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Chemical Constituents and Pharmacology properties of *Aristolochia triangularis*: a south brazilian highly-consumed botanical with multiple bioactivities

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Abstract: *Aristolochia triangularis* Cham., is one of the most frequently used medicinal plant in Southern Brazil. Preparations containing the leaves and/or stems are traditionally used as anti-inflammatory, diuretic, as well as antidote against snakebites. This study screened *A. triangularis* extracts, fractions and isolated compounds for different bioactivities. A weak antiproliferative activity against human lung cancer cell line (A549) was observed only for chloroform fraction obtained from stems (CF_{stems} - CC_{50}: 2.93 µg/mL). Also, a moderate antimicrobial activity against *Staphylococcus aureus* was detected just for chloroform fraction obtained from leaves (CF_{leaves} -13-16 mm inhibition zone). Additionally, two semi-purified fractions (CF_{stems}-4 and CF_{leaves}-4) selectively inhibited HSV-1 replication (IC_{50} values of 0.40 and 2.61 µg/mL, respectively), while only CF_{leaves} showed promising results against *Leishmania amazonensis.* Fractionation of extracts resulted in the isolation of one neolignan (-) cubebin and one lignan (+) galbacin. However, these compounds are not responsible for the *in vitro* bioactivities herein detected. The presence of aristolochic acid I and aristolochic acid II in the crude ethanol extract of stems (CEEstems) and leaves (CEEleaves) was also investigated. The HPLC analysis of these extracts did not display any peak with retention time or UV spectra comparable to aristolochic acids I and II.

Key words: *Aristolochia triangularis*, antiproliferative, antiherpes, antimicrobial, antiprotozoal, aristolochic acids.

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INTRODUCTION

The *Aristolochia* genus (Aristolochiaceae) comprises about 500 species of herbaceous perennials, undershrub or shrubs widespread across Europe, tropical Asia, Africa and South America (Neinhuis et al. 2005). Several *Aristolochia* species have been used worldwide as anti-inflammatory (Muschietti et al. 1996) and to treat rheumatic pains and fevers (Battu et al. 2011) and complications of snakebites (Bhattacharjee and Bhattacharyya 2013).

Several pharmacological activities are attributed to *Aristolochia* species including antibacterial (de Barros Machado et al. 2005, Kumar et al. 2006, Yu et al. 2007, Alviano et al. 2008), antiproliferative (Chaouki et al. 2010), antispasmodic (Zhang et al. 2008), anti-scorpion venon (Izquierdo et al. 2010), anti-snake venon (Samy et al. 2008), insecticidal (Nascimento et al. 2004, Messiano et al. 2008), anti-inflammatory (Battu et al. 2011), and antimycobacterial (Léon-Diaz et al. 2010).

Concerning the chemical composition of *Aristolochia* species, flavonoids (Machado and Lopes 2005, Battu et al. 2011), lignans (Zhai et al. 2004, 2005, De Pascoali et al. 2006, León-Diaz et al. 2010), terpenoids (Wu et al. 2005), aristolochic acids and their esters (Cosyns 2003, Chung et al. 2011), and aristolactams (Marti et al. 2013) are described.

Although aristolochic acids are nitrophenantrene derivatives associated to a high risk of nephrotoxicity and upper urinary tract carcinoma disease (Arlt et al. 2002, Debelle et al. 2008) *Aristolochia triangularis* Cham., known in Brazil as "*cipó-mil-homens*" or "*jarrinha*", is traditionally used for the treatment of inflammatory conditions, diarrhea, asthma, different types of cancer, as well as antidote against snakebites and as diuretic (Simões et al. 1998, Lorenzi and Matos 2002). Regarding to its chemical constituents, Langmann et al. (1979) revealed the presence of tetracyclic diterpenes, kauran derivatives, steroids, lignans and neolignans. In relation to the presence of aristolochic acids in this species, the data reported are conflicting (Ambros and De Siqueira 1971, Langmann 1979, Rücker et al. 1981).

