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Original article

Alkaloids and biological activity of beribá (*Annona hypoglauca*)

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ABSTRACT

Annona hypoglauca Mart., Annonaceae, popularly known as "beribá", was collected in flooded areas of the Amazonian Rain Forest. The crude extract obtained from this species was found to be cytotoxic against human cancer cells. Chemical information on *A. hypoglauca* is scarce. So, the present work aimed the isolation and identification of its alkaloids and to test their cytotoxic activity. Alkaloids were obtained from stem by acid–base partitioning and the remaining alkaloid-free extract was partitioned with organic solvents. Gas chromatography–mass spectrometry GC/MS analysis of total alkaloids allowed the identification of four aporphine alkaloids: actinodaphnine, anonaine, isoboldine and nornuciferine. Total alkaloids were fractionated by column chromatography and were purified by preparative thin-layer-chromatography, which allowed the isolation of two aporphine alkaloids, actinodaphnine and isoboldine, characterized by NMR and CG–MS analyses. This is the first report for the occurrence of actinodaphnine in *Annona* species. All the samples were tested in cytotoxic and antibacterial assays. Total alkaloid extract and its fractions showed antimicrobial activity against *Staphylococcus aureus* and *Enterococcus faecalis*. In the cytotoxicity assay, the crude extract showed a lethal effect against breast and colon cancer cells. Isoboldine-containing FA5 and actinodaphnine-containing FA6 showed activity against breast cancer cell line, while the alkaloid-free fractions did not show significant activity against cancer cell lines.

Introduction

Annonaceae is a pantropical family containing approximately 130 genera and 2300 species of trees and shrubs (Heywood, 1993; Cordell et al., 2001). The tropical American genus *Annona* L. is represented by 140 species, which are recognized mainly by their edible fruits, such as *A. muricata* (graviola), *A. squamosa* (fruta-do-conde), *A. coriaceae* (araticum) and *A. cherimolia* (cherimoya) plants belonging to the genus *Annona* are also used in folk medicine as antitumor, anti-parasite and antidiarrhea (Pimenta et al., 2003), antiprotozoal (Siqueira et al., 2011), anti-inflammatory (Siebra et al., 2009), cytotoxic and anti-ulcerogenic agents (Hamid et al., 2012). Some species have also been pharmacologically studied for their platelet anti-aggregation and antiulcerogenic activities (Villar et al., 1997; Padma et al., 1998). The genus *Annona* is a rich source of isoquinoline – particularly aporphine alkaloids (Leboeuf et al., 1982; Rabêlo et al., 2014) – and acetogenins (Bermejo et al., 2005). The structural diversity of the isoquinoline alkaloids is as wide as the range of its biological activities, which can be used as antimicrobial (Simeón et al., 1990), cytotoxic (Wu et al., 1993), antitumoral (Sonnet and Jacobson, 1971), antiprotozoal (Tempone et al., 2005), antiviral (Montanha et al., 1995) besides many other applications, especially in demonstrating antimicrobial activity against Gram-positive bacteria (Villar et al., 1987; Abbasoglu et al., 1991; Paulo et al., 1992).

Annona hypoglauca Mart. occurs in the flooded areas (*igapós*) of the northern Amazonian forests, as well as in *terra firme* forests. The species can be found as a tree up to 10 m height when occurring in *terra firme* or as a liana when occurring in the *igapós* where it is popularly known as *beribá* (Gottsberger, 1978), or *biribá*, in the Brazilian Amazon region, or as *guanábana huasca* and *tortuga blanca* by other South American communities, or as wild soursop, its English name. Its fruits are eaten by locals and the tea made with the bark is used as medicine against parasites, anemia and chronic diarrhea (Revilla, 2002). *A. mucosa*, *A. sylvatica*, *Duguetia marcgraviana* and *Fusea longifolia* are also known as *biribá*, in Brazil (Reflora, 2016).

Previous biological screening aiming the identification of cytotoxicity of 1277 Amazon plant extracts against human breast cancer cell lines (Suffredini et al., 2007a), prostate cancer cell lines (Suffredini et al., 2006a), colon, lung, central nervous system and leukemia cancer cell lines (Suffredini et al., 2007b) have been made,

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together with the evaluation of the antimicrobial activity of the extracts against some pathogenic microorganisms as *Staphylococcus aureus* (Suffredini et al., 2004), *Enterococcus faecalis* (Castilho et al., 2013, 2014), *Streptococcus mutans* (Barnabé et al., 2014) and *Escherichia coli* (Camargo and Suffredini, 2014). From the previous screening, the crude extract obtained from the stem of *A. hypoglauca* Mart. (Annonaceae), the so-called EB1109, has shown a significant activity against breast cancer cell line MCF-7 (Suffredini et al., 2007a) and against *S. mutans* (Barnabé et al., 2014). Despite these first results, little is known about the chemical composition and biological activities of *A. hypoglauca*, despite a wide literature considering other *Annona* species.

