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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 *Annona 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.

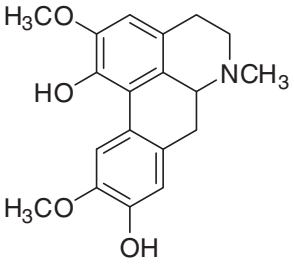
Compound **1**, isoboldine: (^{1}H NMR, 300 MHz, $CDCl_3$, TMS internal standard): δ 7.94 (1H, *s*, H-11), 6.74(1H, *s*, H-8), 6.47 (1H, *s*, H-3), 3.84–3.85 (6H, *s*,*br*, OMe-10 and OMe-2), 2.49 (3H, *s*, N-CH_3) (Soares et al., 2015; Jackson and Martin, 1966); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.74 (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 114.4; 110.1; 106.9; CH_3: 56.1; 53.3; CH_2: 100.6; 42.55; 35.4; 28.3. ^{13}C NMR (75 MHz,$CDCl_3$): δ 146.99 (C-2), 145.65 (C-10), 145.35 (C-9), 141.70 (C-1), 128.09 (C-7a), 125.98 (C-1b), 125.65 (C-3a), 122.74 (C-11a), 116.57 (C-1a), 114.41 (C-8), 110.11 (C-11), 106.96 (C-3), 100.62 (OCH_2O), 56.08 (C-10 OMe), 53.27 (C-6a), 42.56 (C-5), 35.37 (C-7), 28.32 (C-4)) (Stévigny et al., 2002). MS/EI (M+) RT = 27.48: 311 (M^+), 310 (M^+-1), 279, 251, 181.



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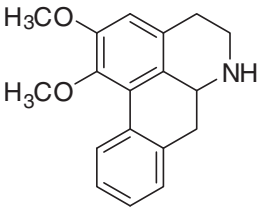

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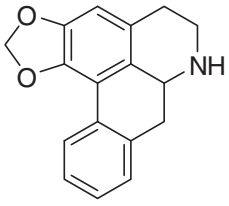

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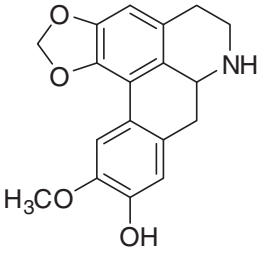

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Spectroscopic analysis

NMR spectra of ^1H and ^{13}C were obtained on a Bruker spectrometer, operating at 200 MHz or at 300 MHz (^1H) and at 75 MHz (^{13}C). GC–MS was performed in an Agilent Series 6890 chromatograph, equipped with a HP-5 column (30 m × 0.25 mm, film of 0.25 μm), the injector was set to 290 °C, the carrier gas (He) flow was adjusted to 1 ml/min, the initial temperature was 95 °C for 2 min, followed by an increment of 8 °C/min until 310 °C, the temperature was maintained for 2 min. The MS detector operated in the electron impact (EI; 70 eV), was used to qualitative evaluate the alkaloid contents.

Biological assays

Samples preparation

Sample preparation for cytotoxic assay: all the samples were diluted to 2 mg/ml in 50% dimethylsulfoxide (DMSO) in water before being evaluated in a single-concentration assay (final test concentration 100 μg/ml). Sample preparation for antibacterial assay: samples were diluted in DMSO50% to the following concentrations of 200, 180, 160, 140, 120, 100, 80, 60, 40 and 20 mg/ml, in order to proportionate final test concentrations of 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 μg/ml. Vehicle was tested for its antimicrobial activity, as well as doxorubicin (Adriamicin®) at a concentration of 25 mM was used as standard drug in the cytotoxicity assay and gentamycin and tetracycline prepared at various concentrations were used as standard drugs in the antibacterial assay. Antibiotic final test concentrations for MIC/MBC assay ranged from 120 μg/ml down to 0.16 μg/ml.