Therefore, as part of our investigations on anti-infective and antitumoral potential of natural products, (De Oliveira et al. 2012, Almeida et al. 2012, Bianco et al. 2013, Guimarães et al. 2013) the *in vitro* biological profile of extracts, fractions and two isolated compounds obtained from *A. triangularis* regarding antiproliferative, antimicrobial, antiherpes and antileishmanial activities were investigated. Moreover, a preliminary examination related to the presence of aristolochic acids in stems and leaves of this medicinal plant by HPLC was also conducted.

MATERIALS AND METHODS

PLANT MATERIAL

Stems and leaves of *Aristolochia triangularis* were collected in Nova Santa Rita (29°52'24"S - 51°15'25"W), Rio Grande do Sul State, Brazil, in March 2013. The plant material was identified by Dr. Sérgio Augusto de Loreto Bordignon, and a voucher specimen was deposited at the Herbarium ICN, voucher number 184663 (Herbarium of the Universidade Federal do Rio Grande do Sul).

EXTRACTION PROCEDURES

Stems and leaves were separated and dried at 40ºC. Thereafter, ethanol maceration (plant: solvent, 1:10, w/v ratio; 2 x 10 days) was carried out. After solvent evaporation, one aliquot of the crude ethanol extract (CEE) was reserved for HPLC analyses while another one was fractionated with chloroform yielding a chloroform fraction (CF) and a residual aqueous fraction (RAF). All these samples were assayed for *in vitro* biological activities.

PHYTOCHEMICAL ANALYSES AND ISOLATION OF THE MAJOR COMPOUNDS FROM CHLOROFORM FRACTIONS

Chloroform fractions (1 g) from stems and leaves (CF_{stems} and CF_{leaves}, respectively) were preliminarily fractionated by flash column chromatography on silica gel (70-230 mesh) using *n*-hexane (200 mL), *n*-hexane: ethyl acetate (1:1, v/v, 200 mL), ethyl acetate (200 mL) and methanol (200 mL) as mobile phase. The fractions were monitored by TLC, and pooled considering their chemical profiles to yield sub-fractions CF-1 to CF-4. Considering that the TLC profiles of stems and leaves were similar, and the yield of CF_{stems} sub-fractions were higher than those of CF_{leaves}, the isolation of major compounds was carried out only with stems sub-fractions. Thus, sub-fraction CF_{stems}-2 (80 mg) eluted with *n*-hexane-ethyl acetate (5:5, v/v,) was fractionated by column chromatography on Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) using ethanol as mobile phase. Two pure compounds were isolated and analyzed by NMR. The identification of the neolignan galbacin (compound **1**, 23 mg) and the lignan cubebin (compound **2,** 16 mg) was carried out by comparing their spectra with spectral data reported in the literature (Lopes and Bolzani 1988, Vieira et al. 1998, Zhang et al. 2008).

BIOLOGICAL ACTIVITY PROFILE

Antiproliferative screening

Human non-small-cell lung cancer A549 cells (ATCC: CCL185) were grown in MEM (Cultilab®, Brazil) supplemented with 5% fetal bovine serum (FBS, Life Technologies®, USA), and maintained at 37°C in a humidified 5% CO_2 atmosphere. Antiproliferative activity was evaluated by the classical colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). Briefly, $1x10^4$ confluent cells were treated with serial dilutions of samples for 72 h at 37°C. After treatment, medium was replaced by MTT solution (Sigma-Aldrich), and cells were further incubated for 4 h. Optical densities were read at 540 nm (Spectra Max M2 (Molecular Devices, Sunnyvale, CA, USA) immediately after the dissolution of formazan crystals with DMSO (Sigma-Aldrich). The concentration of each sample that reduced cell viability by 50% when compared to untreated controls (CC_{50}) was estimated by non-linear regression of concentration–response curves. Paclitaxel (Sigma-Aldrich) was used as positive control.

