



***In vitro* cytotoxic, antifungal, trypanocidal and leishmanicidal activities of acetogenins isolated from *Annona cornifolia* A. St. -Hil. (Annonaceae)**

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Manuscript received on February 8, 2013; accepted for publication on November 11, 2013

ABSTRACT

Annona cornifolia A. St. -Hil. is a small annual perennial tree found in the Brazilian savannah; their green fruit is popularly used in the treatment of ulcers. The acetogenins isolated from the seeds of *Annona cornifolia* previously showed to possess antioxidant activity. In continuation of our investigations on the biological activities of acetogenins, four binary mixtures and ten pure adjacent *bis*-tetrahydrofuran annonaceous acetogenins were evaluated: the cytotoxic (against three human tumor cell lines), antifungal (against *Paracoccidioides brasiliensis*), trypanocidal (against *Trypanosoma cruzi*) and leishmanicidal (against *Leishmania amazonensis*) activities. Acetogenins presented cytotoxic activity confirming their potential use in anti-cancer therapy. Regarding leishmanicidal and trypanocidal activities, an inhibition of 87% of *L. amazonensis* amastigotes and 100% of *T. cruzi* amastigotes and trypomastigotes was observed, when tested at the concentration of 20 $\mu\text{g mL}^{-1}$. Moreover, six acetogenins showed more activity against all the three tested isolates of *P. brasiliensis* than trimethoprim-sulfamethoxazole, a drug used for treating paracoccidioidomycosis. Thus, acetogenins may be an alternative in treating a number of diseases that have a huge impact on millions of people worldwide. This paper reports for the first time the antifungal, leishmanicidal and trypanocidal activities for these acetogenins.

Key words: *Annona cornifolia*, acetogenins, cytotoxic, antifungal, trypanocidal, leishmanicidal.

INTRODUCTION

Several *Annona* species of the Annonaceae family produce edible fruits that are widely consumed in Brazil, such as *Annona squamosa* and *Annona muricata*. *Annona cornifolia* A. St. -Hil., with orange fruits, is a small annual perennial tree

found in the Brazilian savannah; their green fruit is popularly used in the treatment of ulcers (Correa 1984). Annonaceous acetogenins, isolated exclusively from the Annonaceae family until 2008 (Pettit et al. 2008), exhibit a broad range of biological properties (Yang et al. 2009). Our group has already investigated acetogenins from the seeds of *A. crassiflora* (Santos et al. 1994, 1996) and *A.*

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cornifolia (Santos et al. 2006, 2007, Lima et al. 2009, 2010), as well as from the leaves of *Rollinia laurifolia* (Pimenta et al. 2001, 2003, 2005). There are no reports on the study of other parts of *Annona cornifolia*, including the phytochemical studies of the fruit. The fruits of several *Annona* species are an important source of proteins, carbohydrates and amino acids (Correa 1984). Further research may shed light on the use of fruit of *A. cornifolia* in future dietary supplementation.

Leishmaniasis and trypanosomiasis, occurring in tropical and sub-tropical areas around the world, have huge medical, social and economic impact to millions of people (Castillo-Garit et al. 2012). Paracoccidioidomycosis, a systemic disorder caused by the dimorphic fungus, *Paracoccidioides brasiliensis*, occurs in Latin American countries, resulting in more deaths than leishmaniasis in Brazil (Shikanai-Yasuda et al. 2006). In the absence of drug therapy, paracoccidioidomycosis is usually fatal (Shikanai-Yasuda et al. 2006, Johann et al. 2010). In the treatment of cancer, chemotherapeutic agents exhibit severe toxicity and can cause many undesirable side effects. Therefore, it is necessary to find new drugs, with natural products being good candidates. In fact, from 1983 to 1994, more than 60% of approved anticancer drugs were derived from natural products (Newman et al. 2003).

In the present study, we evaluated the cytotoxic, antifungal, trypanocidal and leishmanicidal activities of fifteen adjacent *bis*-tetrahydrofuran (THF) acetogenins: ten pure (**3-8** and **11-14**) and four binary mixtures (**1+2**, **8+9**, **8+10** and **8+15**), isolated from the ethanol extracts of seeds from *A. cornifolia* A. St. -Hil. (Fig. 1).

MATERIALS AND METHODS

GENERAL

Etoposide, amphotericin B, sulforhodamine B, tris[hydroxymethyl]aminomethane buffer, trichloroacetic acid, RPMI medium, methyl thiazolyl tetrazolium and Schneider's medium were purchased

from Sigma-Aldrich (St. Louis, USA). Benzimidazole was obtained from Roche (São Paulo, Brazil), and trimethoprim/sulfamethoxazole from Ducto (Goiás, Brazil). Trypsin-EDTA, RPMI 1640 medium without phenol red and gentamicin were purchased from Gibco (NY, USA). Silica gel 230-400 mesh from Merck (Darmstadt, Germany) was used for column chromatography, and silica gel Merck 60G was used for thin-layer chromatography. All solvents used were of PA and HPLC grade and purchased from Vetec (Brazil) and Sigma, respectively.

