Aldrich, St. Louis, MO, USA). ^{1}H and ^{13}C spectra were recorded, respectively, at 500 and 125 MHz in a Bruker Ascend II 500 spectrometer. Deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) (Sigma-Aldrich, St. Louis, MO, USA) were used as solvent and the residual peak of the non-deuterated solvent was employed as an internal standard. Chemical shifts (δ) are reported in ppm and the coupling constant (*J*) is given in Hz. The gas chromatography with low-resolution electron impact-mass spectrometry (GC-LREIMS) analysis were conducted using an Agilent GC-MS 6850/5975B equipment, a HP-5MS (5% phenyl, 95% polydimethylsiloxane—30 m × 0.25 cm × 0.25 μm film thickness) column and helium as the mobile phase (1 mL min^{-1}). During these analyses, the injector and detector temperatures were 250 °C and 350 °C, respectively. The column temperature program was the following: 150 °C during 5 min, raising 5 °C min^{-1} till 310 °C. The LREIMS analysis employed an ionization voltage of 70 eV and an ion source temperature of 230 °C. Identification was performed by comparing the mass spectra data with NIST library software. Optical rotations were measured in the digital polarimeter JASCO DIP-370 (Na filter, λ = 588 nm) using ethanol (EtOH) as a solvent. The infrared (IR) spectra were measured in mineral oil (Nujol, Mantecorp, Rio de Janeiro, Brazil) in a Infrared Spectrometer model 1750 (Perkin-Elmer, Waltham, MA, USA). The UV spectra were recorded in a HP 8452 A spectrophotometer using methanol (MeOH) as a solvent.

2.2. Plant material

Leaves and steams of *C. echinocarpus* Baill. were collected at Santana do Riacho, Minas Gerais, Brazil, at the State Road 010 in May 2011. A voucher (LBM62) was deposited at the SPF Herbarium (University of São Paulo, Brazil) and Dr Lucimar Motta and Dr Inês Cordeiro identified the species.

2.3. Extraction

Air-dried leaves (25 g) were grounded in the presence of liquid N_2 and underwent maceration with 70% EtOH for seven days. After filtration, concentration under reduced pressure and lyophilization, crude EtOH extract yielded 1.24 g (4.9%) of material. The crude EtOH extract went on the alkaloid isolation by column chromatography (CC) and the acid–base alkaloid extraction methodology described below. In addition, the crude EtOH extract was partitioned using methanol and hexane, yielding methanol (MEP) and hexane phases (HEP).

2.4. Isolation of alkaloids

Part of the crude EtOH extract (0.6 g) was fractionated by CC (silica gel, 230–400 mesh, Merck-Sigma-Aldrich, St. Louis, MO, USA), using mixtures of hexane-ethyl acetate (Hex-EtOAc) and ethyl acetate-methanol (EtOAc-MeOH) with increasing polarity (100:0), to give 10 fractions. Fraction 7 (144.6 mg) was subjected to CC in silica gel using MeOH as a solvent, generating five subfractions (I–V). Subfraction V yielded 5 mg of corydine. Additionally, another part of the crude EtOH extract (0.2 g) was dissolved in HCl 5% and partitioned using CH_2Cl_2. pH was alkalinized using NH_4OH and the alkaloids were extracted twice with CH_2Cl_2 to afford 16.7 mg of norisoboldine.

Corydine (peak **7**). $[\alpha]_D^{25}$ + 201 (EtOH). UV (MeOH) λ/nm (log ε): 220 (3.3), 270 (2.9), 305 (2.6) nm. IR (Nujol®) ν/cm^{-1}: 3200, 1605, 1460, 1390, 1314, 1295, 1264, 1088, 1070, 961, 935, 828, 760. ^{1}H nuclear magnetic resonance (NMR) (500 MHz, $CDCl_3$ + drops of CD_3OD) δ_H: 7.05 (d, J = 7.2 Hz, H-8), 6.85 (d, J = 7.2 Hz, H-9), 6.67 (s, H-3), 3.87 (s, 10-OMe and 11-OMe), 3.71 (s, 2-OMe), 3.45 (m, H-6a), 3.18 (m, H-4_{ax}), 3.08 (m, H-7_{eq}), 3.06 (m, H-5_{ax}), 2.90 (s, Me-N), 2.75 (m, H-5_{eq}), 2.67 (m. H-4_{eq}), 2.37 (m, H-7_{ax}). ^{13}C NMR (125 MHz, $CDCl_3$ + drops of CD_3OD) δ_C: 151.7 (C-10), 149.0 (C-2), 143.7 (C-11), 142.2 (C-1), 130.5 (C-7a), 127.8 (C-1b), 126.3 (C-11a), 124.2 (C-8), 123.7 (C-3a), 119.1 (C-1a), 111.2 (C-3), 110.8 (C-9), 62.6 (C-6a), 61.7 (11-OMe), 56.3 (2-OMe and 10-OMe), 52.6 (C-5), 41.2 (N-Me), 35.3 (C-7), 28.8 (C-4). LREIMS (70 eV): *m/z* (rel. int.): 341 (70), 340 (27), 326 (52), 324 (31), 311 (20), 310 (100), 155 (21), 44 (16), 42 (41).