Antibacterial and antifungal screening

Microorganisms used were gram-positive bacteria [*Clostridium sporogenes* (ATCC 11437), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pneumonie* (ATCC 49619), *Streptococcus pyogenes* (ATCC 19615)]; gram-negative bacteria [*Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonie* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022)]; and fungus [*Candida albicans* (ATCC 10231) and *Candida tropicalis* (ATCC 13803)]. Antimicrobial activity was evaluated by disk diffusion method, as previously described by De Oliveira et al. (2005), with minor modifications. Briefly, filter paper disks (6 mm) were impregnated with 20 µL of sample dilutions (100 mg/mL DMSO) and then placed on Muller-Hinton agar plates (HIMEDIA®), which were inoculated with microorganisms according to the standard protocol described by Clinical Laboratory Standard Institute (2002). Plates were incubated at 35 ± 1°C for 18 h, and the diameters of the inhibition zones were measured. Filter paper disks containing only DMSO were used as negative controls. Standard antibiotic disks were selected according to the sensitivity

of each microorganism tested: ampicillin (10 μg), oxacillin (1μg), ceftazidime (30 μg), imipenen (10 μg), chloramphenicol (30 μg), levofloxacin (5 μg), doxacillin (30 μg) and fluconazole (25 μg).

Antiherpes screening

Vero cells (ATCC:CCL-81) were grown in MEM (Cultilab, Brazil) supplemented with 10% FBS (Life Technologies, USA), and maintained at 37°C in a humidified 5% CO_2 atmosphere. Herpes Simplex Virus type 1 (HSV-1 / KOS strain, Faculty of Pharmacy, University of Rennes I, Rennes, France) viral stocks were prepared as previously described, and titrated based on plaque forming units (PFU) (Burleson 1992).

Initially, cytotoxicity was determined by exposing confluent Vero cells to serial dilutions of samples for 72 h at 37°C. Cell viability was determined by MTT assay, as previously described above. Results were expressed as CC_{50} values, estimated by non-linear regression of concentration-response curves.

Following, antiherpes activity evaluation was carried out by plaque number reduction assay, according to the procedures described by Kratz et al. (2008), with minor modifications. Briefly, $2.5x10^4$ Vero cells were infected with approximately 100 PFU of HSV-1 (KOS strain) for 1h at 37°C. Infected cells were treated with MEM containing 1.5% carboxymethylcellulose in the presence or absence of serial non-toxic dilutions of samples for 72 h at 37°C. After treatment, cells were fixed and stained with naphthol blue-black and viral plaques were counted. The concentration of each sample that reduced plaque number by 50% when compared to untreated controls (IC_{50}) was estimated by non-linear regression of concentration-response curves. The ratio of these indices was used to calculate the selectivity index of each sample ($SI = CC_{50}/IC_{50}$). Acyclovir (Sigma-Aldrich) was used as positive control.

Antileishmanial screening

Macrophage-like THP-1 (ATCC:TIB 202) cells were grown in RPMI-1640 without phenol red (Sigma), supplemented with 10% FBS (Life Technologies, USA), 12.5 mM HEPES, penicillin (100 U/mL), streptomycin (100 μg/mL) and Glutamax (2 mM), at 37°C in a humidified 5% CO_2 atmosphere. *Leishmania amazonensis* (MHOM/BR/77/LTB0016) promastigotes, expressing beta-galactosidase, were grown at 26°C in Schneider's insect medium (Sigma-Aldrich) supplemented with 5% FBS and 2% of human urine.

For the antileishmanial screening against intracellular *L. amazonensis* amastigotes, THP-1 cells (3.0×10^4 per well) were cultivated in 96 well plates with RPMI-1640 medium supplemented as described above, and treated with 100 μg/mL of phorbol 12-myristate 13-acetate (PMA) for 72 h at 37°C to allow THP-1 cells differentiation into non-dividing macrophages (Schwende et al. 1996).

Four day culture promastigotes (4.0×10^6 parasites/mL) were washed with phosphate buffered saline, pH 7.4 (PBS) an incubated in RPMI-1640 supplemented with 10% human AB+ serum for 1 h at 34°C for parasite opsonization. THP-1 cells were then incubated with a parasite/cell ratio of 10:1 for 3 h at 34°C and 5% CO_2. After this period, non-adherent parasites were removed by one wash with PBS, and infected cells were incubated with 180 μL of full supplemented RPMI-1640 medium for more 24 h to allow the transformation of promastigotes into intracellular amastigotes.