The 1D and 2D NMR spectra of acetogenins were performed on Bruker Avance DRX 400 spectrometers (Ettlingen, Germany) in CDCl₃, containing 0.1% tetramethylsilane as the internal chemical shift standard. Electrospray Ionization Mass Spectrometry (ESI-MS) was performed using a Waters MICROMAS Q-TOF (Milford, Massachusetts, USA) equipped with an electrospray ion (ESI) source. A 200 µg mL⁻¹ solution of acetogenins in MeCN-H₂O (1:1) was infused at 2 mL min⁻¹, and the positive mass spectra was acquired with a m/z range between 50 and 1,000 daltons. The cone voltages were optimized for positive ion analysis in the range between 35 and 50 V. In the MS/MS experiments, the parent ion isolation width was 3.8 daltons, and the normalized collision energy was set at 30% for the compounds. Fifty scans were collected from 50 to 700 daltons to generate an average spectrum. Optical rotation was measured on a Perkin Elmer 341 polarimeter (Waltham, Massachusetts, USA). Final purification was performed on a Waters 501 apparatus (Milford, Massachusetts, USA) with a 486 UV-detector and a 746 integrator.

PLANT MATERIAL

The fruits of *Annona cornifolia* A. St. -Hil. were collected from the Curvelo, Minas Gerais, Brazil, from January to March 1998. The species were identified by Dr. R. Mello-Silva and a voucher specimen (BHCB 68114) was deposited at the

Instituto de Ciências Biológicas Herbarium, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

EXTRACTION AND ISOLATION

Extraction of the dried and powdered seeds (850.0 g), by percolation (EtOH, 7 L, 50 h) gave a brown residue (120.0 g, F01), that was dissolved in MeOH/H₂O (3:7) and successively extracted

with C₆H₁₄ and CHCl₃, resulting in 79.5, 7.3 and 21.1 g of hexane (F02), chloroform (F03) and hydroalcoholic (F04) fractions, respectively. Extract and fractions were evaporated to dryness before column chromatography and HPLC.

Acetogenins **3-7** and mixture **1+2** were isolated from F02, and acetogenins **8-15** from F03. The isolation of acetogenins **1-7** and **11-14** are described in previous publications by our group (Santos et