Antibacterial microdilution broth assay

Tests were performed in aseptic conditions (Suffredini et al., 2015). Bacteria inoculum was prepared at the concentration of 1.5×10^2 CFU/ml, starting from a 0.5 McFarland (or 1.5×10^8 CFU/ml), as follows (Younes et al., 2007). *S. aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922) and *E. faecalis* (ATCC 29212) were the bacterial strains tested. Bacteria inoculum of each strain was obtained from fresh 24-h culture colonies grown on Müeller-Hinton agar plates. Each strain was inoculated into 5 ml of Müeller–Hinton broth in order to obtain a concentration of 1.5×10^8 CFU/ml (0.5 McFarland), which was determined by a turbidimeter (Oxoid) adjusted to the 0.5 McFarland concentration. Each inoculum was then diluted in broth medium to 1.5×10^2 CFU/ml. One hundred and ninety microliters of this suspension was transferred to each microplate well. Ten microliters of each treatment were added to the microplate wells and incubated at 35 °C for 18–20 h. Treatments that have visually inhibited bacteria from grow were subcultured in Müeller-Hinton agar plates that were incubated at 35 °C for 18 to 20 h. Observations of bacteria grow or lack of bacteria grow determined which treatment was effective. All samples that showed effectiveness in the single-concentration assay were submitted to the determination of MIC and MBC.

Determination of minimal inhibitory concentration and minimal bactericidal concentration

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for the treatments that showed total growth inhibition in the single-concentration assay using the protocol described above. The same protocol was used to determine MIC and MBC. Samples were prepared in concentrations of 0.2 up to 2 mg/ml, in a 0.200 mg/ml fold (test concentrations ranging from 10 to 100 μg/ml, in 10 μg/ml fold) were evaluated. Turbidity was observed and those identified as having a visual lack of turbidity were subcultured in Müeller–Hinton agar Petri dishes, which were then incubated at 35 °C for 18–20 h. After the incubation, bacteria growth was determined and supported the establishment of MIC's and MBC's as follows: the lower concentration observed as having a lack of turbidity but presenting bacteria growth was determined as the MIC and the lower concentration having a lack of turbidity and did not present any bacteria growth after subculture was considered as the MBC.

Cytotoxicity assay

The original method (Monks et al., 1991) was adapted (Suffredini et al., 2006b) for specific cell lines. Human tumor cell lines (MCF-7, breast adenocarcinoma; KM-12, colon adenocarcinoma; RPMI-8226, multiple myeloma; PC-3, prostate carcinoma; SF-268 glioblastoma and NCI-H460, non-small lung-cell carcinoma), obtained from the National Cancer Institute (Frederick, MD, USA), were maintained in RPMI-1640 medium supplemented with 10% bovine fetal serum, 1% L-glutamine and 1% gentamycin, at 37 °C, 5% CO_2 and 100% relative humidity. Cell density was measured in a Neubawer chamber using the trypan blue exclusion method for each cell line. Cell density (per well per 100 μl) varied according to each cell line, for the assay: PC-3 (7500); KM-12 (15,000); NCI-H460 (7500); SF-268 (15,000) and RPMI-8226 (20,000) (Monks et al., 1991). So, 100 μl of cell suspension was transferred to each well of 96-well plates, following the established cell density described above. Plates were incubated at 37 °C, 5% CO_2 and 100% relative humidity for 24 h. One of the plates were fixed with 50 μl of cold 50% (adherent cells) or 80% (non-adherent cell) trichloroacetic acid (TCA) in order to obtain time zero cell growth, corresponding to the time immediately before treatment addition. So, 100 μl of each

treatment (extract, fractions, isolates or doxorubicin) was added to the wells in sextuplicates. Also, in order to obtain a better experiment control, two wells having only medium plus treatment were added to the plates, as well as 16 wells containing medium plus cells without treatment, in order to establish a regular cell grow (positive control). Plates were kept incubating for 48 h before evaluation. After that, plates were also fixed with TCA at the pre-stipulated concentration. TCA was removed with water and sulforodamine B (SRB) colorimetric assay was performed. According to Voigt (2005), the protein dye SRB assay is currently used for measuring drug-induced cytotoxicity and cell proliferation for binding electrostatically and pH dependent to protein basic amino acid residues of TCA-fixed cells in a linear correspondence to the amount of cells. So, time zero (*T0*), cell control (*C*) and treatment (*T*) optical densities at 515 nm were used to calculate the percentage of cell grow inhibition or, as follows: $[(T - T0)/(C - T0) \times 100 = \%$ of growth]. Results were interpreted by the comparison of cell growth after treatment (*T*) to positive control cells (*C*), both considering cell growth at Time Zero (*T0*). If $100 > T > T0$ was observed the presence of cell growth inhibition; if $T = T0 = 0$ was observed that cells do not grow in relation to the cell density at *T0*; if $T < T0$, it means that treatment is effective enough to cause cell lethality. Treatments were evaluated at a specific test concentration of 100 μg/ml.