Infected cell monolayers were treated by addition of 20 μL of each sample in different concentration (50 μg/mL to 1.56 μg/mL, in triplicate, followed by incubation for 48 h, at 34°C in 5% CO_2. After treatment, cells were carefully washed with PBS and incubated for additional 16 h at 37°C with 250 μL of chlorophenolred-ß-D-galactopyranoside (CPRG) (Sigma-Aldrich) at 100 μM and nonidet P-40 0.1% (NP-40) (Amresco Inc,

USA). Optical density was read at 570/630 nm in an Infinite M-200 multiplate reader (TECAN, Austria). The concentration of each sample that reduced parasite viability by 50% when compared to untreated controls (IC_{50}) was estimated by non-linear regression of concentration-response curves. Amphotericin B (Sigma) was used as positive control.

For cell toxicity evaluation, THP-1 cells were seeded in 96 well plates and incubated for 48 h at 37°C with serial dilutions of samples. The assays were carried out in triplicate, and cell viability was determined by MTT assay as described above.

Statistical analyses

Data analysis was performed with PRISM 5 software (GraphPad Software, San Diego, CA, USA.). CC_{50} and IC_{50} values were calculated by fitting variable slope normalized sigmoidal concentration-response curves. Data are presented as means ± standard deviations with 95% confidence interval (n = 3).

HPLC ANALYSES

HPLC analyses of CEE obtained from stems and leaves was conducted to detect the presence of aristolochic acids AA-I and AA-II, and were performed in a LC PerkinElmer Series 200, composed by a Diode Array Detector (DAD), quaternary pump, autosampler, and online degasser. The separation was achieved on a Perkin Elmer Brownlee Choice C_{18} column (250 mm x 4.6 mm i.d. x 5 µm), and the mobile phase was a gradient of solvent A (acetonitrile-acetic acid 0.2%) and solvent B (acetic acid 0.2%) as follows: 20-40% A (0-32 min), isocratic 40% A (32-67 min), and gradient 40-90% A (67-95 min). The flow rate was kept constant at 1.0 mL/min. The chromatograms were recorded at 254 nm, and the UV spectra were monitored over a range of 200-500 nm. Standards of AA-I and a mixture of AA-I and AA-II were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Peaks were characterized by comparing retention times of the samples and their UV spectra with reference standards and by co-injection of authentic samples. All analyses were performed in triplicate.

RESULTS AND DISCUSSION

In South Brazil, according to a study conducted by EPAGRI (Santa Catarina Agricultural Agency) (Silva-Junior and Salerno 2012), *A. triangularis* was cited as the most frequently used medicinal plant in Santa Catarina State. In this way, a multiple *in vitro* biological screening of extracts, fractions and two isolated compounds from this plant was performed. Considering the controversial literature data about the presence of aristolochic acids in *A. triangularis*, the existence of these substances in the crude ethanol extract (CEE) of this plant was also investigated.

PHYTOCHEMICAL ANALYSES AND ISOLATION OF MAJOR COMPOUNDS

Chloroform fractions (CF_{stems} and CF_{leaves}) were submitted, separately, to flash column chromatography procedures yielding four fractions (CF-1, CF-2, CF-3 and CF-4), which were analyzed by TLC. It was observed the presence of two major compounds **1** and **2** in CF_{stems}-4, which could be isolated by chromatographic fractionation over silica gel and Sephadex LH-20. These compounds were characterized comparing their NMR spectra with spectral data previously reported (Lopes and Bolzani 1988, Vieira et al. 1998, Zhang et al. 2008). Compounds **1** (23 mg) and **2** (16 mg) were identified as the neolignan (+) galbacin (*5-[(2R,3R,4R,5R)-5-(1,3-benzodioxol-5-yl)-3,4-dimethyloxolan-2-yl]-1,3-benzodioxole* (**1**): ^{1}H-NMR ($CDCl_3$, 125 MHz) δ 5.94 (2H, *s*, -O-CH_2-O-), δ 4.61(2H, *d*, *J*-9.5 Hz, H-2 and H-5), δ 1.75 (2H, *m*, H-3 and H-4), δ 1.01 (6H, *s*, two methyl groups)), and the lignan (-) cubebin [*(2R,3R)-2,3-di-(3,4-*