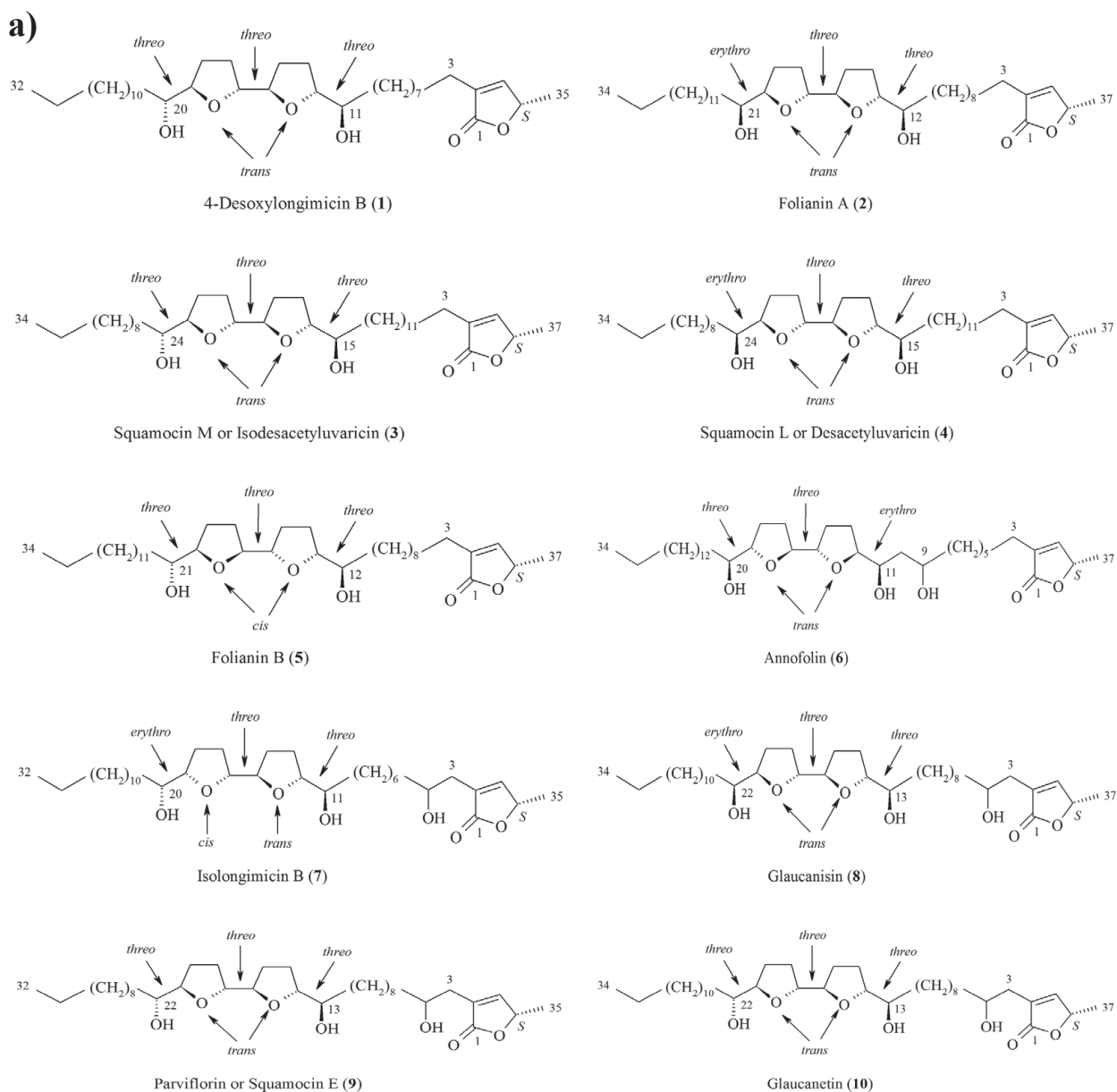


Figure 1 - Acetogenins isolated from *Annona cornifolia*. **A)** Acetogenins 1-10, **B)** Acetogenins 11-15.

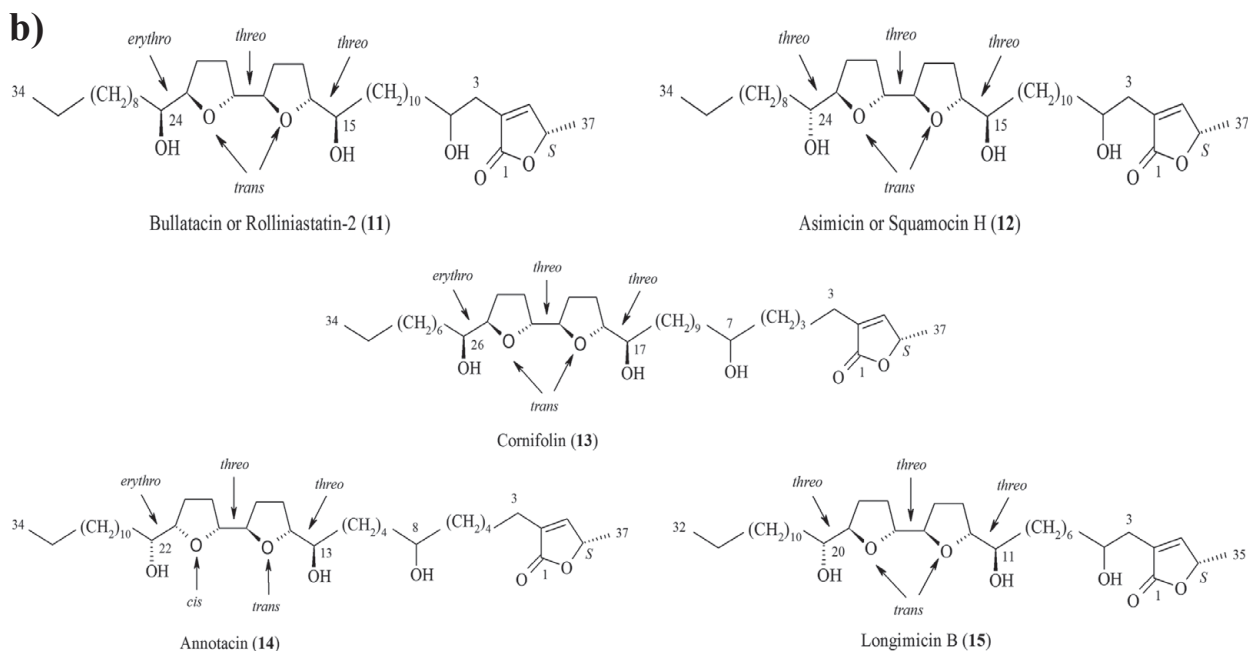


Figure 1 (continuation) - Acetogenins isolated from *Annona cornifolia*. **A)** Acetogenins 1-10, **B)** Acetogenins 11-15.

al. 2006, Lima et al. 2009, 2010). F02 was chromatographed leading to 15.1 mg of a mixture of acetogenins 4-desoxylongimicin B + folianin A (**1+2**), 38.7 mg of squamocin M or isodesacetyluvaricin (**3**), 38.0 mg of squamocin L or desacetyluvaricin (**4**), 14.7 mg of folianin B (**5**), 40.0 mg of annofolin (**6**), and 6.0 mg of isolongimicin B (**7**). The acetogenins bullatacin or rolliniastatin-2 (**11**) (10.5 mg), asimicin or squamocin H (**12**) (3.5 mg), cornifolin (**13**) (30.4 mg), and annotacin (**14**) (12.0 mg) were obtained from F03. The isolation of acetogenins **8-10** and **15** are described below. Chloroform fraction (F03, 2.0 g) was fractionated by normal phase MPLC (with $C_6H_{14}/\Phi CH_3/EtOAc/MeOH$ pure or in mixtures of increasing polarity), yielding 54 fractions of 75 mL each, combined according to their TLC patterns, yielding 10 groups of fractions. G-7 (620.0 mg, positive to Kedde reagent) underwent column chromatography (1.5 x 25 cm, 50.0 g SiO_2 230-400 mesh, $CH_2Cl_2/MeOH$ 97:3), yielding 52 fractions of 15 mL each, combined according to their TLC patterns in 19 subgroups of fractions. Subgroup 2 (45.4 mg) was submitted to