Results and discussion

The alkaloid yield from *A. hypoglauca* stems was 0.28%. Previous studies (Debourges et al., 1987) relate yields ranging from 0.3% and 0.2% for *Duguetia* species, while (Fischer et al., 2004) describes yields of 0.5% for *Annona*. The yield of total alkaloids here obtained from *A. hypoglauca* is somewhat among the expected values.

TA fraction was analyzed by gas GC–MS, and the presence of four aporphine alkaloids could be determined: isoboldine (**1**) isolated from FA5.2, nornuciferine (**2**) and anonaine (**3**), identified as mixture in fraction FA5.3 and actinodaphnine (**4**), isolated from FA6.2. Compounds were characterized by the comparison of their fragmentation pattern in the mass spectra (MS) to the library from the equipment (Wiley 25) and to the literature data. Major compound (*Rt* 20.12 min) showed the following fragmentation pattern: *m/z* 281 ($M^{+\cdot}$), 280 (M^+-1), 266 (M^+-15), 250 (M^+-30), 252 (M^+-29), 237, 221, 178, 165, 152 which is compatible to nornuciferine (**2**). The peak at *m/z* 280 with 100% intensity is characteristic of aporphines, which easily lose the hydrogen next to the NH group at C-6a (Ohashi et al., 1963). Nornuciferine (**2**) was isolated from *A. hayesii* stem as the second major compound (Rasamizafy et al., 1987). This alkaloid was also reported to occur in *Annona* and in other Annonaceae genus, such as *Enantia*, *Guatteria*, *Isolona*, *Pseudovaria* and *Xylopia* (Lebouef et al., 1982; Lúcio et al., 2015). The compound having *Rt* 21.32 min was characterized as anonaine (**3**), by comparison of its fragmentation pattern (*m/z* 265 ($M^{+\cdot}$), 264 (M^+-1), 236 (M^+-29), with the literature (Jackson and Martin, 1966; Bhakuni et al., 1972).

Compound showing *Rt* 27.48 min, *m/z* 311 ($M^{+\cdot}$), 310 (M^+-1), 282 (M^+-29), 279 (M^+-31), 266 (M^+-15), 251, 181 was identified as actinodaphnine (**4**), which fragmentation pattern is in accordance to previous report (McLafferty and Stauffer, 1989). Isoboldine (**1**) was characterized at *Rt* 27.9 min and by its fragmentation pattern, *m/z* 327 ($M^{+\cdot}$), 326 (M^+-1), 310 (M^+-17), 284 (M^+-43), 269 (M^+-58), 253 (M^+-74) (Jackson and Martin, 1966). Isoboldine (**1**) previously isolated from five *Annona* species: *A. cherimola, A. glabra, A. montana, A. salzmanni* and *A. senegalensis* (Lebouef et al., 1982; Simeón et al., 1990; Philipov et al., 1994). In the present work, both isoboldine and actinodaphnine were also isolated from TA, as described below.