methylenedioxybenzyl)-butyrolactol] (**2**): ^{1}H-NMR ($CDCl_3$, 125 MHz) δ 6.47 - 6.50 (6H, *m*, Aryl-H), δ 5.90 (4H, *s*, (2x) -O-CH2-O-), δ 5.23 (1H, *brs*, H-9'), δ 3.82-3.55 (1H, *m*, H-9'), δ 2.64-2.40 (4H, *m*, (2x) H-7 and (2x) H-6), δ 2,10 (2H, *m*, H-8 and H-8'), respectively (Figure 1). Lignans and neolignans with great structural diversity were described for *Aristolochia* species (Zhai et al. 2005, De Pascoali et al. 2006, León-Diaz et al. 2010). In this way, our results corroborate these reported data as well as the results obtained by Langmann et al. (1979) with *A. triangularis* extracts, who also isolated cubebin and galbacin.

ANTIPROLIFERATIVE SCREENING

The antiproliferative activity against the human lung cancer cell line (A549) is summarized in Table I.

Results showed that CF_{stems} was the most cytotoxic sample. CF fractions, from stems and leaves, showed similar antiproliferative effects, but were less cytotoxic than the respective crude extracts. In addition, compounds **1** and **2** were less cytotoxic when compared to CF fractions.

The antitumor potential of *Aristolochia* species and related compounds has been previously described (Chaouki et al. 2010, Hedge et al. 2010, Marti et al. 2013, Zhou et al. 2013). For *A. triangularis*, antiproliferative effects were described for dichloromethane extract against human epidermoid carcinoma (KB cells) (Mongelli et al. 2000). Moreover, several neolignans have been isolated from *Aristolochia* species, and showed a diverse pattern of cytotoxicity against various cancer cell lines (Zhou et al. 2013). As far as we are aware, this is the first report of antiproliferative activity for *A. triangularis* against A549 cells. Concerning the antiproliferative activity of compounds **1** and **2**, the results indicated a weak action suggesting that these compounds, individually, are not responsible for the detected activity. Indeed, few studies demonstrated that galbacin and cubebin, alone, showed merely moderate antiproliferative activity against other cancer cell lines (Lee et al. 2004, Nascimento et al. 2004).

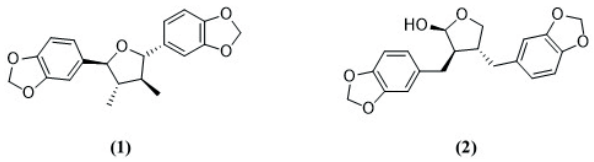

Figure 1 - Structure of the neolignan and lignan isolated from *Aristolochia triangularis*, galbacin (**1**) and cubebin (**2**).

ANTIBACTERIAL AND ANTIFUNGAL SCREENING

CEE_{stems} and CF_{stems}-4 showed weak antimicrobial activity against *S. aureus* (9-12 mm inhibition zone). The most interesting results were obtained with CF_{leaves} (13-16 mm inhibition zone). Compounds **1** and **2** were inactive against all bacterial and fungal species evaluated.

Few studies reporting antimicrobial activity of plants from *Aristolochia* genus have been published (Camporese et al. 2003, Machado and

TABLE I
Antiproliferative activity against A549 cells of *Aristolochia triangularis* extracts, fractions and compounds 1 and 2.

Samples	Stems		Leaves	
	CC_{50} (µg/mL)	CI 95%	CC_{50} (µg/mL)	CI 95%
CEE	9.21	7.97-10.65	> 50	-
CF	2.93	2.25-3.81	12.96	11.54-14.55
CF-1	22.94	14.45-36.42	43.97	31.95-61.29
CF-2	22.04	15.46-31.42	24.53	14.66-41.04
CF-3	16.74	6.27-44.72	13.80	4.79-39.74
CF-4	7.24	0.43-12.31	7.22	4.17-12.51
1	13.42		7.51-23.99	
2	23.51		7.19-60.51	

CI 95%: confidence interval at 95%; Paclitaxel - CC_{50} = 0.72; CI = 0.42-1.24; CEE: crude ethanol extract; CF: chloroform fraction; CF-1: *n*-hexane; CF-2: *n*-hexane-ethyl acetate (5:5); CF-3: Ethyl acetate; CF-4: methanol; 1: galbacin; 2: cubebin.