a RP-HPLC (Shim-pack C18 5.0 μm , 20 x 250 mm cartridge column, flow rate 15 mL min^{-1} , MeCN/ H_2O 70:30, detection 220 nm), leading to glaucanisin (**8**) (6.0 mg), to glaucanisin + parviflorin (**8+9**) (14.0 mg) and to glaucanisin + glaucanetin (**8+10**) (14.9 mg). Subgroup 7 (90.1 mg), from G-7, led to 15 subfractions, after being submitted to purification by RP-HPLC (Supelco SPLC-18 5.0 μm , 10 x 250 mm cartridge column, flow rate 2.5 mL min^{-1} , MeCN/ H_2O 60:40, detection 220 nm). Subgroup 11 (84.7 mg), from G-7, by HPLC (same conditions as above) gave mixture of glaucanisin + longimicin B (**8+15**) (14.0 mg).

CYTOTOXIC ASSAY

The cytotoxic potential of acetogenins against the human melanoma (UACC-62), renal carcinoma (TK-10) and breast cancer (MCF-7) cell lines was evaluated in November 2001, using the sulforhodamine B (SRB) assay adopted by the National Cancer Institute, USA (Monks et al. 1991). All cells were cultured in RPMI medium supplemented with 5% FBS and gentamicin, at 37°C

with 5% CO₂. Shortly before reaching confluence, the cells were detached with trypsin-EDTA and seeded into 96-well plates so that 100 µL in each well contained 10,000 UACC-62 and MCF-7 cells or 15,000 TK-10 cells. Acetogenins dissolved in DMSO 1% were tested firstly in triplicate at 20 µg mL⁻¹, and after at different concentrations, acetogenins that inhibits 75% of growth human tumor cell lines. After 48 hours in the presence of the compound, the cells were fixed by adding 50 µL of cold 50% (w/v) trichloroacetic acid to each well and incubating the plate at 4°C for 1 h. The supernatant was then discarded and the cells were washed five times with water. After drying at room temperature, 50 µL of SRB solution (0.4% w/v in 1% acetic acid) was added to each well, and the plate incubated for 30 min at 4°C. Unbound SRB was removed by washing five times with 1% acetic acid and the plates were dried at room temperature overnight. The plates were read at 515 nm after dissolution of the dye with Tris buffer (tris[hydroxymethyl] aminomethane). Etoposide was used as the positive control and 0.2% DMSO as the negative control. Each experiment was performed in triplicate. The concentration of the compound that produces 50% of growth inhibition (IC₅₀, cytostatic effect) and the concentration of the compound that kills 50% of cells (LD₅₀, cytotoxic effect) were obtained from data of three independent experiments by non-linear regression using SOFTmax pro 5.3.

ANTIFUNGAL ACTIVITY

Antifungal activity was evaluated in February 2004 and repeated in March 2011, with similar results. *In vitro* antifungal activity against *Paracoccidioides brasiliensis*, strains Pb-01 (ATCC- MYA-826), Pb-18 and Pb-18 virulent isolates (from the fungal collection of the Faculty of Medicine of the Universidade de São Paulo, SP, Brazil) were prepared in accordance with the guidelines in the CLSI document M27-A3 (CLSI 2008) and modified according to the suggestions of Johann

et al. (2010), to obtain a final suitable inoculum dilution for each strain. After homogenization by vortexing, the transmittance of the suspension was measured at wavelengths of 530 nm and adjusted to 69 to 70%.

The Minimal Inhibitory Concentration (MIC) was obtained from broth microdilution testing performed in accordance to described methods (CLSI 2008, Johann et al. 2010). Amphotericin B and trimethoprim-sulfamethoxazole were included as positive antifungal control. Their stock solutions were prepared in DMSO and water, respectively.

The Minimal Fungicidal Concentration (MFC) values for acetogenins were determined as follows: from the microtiter plate used to determine the MIC values, the test wells that showed: a) complete fungal growth inhibition (clear wells), b) growth similar to that of the no-drug control well, and c) growth control wells, were selected for the assay to determine the MFC. The MFC was determined as the lowest drug concentration at which fewer than three colonies were able to grow (Espinel-Ingroff et al. 2001).

TRYPANOCIDAL ACTIVITY

In vitro assay with amastigote and trypomastigote forms of *Trypanosoma cruzi* was performed in October 2003 and repeated in June 2013, with similar results, according to protocols established by Buckner et al. (1996) with modifications (Romanha et al. 2010). Briefly, parasites and culture procedures: *T. cruzi* (Tulahuen strain) expressing the *Escherichia coli* β-galactosidase gene were grown on monolayer of mouse L929 fibroblasts. Cultures to be assayed for β-galactosidase activity were grown in RPMI 1640 medium (pH 7.2–7.4) without phenol red plus 10% foetal bovine serum and glutamine. For the bioassay, 96 well tissue culture microplates were seeded with L929 fibroblasts in 80 µL with a density of 4.0 x 10³ fibroblasts per well and incubated overnight at 37°C and 5% CO₂. β-galactosidase-expressing