The alkaloid fractions (FA5 and FA6), originated from column chromatography (CC), were purified by PTLC and yielded isoboldine (**1**) as a brown amorphous solid. Isoboldine structure was proposed based on its molecular weight (M 327 Da), that was determined by GC–MS, and it is compatible with the molecular formula $C_{19}H_{21}O_4N$. Moreover, ^1H and ^{13}C NMR chemical shift values were in accordance to those reported in the literature (Jackson and Martin, 1966), the singlets at 6.47 and 6.74 ppm were due to the hydrogens in carbons 3 and 8; respectively; a broad singlet at 3.83–3.84 ppm (6H) relative to the methoxy groups found carbons 2 and 10 and a singlet at 2.49 ppm, relative to the hydrogens in the NCH_3 group. Jackman et al., 1979; Soares et al., 2015 have assigned the carbons signals in the NMR spectra for this compound, and it is possible to verify again the level of accordance between the data here obtained and the literature results. Isoboldine (**1**) was previously isolated from *A. cherimola* (Simeón et al., 1990), *A. glabra*, *A. montana* and from several other Annonaceae species. However, actinodaphnine (**4**) has been exclusively isolated from *Guatteria scandens* (Hocquemiller et al., 1983), and it is being here reported as novelty for the *Annona* genus. This alkaloid was reported to occur ubiquitously in Hernandiaceae and Lauraceae species (Guinaudeau et al., 1983; Hocquemiller et al., 1983; Sulaiman et al., 2011).

Nornuciferine (**2**) and anonaine (**3**) (Hasrat et al., 1997) had their structures proposed based on chemical shift values recorded in a 200 MHz spectrometer, once they were found as a mixture in

Table 1
Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) from the crude extract obtained from the stem of *Annona hypoglauca* Mart. and its alkaloid fractions against three bacteria strains.

Extracts and fractions	*Staphylococcus aureus* ATCC 29213		*Enterococcus faecalis* ATCC 29212		*Escherichia coli* ATCC 25922
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)
Crude extract	>100	>100	>100	>100	>100
FHex	>100	>100	>100	>100	>100
DCM/MeOH	>100	>100	>100	>100	>100
FEAC	>100	>100	>100	>100	>100
FBuOH	>100	>100	>100	>100	>100
Total alkaloids	60	70	50	50	>100
FA4.4	NT	NT	NT	NT	NT
FA5[a]	70	70	40	40	>100
FA6[a]	70	80	40	40	90
Gentamycin	0.20	0.20	8	8	0.40
Tetracyclin	0.50	0.50	32	32	2

MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration, NT, not tested. FHex, fraction hexane, DCM/MeOH, fraction dichloromethane and methanol, FEAC, fraction ethyl acetate, FBuOH, fraction butanol.

[a] FA5 and FA6 are column fractions obtained from the total alkaloids containing as major components isoboldine and actinodaphnine, respectively.

fraction FA5.3 not all hydrogens could be assigned. So, nornuciferine showed a doublet at 8.32 ppm (J = 7.8 Hz) corresponding to the H at carbon 11, a singlet at 6.58 ppm for the H at carbon 3, and singlets at 3.59 and 3.32 ppm relative to the two methoxy groups 1- and 2-OCH_3. The other alkaloid, anonaine, showed a doublet at 8.00 ppm (7.8 Hz) relative to the H at carbon 11, a singlet at 6.68 relative to the H at carbon 3 and two doublets at 5.89 and 6.28 belonging to the methylenedioxy at carbons 1 and 2(1-O-CH_2-O-2).

Actinodaphnine (**4**) was isolated from FA6.2 as a light brown amorphous solid. The molecular weight (M 311 Da) was determined by GC–MS and it was compatible with the molecular formula $C_{18}H_{17}O_4N$. The structure of actinodaphine (**4**) was attributed based on the comparison of NMR spectral data with values reported in the literature (Stévigny et al., 2002). It is possible to observe the presence of two apparent singlets at 5.86 and 6.01 ppm that were attributed to the hydrogens in the methylenedioxy in position 1, 2. The presence of a singlet referent to 3H in 3.85 ppm may correspond to a methoxy group as a substituent at carbon 10. The absence of a singlet with integration to 3H next to 2.82 ppm eliminates the possibility of a NCH_3 group, confirming that compound **4** is a noraporphine. With these observations, it was possible to attribute that compound **4** is actinodaphnine. The attributions of the J values were compared to results found in the literature (Stévigny et al., 2002). The purity grade of the sample permitted its analysis with different carbon NMR experiments to confirm the structure. Actinodaphnine was first isolated from Annonaceae species such as *G. scandens* as a brown crystal (Guinaudeau et al., 1983; Hocquemiller et al., 1983).