Lopes 2005). From *A. triangularis*, there is only one report concerning the antibacterial activity of its roots against *S. aureus* (Mesa-Alicia et al. 1950).

ANTIHERPES SCREENING

In this study, the antiviral activity against Herpes Simplex Virus type 1 (HSV-1, KOS strain) was also evaluated. Cytotoxic effects on Vero cells, which are permissive to herpesvirus replication, were preliminarily investigated by MTT assay. Only non-cytotoxic concentrations were used for the antiviral screening. The results indicated that only semi-purified fractions CF_{stems}-4 and CF_{leaves}-4 could inhibit viral replication (99.0 and 97.9%, respectively). These results agree with the findings of Garcia et al. (1990), who showed that a crude ethanolic extract from *A. triangularis* did not present antiherpes activity. Furthermore, the antiherpes activity of both semi-purified fractions CF_{stems}-4 and CF_{leaves}-4 inhibited viral replication in a concentration-dependent manner with IC_{50} values of 0.40 and 2.61 µg/mL, respectively (Figure 2), and selectivity indices of 5.0 and 4.1, respectively. Compounds **1** and **2** did not show antiviral activity (inhibition < 10%).

These findings could be regarded as promising for semi-purified fractions or extracts of medicinal plants (Cos et al. 2006). In addition, since compounds **1** and **2** were inactive, the antiherpes activity detected is possibly related to the synergistic effects of multiple compounds present in the extracts or fractions (Williamson 2001, Wagner 2010), although the antiherpes activity of other neolignans and lignans has been described (Sawasdee et al. 2013).

ANTILEISHMANIAL SCREENING

CF_{stems} showed promising results against *L. amazonensis* (85.3% growth inhibition at 50 µg/mL) (Table II). This fraction was also tested against the intracellular forms of *L. amazonensis* showing a selectivity index (SI) of 31.80, which could be also regarded as promising (Cos et al. 2006). Additionally, CF_{stems}-1 to CF_{stems}-4 were tested, but they were less active. In relation to the isolated compounds, only **1** was active showing a moderated growth inhibition at 50 µg/mL, and an IC_{50} of 16.69 µg/mL.

Tempone et al. (2008) reported leishmanicidal and trypanocidal activities for *Aristolochia cymbifera,* also popularly known in Brazil as "cipó-mil-homens". Another study conducted by Sartorelli et al. (2010) strengthened these results, and correlated the detected trypanocidal activity to the presence of diterpenes and lignans in this species. Thus, our results are, at least in part, in agreement with the literature.

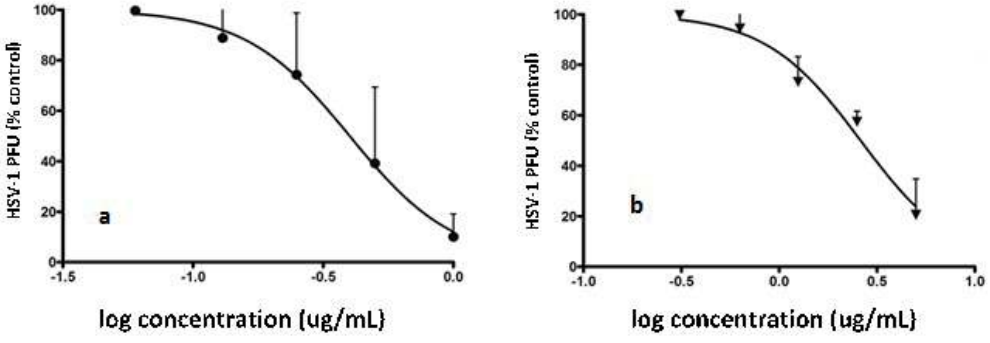

Figure 2 - Antiherpes activity (HSV-1, KOS strain) of the semi-purified fractions CF_{stems}-4 **(a)** and CF_{leaves}-4 **(b)** from *Aristolochia triangularis*.