trypomastigotes in 20 μL of medium were then added at a density of 4.0×10^4 per well. After 2 h of contact, the medium with trypomastigotes that had not penetrated the cells was discarded and replaced by 200 μL of fresh medium. After 48 h, the medium was discarded again and replaced by 180 μL of fresh medium and 20 μL of a solution of acetogenins at concentration of $20 \mu\text{g mL}^{-1}$. Each compound was tested in triplicate. After seven days of culture development, chlorophenol red β -D-galactopyranoside at 100 μM and Nonidet P-40 at 0.1% were added to the plates and the plates were then incubated overnight at 37°C . The absorbance was measured at 570 nm in an automated microplate reader. Benznidazole at its half maximal inhibitory concentration (IC_{50}) ($1 \mu\text{g/mL} = 3.8 \mu\text{M}$) was used as a positive control. The results were expressed as the percentage of parasite growth inhibition.

Cytotoxic test for determination of the Selectivity Index (SI) was performed in June 2013. *In vitro* cytotoxic test was also carried out to determine the toxicity of the compounds over L929 cells by alamarBlue[®]. The same cell number, time of the cells development and time of compound exposure were used for the β -galactosidase assay. After 96 hours of compounds exposure the alamarBlue[®] was added and the absorbance at 570 and 600 nm was measured after 4-6 h. The cell viability was expressed as the percentage of difference in the reduction between treated and untreated cells (Romanha et al. 2010). IC_{50} values were calculated by linear interpolation and the Selectivity Index (SI) was determined based on the ratio of the IC_{50} value in the host cell divided by the IC_{50} value of the parasite ($\text{IC}_{50}/\text{IC}_{50}$ ratio).

LEISHMANICIDAL ACTIVITY

Assays with amastigote-like forms of *Leishmania (Leishmania) amazonensis* were performed in September 2003, using the MTT (methyl thiazolyl tetrazolium)-based colorimetric assay (Callahan et al. 1997). Amphotericin B was used

as the positive control. Promastigotes forms of *L. amazonensis* (strain IFLA/BR/196/PH-8) were obtained from lesions of experimentally infected hamsters. The parasites were incubated for 9 days at 26°C in Schneider's medium, buffered at pH 7.2. The promastigotes forms were then stimulated to differentiate into amastigote-like forms by rising the incubation temperature to 32°C and lowering the pH of the medium to 6.0. After 7 days under these conditions, 90% of the parasites differentiated. The parasite concentration was adjusted to 1×10^8 cells mL^{-1} , and 90 μL were added to each well of 96-well plates, followed by 10 μL of the solutions containing the samples ($20 \mu\text{g mL}^{-1}$) and control drug ($0.2 \mu\text{g mL}^{-1}$ amphotericin B). Only compounds that caused at least 75% of inhibition were tested again in different concentration. The plates were incubated at 32°C for 72 h and the number of parasites was estimated using the MTT based colorimetric assay. The LD_{50} (lethal dose that kills 50% of cells) was obtained from data of three independent experiments by non-linear regression using the SOFTmax pro 5.3.

STATISTICAL ANALYSES

Measurements were conducted in triplicate. All the data are shown as mean standard deviation (SD). The values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The identification (by IR, ^1H and ^{13}C NMR and MS spectra) of acetogenins **1-2**, **5-7**, and **13-14**, isolated for the first time from *Annona cornifolia*, are described in previous publications by our group (Santos et al. 2006, Lima et al. 2009, 2010). Spectrometric data of the known acetogenins (new for this specie) **3-4** and **11-12** are described by Lima et al. (2010). The acetogenins squamocin L (**4**) and squamocin M (**3**) were isolated by Sahai et al. (1994). The squamocin L was identical with desacetyluvaricin, isolated from *Uvaria accuminata* as well as *Annona glabra* seeds. The squamocin M

was identical with isodesacetylvaricin, isolated from *Uvaria narum* (Sahai et al. 1994, Zeng et al. 1996, Cavé et al. 1997). Acetogenins **8-10** and **15** were compared to other molecules in the literature: glaucanisin (**8**) (Waechter et al. 1995), parviflorin or squamocin E (**9**) (Gallardo et al. 1998), glaucanetin (**10**) (Waechter et al. 1997) and longimicin B (**15**) (Ye et al. 1996).

The cytotoxic activities of acetogenins **1-15** at 20 µg mL⁻¹ are shown in Table I. IC₅₀ and LD₅₀ were established only for those that inhibited 75% of the cells at the concentration of 20 µg mL⁻¹ (Table II). These acetogenins exhibited significant cytotoxic activity and selectivity for the tumor cell lines, being more active against MCF-7. Annofolin (**6**) presented lower LD₅₀ values than the etoposide for all the lines tested, while annofolin (**6**), isolongimicin B (**7**) and the mixtures **8+9**, **8+10** and **8+15** showed lower IC₅₀ values than the etoposide for the TK-10 cell line. The mixtures showed higher cytostatic activity than pure glaucanisin (**8**) against TK-10 and UACC-62 cells, suggesting a potentiation of the effect by the two acetogenins.