EB1109, hexane fraction, DCM/MeOH fraction, ethyl acetate fraction butanol fraction (Table 1) did not show a significant antibacterial activity, considering that crude extracts and fractions should have antibacterial activity < 100 μg/ml (Padma et al., 1998) to be considered as a potential antimicrobial natural product agent. On the other hand, TA, FA5 (major compound isoboldine (**1**), other compounds nornuciferine (**2**) and anonaine (**3**)) and FA6 (actinodaphnine (**4**)) showed a moderate activity against Gram-positive bacteria (*S. aureus* and *E. faecalis*), MIC as low as 70 and 40 μg/ml, respectively, and gives us a prospection of good antibacterial activity to actinodaphnine. Almost all samples were inactive against the Gram-negative organism (*E. coli*), only FA6 (MIC > 90 μg/ml) showed some activity. Previous reports showed that isoboldine (**1**) was not considered as having antibacterial activity (Villar et al., 1987; Bermejo et al., 2005) whereas anti-*S. aureus* activity has been confirmed to actinodaphnine (**4**) (Hoet et al., 2004), supporting the present findings. According to the present achievements, the fractionation of EB1109 up to FA5 and FA6 led to an improvement of the antibacterial activity and confirmed actinodaphnine as a potential antibacterial agent. The antimicrobial activity against Gram-positive bacteria of different isoquinoline compounds have already been studied, regarding their structure–activity relationships. In these studies, aporphines demonstrated lower or no activity. Only nor-aporphines bearing a 1,2-methylendioxy group could be considered active. *A. hypoglauca* accumulated at least two aporphine alkaloids, anonaine (**3**) and actinodaphnine (**4**), that contain the structural requirements likely to be the responsible for the results observed.

Results shown in Fig. 1 and Table 2 describe the percentage of growth and lethality observed for all treatments against cancer cell lines. EB1109 showed cytotoxicity against breast (MCF-7) and colon (KM-12) cancer cell lines. The percentage of lethality found for breast cell lines was −32.80%. This negative value shall be interpreted based on the time zero (T0) optical density related to cell growth before treatment addition, so EB1109 not only killed 100% of these cells (when compared to a cell growth control without treatment), but also killed a number of the cells that grew before treatment. EB1109 has also shown lethality against KM-12