The present study aims to report the isolation of aporphine alkaloids from the stems of *A. hypoglauca*, as well as to report the cytotoxic activity of EB1109 and its fractions. As literature also indicates a putative antimicrobial activity related to the *Annona* alkaloids, all samples were also tested against the pathogenic bacteria.

Materials and methods

Plant material

The stem of *A. hypoglauca* Mart., Annonaceae, was collected in water-flooded forests (named *igapó* forest), Amazon rain forest, Manaus, (Lat. 2°58′ and Long. 60°27′), Anavilhanas Ecological Station). A voucher specimen was deposited under identification number [AAOliveira, 3577 (UNIP Herbarium] and was identified by Dr. Mateus L. B. Paciencia. Plant collection was done under Brazilian Government plant collect license number MMA/ICMBio/SISBIO#14895 and license to access genetic material Ibama/MMA/CGen#012A-2008.

Extraction procedures

Crude extract: The stems of the plant (1.074 kg) were air-dried at 40 °C in an air-circulating oven. Plant material was ground in a hammer mill before being submitted to 24 h maceration with 10 l of a mixture containing dichloromethane:methanol (1:1, v/v). The resulting organic extract was concentrated to dryness (46.14 g) and yielded 4.3% of EB1109 (Younes et al., 2007).

Total alkaloid fraction (TA): EB1109 (5 g) were extracted with 30 ml 0.1 M phosphoric acid under agitation for 30 min. Extraction was repeated four times. Acid solutions were filtered and reunited to the same funnel. Acid solution was three times extracted with 50 ml hexane in order to have non-polar compounds removed. The acid solution was brought to pH ~ 9 with ammonia hydroxide solution (25%, w/w) and was partitioned with portions of 50 ml chloroform, until negative to Dragendorff's reagent. The organic phase was dried with anhydrous sodium sulphate and concentrated to give the total alkaloid (TA) fraction (3.1 g; 0.28%). TA yield was obtained considering the crude plant material.

Alkaloid-free extracts: The cake, considered as the remaining insoluble material resulted from the alkaloid extraction, was dried and the remaining solids were dissolved in a series of solvent systems. Hexane (30 ml) was added to the cake and the system remained under agitation for 30 min. After that, the system was decanted and filtered. This procedure was repeated three times under the same conditions. The combined hexane solutions were evaporated and originated fraction hexane (FHex, 2.64 g; 0.25%). A second dissolution done in a similar way was performed with a mixture of dichloromethane (DCM) and methanol (MeOH) (1:1, v/v), resulting fraction DCM/MeOH (DCM/MeOH; 1.71 g; 0.16%). The third dissolution was made with ethyl acetate, resulting in fraction FEAC (0.21 g; 0.02%). Finally, the remaining cake material was partitioned with a 20% ethanol:H_2O solution and extracted with three portions of 250 ml each of butanol, originating the fraction butanol (FBuOH; 0.65 g; 0.06%). Fractions yields were obtained considering the crude plant material.

Isolation of alkaloids

TA (2 g) was fractionated by column chromatography (70 cm length x 40 mm diameter) on 50 g normal phase silica gel 60. Elution was made with the following solvent mixtures: DCM and MeOH in order of increasing polarity, as follows: 100% DCM (200 ml), DCM:MeOH 9:1 (400 ml), DCM:MeOH 8:2 (400 ml), DCM:MeOH 7:3 (400 ml), DCM:MeOH 1:1 (400 ml) and 100% MeOH (200 ml). The elution resulted in the following fractions: 100% DCM yielded fraction 1 (FA1, 7.6 mg; 0.0011%); DCM:MeOH 9:1 (v/v) yielded fractions 2 (FA2, 0.4 mg; 0.00005%) and 3 (FA3, 738.4 mg; 0.1055%); DCM:MeOH 8:2 (v/v) yielded fractions 4 (FA4, 161.4 mg; 0.0231%) and 5 (FA5, 176.1 mg; 0.0252%); DCM:MeOH 7:3 (v/v) yielded fractions 6 (FA6, 395.8 mg; 0.0565%) and 7 (FA7, 43.4 mg; 0.0062%); and finally fractions 8 (FA8, 26.9 mg; 0.0038%), 9 (FA9, 22.1 mg; 0.0031%) and 10 (FA10, 16.0 mg; 0.0023%) were eluted with DCM:MeOH 1:1 (v/v). Fractions yields were obtained considering the crude plant material.