Yang et al. (2009) established that (i) adjacent *bis*-THF acetogenins are the most potent antitumor agents among this class of compounds, (ii) if all other structural features are identical, C-35 acetogenins are more active than those with 37 carbon atoms; (iii) for better activity, the distance between the OH-flanked THF and the γ-lactone must be 13 carbon atoms, and (iv) acetogenins with stereochemical arrangement of *threo/trans/threo/trans/threo* around THF rings are less potent than those with stereochemical arrangement of *threo/trans/threo/trans/erythro*. However, annofolin (**6**), the most active agent in this work, is interestingly a C-37 acetogenin with 9 carbon atoms between the OH-flanked THF and the γ-lactone, presenting a stereochemical arrangement of *erythro/trans/threo/trans/threo* around THF rings.

The ten pure and the four mixtures of acetogenins were tested against three isolates of *Paracoccidioides brasiliensis* (Pb01, Pb18 and Pb18 virulent) at 1.17-150 µg mL⁻¹, their Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) being determined.

TABLE I
In vitro effect of acetogenins from *Annona cornifolia*, tested at 20 µg mL⁻¹, against human cancer cell lines, *Trypanosoma cruzi* and *Leishmania amazonensis*.

Acetogenins	MCF-7 (%)	TK 10 (%)	UACC 62 (%)	<i>T. cruzi</i> (%)	<i>L. amazonensis</i> (%)
1 + 2	93.0 ± 11.3*	77.5 ± 24.7*	75.5 ± 12.0*	76.2 ± 5.1*	58.5 ± 3.0*
3	116.0 ± 2.0*	85.5 ± 12.0	40.5 ± 0.7*	64.1 ± 6.5*	41.7 ± 1.1*
4	66.3 ± 5.8*	38.5 ± 4.7*	36.5 ± 0.7*	40.7 ± 6.2*	35.3 ± 2.5*
5	69.3 ± 8.5*	44.5 ± 3.5*	39.0 ± 2.8*	23.4 ± 1.0*	56.7 ± 6.2*
6	103.0 ± 13.8	91.0 ± 9.9*	81.0 ± 8.5*	97.6 ± 3.4*	87.0 ± 0.0*
7	109.6 ± 11.0*	81.5 ± 6.3	77.5 ± 3.5*	36.8 ± 2.2*	57.5 ± 4.1*
8	109.0 ± 0.5*	75.6 ± 2.1*	75.0 ± 5.4*	17.1 ± 2.0*	34.8 ± 5.4*
11	50.0 ± 1.0*	21.0 ± 1.4*	28.0 ± 1.4*	24.5 ± 1.0*	55.3 ± 7.2*
12	53.3 ± 4.7*	19.5 ± 2.1*	32.0 ± 1.4*	20.7 ± 3.4*	55.5 ± 6.3*
13	30.0 ± 5.3*	17.0 ± 0.8*	19.0 ± 0.7*	2.0 ± 0*	55.6 ± 5.0*
14	103.3 ± 3.5	75.5 ± 7.1*	75.1 ± 1.4*	51.9 ± 5.5*	76.9 ± 5.1*
8 + 9	105.0 ± 12.1	91.2 ± 13.3*	76.5 ± 1.4*	77.2 ± 10.2*	49.6 ± 2.3*
8 + 10	107.0 ± 5.2	79.3 ± 4.8*	80.5 ± 1.8*	100.0 ± 5.1*	46.2 ± 1.0*
8 + 15	108.0 ± 10.4	88.0 ± 2.9*	80.0 ± 1.4*	38.6 ± 5.4*	0.0
Etoposide^a	105.0 ± 16.4	83.5 ± 2.0	88.0 ± 2.8	-	-
Amphotericin B^a	-	-	-	-	79.0 ± 1.0
Benznidazole^a	-	-	-	73.0 ± 0.0	-

^aPositive control: Etoposide was tested at 8 µg mL⁻¹, amphotericin B at 0.2 µg mL⁻¹, and benznidazole at 1 µg mL⁻¹.

Each value in the table is the mean ± standard deviation (n = 3).

*p < 0.05 compared with positive control.

TABLE II
In vitro cytotoxicity, trypanocidal and leishmanicidal activities of acetogenins from *Annona cornifolia*.