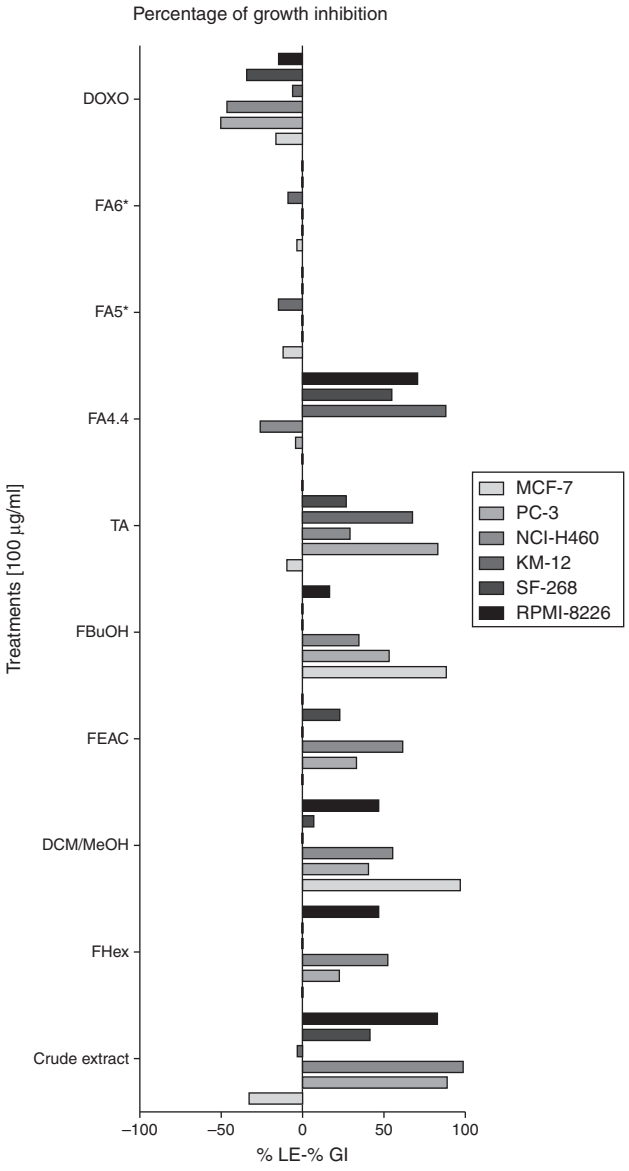

Fig. 1. Cytotoxic activity observed after treatments with crude extract, total alkaloids (TA), alkaloid-free fractions described as hexane fraction (FHex), butanol fraction (FBuOH), dichloromethane/methanol fraction and ethyl acetate fraction (FEAC), from stem of *Annona hypoglauca* against six human tumor cell lines. MCF-7: breast carcinoma; PC-3: prostate carcinoma; NCI-H460: non-small cells lung carcinoma; KM-12: colon adenocarcinoma; SF-268: glioblastoma and RPMI-8226: multiple myeloma. * FA4.4, FA5 and FA6: column fractions obtained from TA containing as major components isoboldine and actinodaphnine, respectively. NI: no inhibition; NT: not tested. Graphic is interpreted as the percentage of growth observed for the six cells after being treated. Calculations use the formula $[\{(T-T0)/(C-T0)\times 100\}-100 = \%$ of growth inhibition]. Results are interpreted by the comparison of cell growth after treatment (T) to the cell growth at Time Zero ($T0$). If $100 > T > T0$ it means that treatment prevented a percentage of cells from grow; if $T = T0 = 0$ it means that treatment is effective to avoid cells from grow, in relation to $T0$; if $T < T0$ it means that treatment is effective enough to cause lethality.

(−2.5%) and cytotoxicity against central nervous system cell line SF-268 (41.5% growth inhibition), prostate cancer cell line PC-3 (88.6% of growth inhibition), leukemia cell line RPMI-8226 (82.7% of growth inhibition) and lung cancer cell line NCI-H461 (98.7% of growth inhibition). Total alkaloid fraction showed expressive cytotoxicity against MCF-7 (−8.90% of lethality) and has also shown cytotoxicity against SF-268 (26.7% growth inhibition), against NCI-H461 (29.0% of growth inhibition) and against KM-12 (67.6% of growth inhibition). It was noticed that both EB1109 and TA

Table 2
Percentage of growth of cancer cell lines MCF-7 (breast), PC-3 (prostate), NCI-H460 (lung), KM-12 (colon), SF-268 (central nervous system) and RPMI-8226 (leukemia), after treatment with organic extract of stem of *Annona hypoglauca* and its fractions. Negative numbers mean lethality to cell lines in relation to cell growth control and Time zero growth control (cell growth before treatment addition).

	MCF-7	PC-3	NCI-H460	KM-12	SF-268	RPMI-8226
Crude extract	−32.80	11.4	1.3	−2.5	58.5	17.3
FHex	NI	77.4	48.1	NI	NI	53.5
DCM/MeOH	2.90	59.6	44.9	NI	93.7	53.4
FEAC	Not tested	67.1	38.6	NI	77.4	NI
FBuOH	11.90	46.6	65.6	NI	NI	83.8
TA	−8.90	17.04	71.7	32.4	73.3	NI
FA4.4	Not tested	−3.8	−26.0	11.7	45.4	29.7
FA5[a]	−11.60	Not tested	Not tested	−14.4	Not tested	Not tested
FA6[a]	−3.10	Not tested	Not tested	−8.4	Not tested	Not tested
DOXO	−16.31	−50.0	−46.2	−5.1	−34.0	−14.3

FHex, fraction hexane; DCM/MeOH, fraction dichloromethane and methanol; FEAC, fraction ethyl acetate; FBuOH, fraction butanol; TA, total alkaloid fraction; DOXO, doxorubicin.