TABLE II
Antileishmanial screening (50 µg/mL) against *Leishmania amazonensis*.

Samples	Growth inhibition (%)	IC_{50} (µg/mL)	CI 95% (µg/mL)	CC_{50} (µg/mL)	CI 95% (µg/mL)	SI
CEE_{stems}	NI	-	-	-	-	-
CF_{stems}	85.3 ± 0.5	2.28	0.86 - 6.07	72.5	45.21 - 116.2	31.80
CF_{stems}-1	23.8 ± 7.1	-	-	-	-	-
CF_{stems}-2	59.7 ± 9.2	56.13	54.50 - 57.80	154.6	150.8 - 158.5	2.75
CF_{stems}-3	13.1 ± 5.3	-	-	-	-	-
CF_{stems}-4	NI	-	-	-	-	-
CEE_{leaves}	NI	-	-	-	-	-
CF_{leaves}	NI	-	-	-	-	-
CF_{leaves}-1	59.8 ± 5.5	12.82	9.70 - 16.95	268.9	214.0 - 337.8	20.98
CF_{leaves}-2	NI	-	-	-	-	-
CF_{leaves}-3	65.7 ± 2.2	12.06	7.38 - 19.69	49.53	6.05 - 405.6	4.11
CF_{leaves}-4	47.8 ± 5.2	-	-	-	-	-
1	59.47 ± 4.7	16.69	13.61 - 20.45	> 50	-	> 3.00
2	9.69 ± 4.0	-	-	-	-	-

NI: no inhibitory activity; CI 95%: confidence interval at 95%; Positive control (Amphotericin B, 2 µM); $IC_{50:}$ concentration that show parasite growth inhibition in 50%; CC_{50}: concentration that reduces cell viability by 50%; SI: selectivity index (CC_{50}/IC_{50}); 92.8 ± 1.7%. CEE_{stems}: crude ethanolic extract; CF_{stems}: chloroform fraction; CF_{stems}-1: *n*-hexane; CF_{stems}-2: *n*-hexane-ethyl acetate (5:5); CF_{stems}-3: ethyl acetate; CF_{stems}-4: methanol; compound 1: galbacin; compound 2: cubebin; CEE_{leaves}: crude ethanolic extract; CF_{leaves}: chloroform fraction; CF_{leaves}-1: *n*-hexane; CF_{leaves}-2: *n*-hexane-ethyl acetate (5:5); CF_{leaves}-3: ethyl acetate; CF_{leaves}-4: methanol.

HPLC ANALYSES OF CRUDE ETHANOL EXTRACT (CEE)

Considering the reported contradictory results concerning the presence of aristolochic acids (AA) in *A.triangularis* (Ambros and De Siqueira 1971, Langmann 1979, Rücker et al. 1981), its popular utilization in South Brazil for different purposes, and the possible toxic effects associated with these acids (Arlt et al. 2002, Debelle et al. 2008), we performed HPLC analyses the crude ethanol extract of stems and leaves.

Under the chromatographic conditions employed, AA-I and AA-II standards showed retention times of 62.7 min and 53.5 min, respectively (Figure 3). Chromatograms from CEE_{stems} (Figure 3a) and CEE_{leaves} (Figure 3b) did not display any peak with retention time comparable to that of AA-II (limits of detection and quantitation of 0.99 and 3.03 µg/mL for AA-I, and 0.13 and 0.39 µg/mL for AA-II). On the other hand, the chromatograms revealed the presence of a compound with a similar retention time of AA-I at 62 min, especially in CEE_{stems}. However, this putative compound showed important differences in its UV spectra (λ 275 and 225 nm) when compared to those of AA-I (λ 320 and 270 nm). Therefore, according to our data, *A. triangularis* did not show the presence of AA-I and AA-II in CEE_{stems} and CEE_{leaves}. Thus, these findings agree with those published by Langmann (1979) and Rücker et al. (1981), who evaluated two different samples of *A. triangularis* by distinct chromatographic techniques, TLC and GC/MS, respectively, and also did not detected the presence of AAs.