Norisoboldine (peak **8**). $[\alpha]_D^{25}$ + 45 (EtOH). UV (MeOH) λ/nm (log ε): 304 (4.2), 280 (4.0), 219 (4.5) nm. IR (nujol) ν/cm^{-1}: 3208, 1602, 1450, 1381, 1300, 1250, 1072, 955, 829, 760. ^{1}H NMR (500 MHz, $CDCl_3$ + CD_3OD) δ_H: 8.06 (s, H-11), 6.80 (s, H-8), 6.57 (s, H-3), 3.92 (s, 2-OMe), 3.91 (s, 10-OMe), 3.47 (m, H-6a), 3.06 (m, H-4_{ax}), 3.05 (m, H-5_{ax}), 2.73 (m, H-5_{eq}), 2.72 (m, H-4_{eq}), 2.70 (m, H-7_{eq}), 2.05 (m, H-7_{ax}). ^{13}C NMR (125 MHz, $CDCl_3$ + CD_3OD) δ_C: 145.7 (C-10), 145.0 (C-9), 141.8 (C-1), 128.0 (C-7a), 126.2 (C-1a), 123.9 (C-11a), 114.3 (C-3 and C-8), 109.0 (C-11), 56.3 (10-OMe), 56.2 (2-OMe), 53.3 (C-6a), 42.5 (C-5), 35.3 (C-7), 27.5 (C-4). LREIMS (70 eV): *m/z* (rel. int.): 313 (69), 312 (100), 311 (9), 298 (17), 296 (5), 284 (5), 283 (5), 282 (9), 269 (5).

2.5. Anti-HIV activity

The inhibitory activity of the isolated substances over the human immunodeficiency virus-1 reverse transcriptase enzyme (RT-HIV-1) was analyzed using the reverse transcriptase assay, a colorimetric kit (Roche Diagnostics, Basel, Suíça), according to the manufacturer's instructions. Briefly, all samples were dissolved in 10% dimethyl sulfoxide (DMSO) prepared using diethylpyrocarbonate (DEPC) water to reach concentrations of 100, 250 and 500 μg mL^{-1} of crude EtOH extract and phases and concentrations of 5 to 450 μg mL^{-1} of the isolated substances. Twenty milliliters of the diluted samples was incubated for 1 h at 37 °C, along with 1 μL of the reverse transcriptase enzyme, 19 μL of buffer (50 mM Tris, 80 Mm potassium chloride, 2.5 mM DTT, 0.75 mM EDTA and 0,5% Triton X-100, pH 7.8) and 20 μL of the oligo + polyA solution (50 mM Tris–HCl, pH 7.8; DIG-dUTP, biotin-dUTP and dTTP + template/primer poli (A) oligo (dT)15 (9 A260 nm mL^{-1})). Sixty milliliters was transferred to a microplate containing streptavidin and was incubated for 1 h at 37 °C. After that, the microplate was washed twice with buffer and 198 μL of the incubation buffer (pH 7.8, 50 mM Tris, 319 mM potassium chloride, 33 mM magnesium chloride and 11 mM DTT) plus 2 μL of anti-DIG-POD (antibody anti-digoxigenin-peroxidase) were added. The microplate was washed five times with buffer and 200 μL of ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) was added; the solution was incubated for 15 min at 25 °C. The absorbance was analyzed at 405 and 490 nm. Three controls used were: 10% DMSO instead of the samples (S), with no addition of RT (Blank—B); 10% DMSO instead of the samples with the addition of RT