[a] FA5 and FA6 are column fractions obtained from the total alkaloids containing as major components isoboldine and actinodaphnine, respectively.

inhibited breast cancer cell lines, and that may indicate that the alkaloids are responsible to the cytotoxicity against breast cancer cell line. None of the fractions obtained from the cake showed lethality, although have shown cytotoxicity, as DCM/MeOH fraction, which showed growth inhibition of 97.1% against breast cancer cell line and BuOH fraction, which inhibited in 88.1% the growth of the same cell. FA5 and FA6 were tested only against MCF-7 and KM-12. It was observed a lethality of 11.6% against MCF-7 breast cancer cell line and 14.4% against colon cancer cell line KM-12 for fraction FA5. Isoboldine is the major compound of fraction FA5, which contains yet nornuciferine and anonaine. Nornuciferine showed leishmanicidal activity against *Leishmania mexicana* and *L. panamensis* (Montenegro et al., 2003) Anonaine, as isolated from diverse Annonaceae and Magnoliaceae species is found to be active as antiplasmoidal, antimicrobial, antioxidant, anticancer, antidepressant and vasorelaxant (Li et al., 2013). *In silico* analysis of anonaine led to the identification of its potential to inhibit topoisomerase II, one of the crucial targets against cancers (Singh et al., 2016). FA6 has also shown a lethal effect, although weaker, against the cell lines of breast cancer MCF-7 (−3.1%) and colon cancer KM-12 (−8.4%). Actinodaphnine (**4**) was found in this fraction. Aporphinoid alkaloids bearing a methylenedioxy group, such as actinodaphnine (**4**), exhibited a high affinity to the DNA molecule demonstrating a cytotoxic activity *in vitro* and a non-specific inhibition of the Topoisomerase I activity through DNA intercalation (Hoet et al., 2004). In our findings, fraction FA5, which contains isoboldine (**1**), nornuciferine (**2**) and anonaine (**3**), was more efficient than FA6, the fraction containing actinodaphnine. Further studies related to structure–activity relationship are needed to elucidate differences in biological activity related to *Annona* alkaloids. Moreover, fraction FA4.4, obtained from fraction FA4 by CCD, was tested and results showed its significant lethality against lung cancer cell line NCI-H460 (−26.0%) and prostate cancer PC-3 (−3.8%), and showing a growth inhibition against the cell lines of CNS cancer SF-268 (54.6%), leukemia RPMI-8226 (70.3%) and colon cancer KM-12 (88.3%). It is clear that the total alkaloid fraction and their fractions FA5 and FA6 have shown an excellent tumor cell lethality in comparison to any of the fractions obtained from the cake, and clearly, the alkaloids are responsible for the significant cytotoxic activity, but maybe the presence of other compounds may induce some synergism observed in the extreme good activity of the crude extract. Also, fraction FA4.4 has shown a different cytotoxicity profile, because it was the only fraction among all tested that revealed an extreme good activity against lung cancer cell line, although no alkaloid has been identified from that fraction so far. The stronger effect observed for the crude extract might be explained by a synergism among the alkaloids and the other metabolites present in the extract.

Conclusions

The present results demonstrated that *A. hypoglauca* showed an improvement of its antibacterial activity, as fractionation was performed, maybe due to the presence of actinodaphnine. Actinodaphnine, the alkaloid present in FA6, may also be the responsible for the fraction cytotoxicity, but the presence of isoboldine, nornuciferine and anonaine may have synergistically contributed to the expressive lethality to breast cancer cells, if compared to FA6. This is the first report for the occurrence of actinodaphnine in *Annona* species. Also, EB1109, TA, FA5 and FA6 showed significant cytotoxic activity against breast and colon cancer cell lines. Finally, the identification of such enthusiastic results led us to pursue new evidences of biological activities related to *A. hypoglauca*, as well as to achieve results on its toxic activities in the future.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors contributions

MVNR executed all the experiments; IECD made NMR analyses; IBS executed/designed cytotoxicity assays and manuscript writing and translation; PRHM writing/translation of the manuscript, designed the chemical experiments, conducted as a supervisor.

Conflicts of interest

The authors declare no conflicts of interest.

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