Fractions FA4, FA5 and FA6 were again fractionated using preparative thin layer chromatography (PTLC) precoated with 1 mm of silica gel $60F_{254}$, without being activated and a solvent mixture of $CHCl_3$:MeOH (92:8) as mobile phase. Spots were observed under U.V. light (λ = 366 nm) and revealed after reaction with Dragendorff's reagent. Bands related to each isolated compound were eluted with DCM and were then submitted to RMN analysis. Fraction FA4 originated four fractions, FA5 originated three fractions, and FA6 originated six fractions. Compound **1** was then isolated from FA5.2 using PTLC as a brown amorphous solid (3.7 mg). Compounds **2** and **3** were analyzed by ^{1}H NMR as a mixture. Lastly, compound **4** was isolated from FA6.2, obtained from preparative TLC, as a light brown amorphous solid (7.8 mg). Four alkaloids were identified.

Compound **1**, isoboldine: (^{1}H NMR, 300 MHz, $CDCl_3$, TMS internal standard): δ 7.94 (1H, *s*, H-11), 6.74(1H, *s*, H-8), 6.47 (1H, *s*, H-3), 3.84–3.85 (6H, *s,br*, OMe-10 and OMe-2), 2.49 (3H, *s*, N-CH_3) (Soares et al., 2015; Jackson and Martin, 1966); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.74 (C-2), 144.90 (C-9), 144.43 (C-10), 140.47 (C-1), 129.7 (C-7a), 124.4 (C-11a), 119.7 (C-1a), 113.87 (C-11), 111.55 (C-8), 108.64 (C-3), 62.51 (C-6a), 56.16 (C-2 OMe), 56.09 (C-10 OMe), 53.36 (C-5), 43.80 (C-6 N-CH_3), 33.9 (C-7), 28.79 (C-4)) (Jackman et al., 1979). MS/EI (M+) RT = 27.99: 327 (M^+), 326 (M^+-1), 310 (M^+-17), 284 (M^+-43), 269 (M^+-58), 253 (M^+-74).

Compound **2**, nornuciferine (present in fraction FA5.3): (^{1}H NMR, 200 MHz, $CDCl_3$, TMS internal standard): δ 8.32 (1H, *d*, *J* = 7.8 Hz, H-11), 7.28 (1H, *m*, H-8, H-9, H-10), 6.58 (1H, *s*, H-3), 3.59 (3H, *s*, 2-OCH_3), 3.32 (3H, *s*, 1-OCH_3) (Dutra et al., 2012; Hasrat et al., 1997). MS/EI (M+) RT = 20.12: 281 (M^+), 280 (M^+-1), 266 (M^+-15), 250 (M^+-31), 237 (M^+-43), 221, 178, 165, 152.

Compound **3**, anonaine (present in fraction FA5.3): (^{1}H NMR, 200 MHz, $CDCl_3$, TMS internal standard): δ 8.00 (1H, *d*, *J* = 7.8 Hz; H-11), 7.28–7.14 (1H, *m*, H-8, H-9, H-10), 6.68 (1H, *s*, H-3), 5.89 (1H, *d*, *J* = 0.8 Hz, 1-OCH_2O-2) and 6.28 (1H, *d*, *J* = 0.8 Hz, 1-OCH_2O-2,) (Costa et al., 2012; Hasrat et al., 1997). MS/EI (M+) RT = 21.32: 265 (M^+), 254 (M^+-1), 236 (M^+-29).

Compound **4**, actinodaphnine: (^{1}H-NMR, 300 MHz, $CDCl_3$, TMS internal standard): δ 7.56 (1H, *s*, H-11), 6.73(1H, *s*, H-8), 6.45 (1H, *s*, H-3), 6.01 (1H, app. *s*, 1-OCH_2O-2), 5.86 (1-H, app. *s*, 1-OCH_2O-2), 3.92 (1H, *dd*, *J* = 13.92, 5.16 Hz, H-6a), 3.85 (3-H, *s*, OMe-10), 3.46 (1H, *s,br*, NH-6), 3.38 (1H, *d* br, *J* = 7.77, H-5_{eq}), 2.97 (2H, *d* br, *J* = 8.88, H-5_{ax}, H-4_{eq}), 2.82 (1H, *d* br, *J* = 13.92, H-7_{eq}), 2.61 (2H, *d* br, *J* = 12.05, H-4_{ax}, H-7 $_{ax}$); ^{13}C-DEPT 135 (300 MHz, $CDCl_3$): CH: δ

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