Acetogenins	Cytotoxic activity (μM)						<i>T. cruzi</i>		<i>L. amazonensis</i>
	MCF-7		TK-10		UACC-62		IC ₅₀ ^a (μM)	SI ^c	LD ₅₀ ^b (μM)
	IC ₅₀ ^a	LD ₅₀ ^b	IC ₅₀ ^a	LD ₅₀ ^b	IC ₅₀ ^a	LD ₅₀ ^b			
1 + 2	5.9*	$> 1.7 \times 10^2$	$5.7 \times 10^{1*}$	$1.9 \times 10^{2*}$	$9.2 \times 10^{1*}$	$> 1.7 \times 10^2$	$1.2 \times 10^{-1*}$	1	-
3	$< 3.1 \times 10^{-1}$	$> 1.6 \times 10^2$	$4.1 \times 10^{1*}$	$> 1.6 \times 10^2$	nt	nt	$1.0 \times 10^{-1*}$	1	-
6	$< 3.0 \times 10^{-1}$	$7.8 \times 10^{1*}$	6.4*	$1.4 \times 10^{2*}$	$1.5 \times 10^{1*}$	$1.1 \times 10^{2*}$	$1.1 \times 10^{-1*}$	1	6.4×10^1
7	$< 3.2 \times 10^{-1}$	$2.1 \times 10^{2*}$	$1.7 \times 10^{1*}$	$2.1 \times 10^{2*}$	$2.9 \times 10^{1*}$	$> 1.7 \times 10^2$	-	-	-
8	$< 3.0 \times 10^{-1}$	$> 1.6 \times 10^2$	$9.5 \times 10^{1*}$	$> 1.6 \times 10^2$	$1.7 \times 10^{2*}$	-	-	-	-
14	$< 3.0 \times 10^{-1}$	$2.1 \times 10^{2*}$	$9.5 \times 10^{1*}$	$> 1.6 \times 10^2$	$1.5 \times 10^{2*}$	-	1.7	1	7.2×10^1
8 + 9	$< 3.2 \times 10^{-1}$	$> 1.7 \times 10^2$	5.3*	$> 1.7 \times 10^2$	$3.5 \times 10^{1*}$	$> 1.7 \times 10^2$	$1.3 \times 10^{-1*}$	1	-
8 + 10	$< 3.0 \times 10^{-1}$	$> 1.6 \times 10^2$	$2.1 \times 10^{1*}$	$> 1.6 \times 10^2$	$2.2 \times 10^{1*}$	$2.0 \times 10^{2*}$	1.7	1	-
8 + 15	$< 3.2 \times 10^{-1}$	$> 1.7 \times 10^2$	9.6*	1.7×10^2	$2.3 \times 10^{1*}$	$> 1.7 \times 10^2$	-	-	-
Etoposide^d	$< 3.2 \times 10^{-1}$	$> 1.7 \times 10^2$	3.4×10^1	$> 1.7 \times 10^2$	5.3	$> 1.7 \times 10^2$	-	-	-
Benznidazole^d	-	-	-	-	-	-	3.8	625	-

^aconcentration that inhibits 50% of growth (cytostatic effect); ^bconcentration that kills 50% of the cells or parasites (cytotoxic effect); ^cSelectivity Index (SI) is the ratio of IC₅₀ on normal cell line to the IC₅₀ on parasite; ^dpositive control.

nt: not tested

* $p < 0.05$ compared with positive control.

MIC and MFC values were found to be equal and are presented in Table III. Acetogenins **5-12** showed antifungal activity at concentrations $\leq 150 \mu\text{g mL}^{-1}$, thus being more active than trimethoprim-sulfamethoxazole, the first class of drugs used to treat paracoccidioidomycosis, but less active than amphotericin B. Cornifolin (**13**) was inactive at this concentration against all three isolates. Annofolin (**6**), isolongimicin B (**7**) and bullatacin (**11**) showed the highest levels of activity against the three isolates of *P. brasiliensis*. Pb18 exhibited some selectivity, being more sensitive to squamocin M (**3**) and folianin B (**5**).

There are few studies in the literature reporting the activity of natural products against the pathogenic fungus *P. brasiliensis*. The natural product (R)-goniothalamin and its synthetic enantiomer were evaluated against a panel of microorganisms that included three strains of *P. brasiliensis* (Pb-01, Pb-18 and Pb-B339). Their MIC values were in the range between 7 and 22 $\mu\text{g mL}^{-1}$ for *P. brasiliensis* (Fátima et al. 2008). In this study, annofolin (**6**) and isolongimicin B (**7**) showed

higher activity than the goniotalamin enantiomers against *P. brasiliensis*. In the experiment carried out by Lima et al. (2011), the authors tested the *in vitro* susceptibility of twelve clinical *P. brasiliensis* isolates to 9-hydroxy-folianin, an adjacent bis-THF acetogenin. The results demonstrated that all isolates were susceptible to 9-hydroxy-folianin, with a MIC of 3.4 to 27.7 $\mu\text{g mL}^{-1}$. In our study, annofolin (**6**) was more active than 9-hydroxy-folianin against the three isolates of *P. brasiliensis*.

The acetogenins, at 20 $\mu\text{g mL}^{-1}$, were also tested against the amastigote and trypomastigote forms of *T. cruzi*. The results are expressed in percentages of parasite growth inhibition (Table I). Squamocin M (**3**), annofolin (**6**), annotacin (**14**) and the mixtures (**1+2**), (**8+9**) and (**8+10**) inhibited more than 50% of amastigote and trypomastigote forms of *T. cruzi*; their IC₅₀ values are given in Table II.

All the acetogenins showed similar or greater trypanocidal effects as compared to benznidazole (IC₅₀ values less 1.7 μM), but showed toxicity at the same concentration, causing 50% of cell death (L929 cells). Thereby, no selective toxicity

TABLE III
In vitro antifungal activity of acetogenins from *Annona cornifolia* against *Paracoccidioides brasiliensis*.

Acetogenins	MIC and MFC ($\mu\text{g mL}^{-1}$) ^a		
	Pb01	Pb18	Pb18 virulent
1+2	nd	nd	nd
3	> 150.0	75.0*	> 150.0
4	150.0*	150.0*	> 150.0
5	150.0*	75.0*	150.0*
6	9.3*	9.3*	9.3*
7	18.7*	18.7*	18.7*
8	150.0*	75.0*	75.0*
11	37.5*	37.5*	37.5*
12	150.0*	150.0*	150.0*
13	> 150.0	> 150.0	> 150.0
14	150.0*	> 150.0	> 150.0
8 + 9	nd	nd	nd
8+10	nd	nd	nd
8+15	nd	nd	nd
Amphotericin B ^b	0.12	0.062	0.062
Trimethoprim-sulfamethoxazole ^b	300	300	300

^a MIC: Minimal Inhibition Concentration; MFC: Minimal Fungicidal Concentration; ^b positive control. nd: no detected activity at $150.0 \mu\text{g mL}^{-1}$.

*p < 0.05 compared with positive control.

to the parasites was observed (Selectivity Index, SI = 1) when a tissue culture assay was used. The acetogenins **3**, **6**, **14** and the mixtures (**1+2**), (**8+9**) and (**8+10**) showed growth inhibition of the parasites in the range of 51.9-100.0% (Table I). This level of activity is considered to be significant (Croft et al. 1988). Some studies confirmed the trypanocidal activity of the acetogenins against *T. cruzi* parasites (Waechter et al. 1998, González-Coloma et al. 2002).

The activity of all acetogenins against *L. amazonensis* amastigotes was evaluated (also at $20 \mu\text{g mL}^{-1}$). Those that presented inhibition higher than 75% had their LD₅₀ values determined (Table II). Annofolin (**6**) and annotacin (**14**), both C-37 bis-THF acetogenins, were the most active, presenting LD values of 6.4×10^1 and $7.2 \times 10^1 \mu\text{M}$, respectively, indicating the relevance of these compounds in the search for new leishmanicidal drugs.

Few works report the leishmanicidal activity of acetogenins with one or two THF rings against the promastigote and amastigote forms

of *Leishmania* (Waechter et al. 1998, Raynaud-Le Grandic et al. 2004, Vila-Nova et al. 2011). According to Raynaud-Le Grandic et al. (2004), the stereostructural arrangement of *threo/trans/threo/trans/erythro* around the THF ring favors leishmanicidal activity. Annofolin (**6**), with the stereostructural arrangement *erythro/trans/threo/trans/threo*, exerted the highest level of leishmanicidal activity among the acetogenins tested in this work.

CONCLUSIONS

Although the cytotoxic activity of acetogenins has already been described, the high level of this activity observed in these compounds confirms their potential for being used in anti-cancer therapy. Regarding leishmanicidal and trypanocidal activities, an inhibition of 87% of *L. amazonensis* amastigotes and 100% of *T. cruzi* amastigotes and trypomastigotes was observed, when tested at the concentration of $20 \mu\text{g mL}^{-1}$. Moreover, six acetogenins showed more activity against all the three tested isolates of

P. brasiliensis than trimethoprim-sulfamethoxazole, a drug used for treating paracoccidioidomycosis. Thus, acetogenins hold great promise in treating a number of diseases that have a huge impact on millions of people worldwide.

ACKNOWLEDGMENTS

We are grateful to Dr. Renato Mello-Silva for the botanical identification of this plant species.

RESUMO

Annona cornifolia A. St. -Hil. é uma pequena árvore anual perene encontrada no cerrado brasileiro; seu fruto verde é usado popularmente no tratamento de úlceras. As acetogeninas isoladas a partir das sementes de *Annona cornifolia* anteriormente apresentaram atividade antioxidante. Continuando as nossas investigações sobre as atividades biológicas das acetogeninas, quatro misturas binárias e dez acetogeninas bis-tetra-hidrofurânicas adjacentes puras foram avaliadas quanto às atividades: citotóxica (sobre três linhagens de células tumorais humanas), antifúngica (sobre *Paracoccidioides brasiliensis*), tripanocida (sobre *Trypanosoma cruzi*) e leishmanicida (sobre *Leishmania amazonensis*). As acetogeninas apresentaram atividade citotóxica, confirmando seu uso potencial na terapia anticâncer. Quanto às atividades leishmanicida e tripanocida, foi observada uma inibição de 87% das formas amastigotas de *L. amazonensis* e 100% de formas amastigotas e tripomastigotas de *T. cruzi*, quando testadas a uma concentração de 20 µg mL⁻¹. Além disso, seis acetogeninas mostraram maior atividade sobre todas as três cepas testadas de *P. brasiliensis* que o trimetoprim-sulfametoxazol, um medicamento usado no tratamento da paracoccidioidomicose. Assim, as acetogeninas podem ser uma alternativa no tratamento de uma série de doenças que têm um enorme impacto em milhões de pessoas no mundo. Este trabalho relata pela primeira as atividades antifúngica, leishmanicida e tripanocida para estas acetogeninas.

Palavras-chave: *Annona cornifolia*, acetogeninas, citotóxica, antifúngica, tripanocida, leishmanicida.

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