CONCLUSIONS

The biological screening performed with extracts, semi-purified fractions and isolated compounds showed multiple *in vitro* activities. In summary,

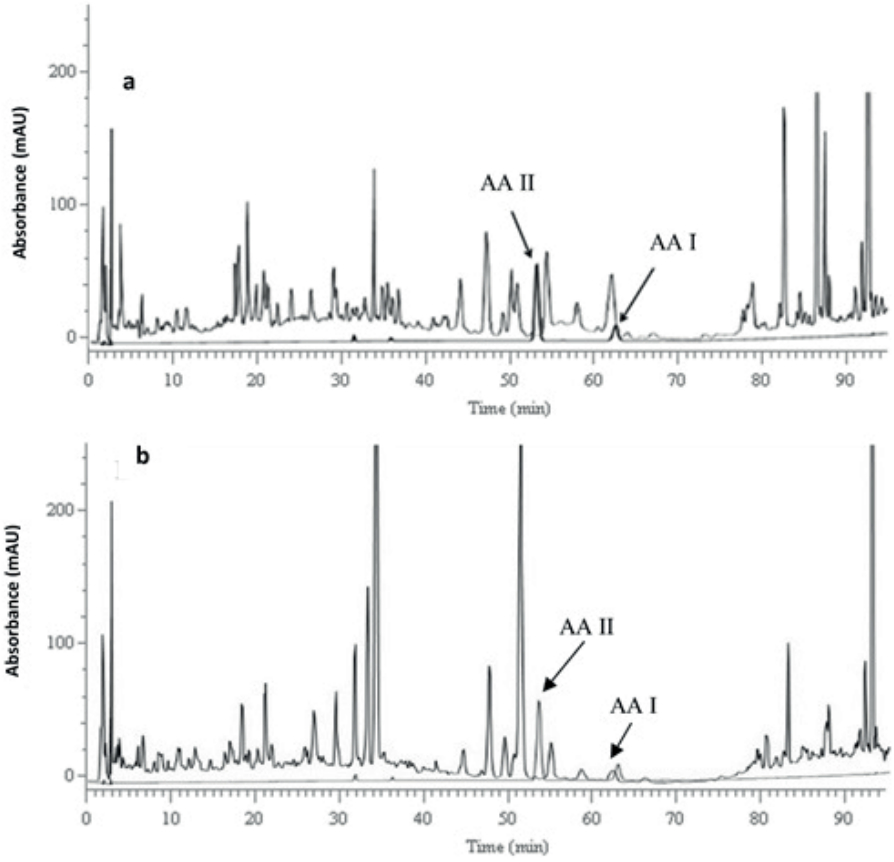

Figure 3 - HPLC chromatograms, at 254 nm, of crude ethanolic extract (CEE) from stems **(a)** and leaves **(b)** of *Aristolochia triangularis* with reference standards: aristolochic acid I (AA-I, 62.7 min) and aristolochic acid II (AA-II, 53.5 min).

we found that chloroform fractions from stems and leaves of *A. triangularis* showed in all tested assays results that could be regarded as promising for semi-purified fractions or extracts of medicinal plants. However, the neolignan/lignan isolated (galbacin and cubebin) did not show anti-HSV-1 activity and displayed only a weak antiproliferative activity and the neolignan galbacin was active against *L. amazonensis,* showing a moderated growth inhibition at 50 µg/mL, and an IC_{50} of 16.69 µg/mL. Further pharmacological evaluation of *A. triangularis* remains highly desirable, especially in the light of its high usage among the population and the paucity of scientific data.

Finally, in view of the abundant studies reporting the toxicity of aristolochic acid derivatives and that they are frequently reported for *Aristolochia* species, the presence of aristolochic acid I and aristolochic acid II was also investigated in the crude ethanol extract of stems and leaves, and these compounds could not be detected by the HPLC analysis. Thus, these findings agree with those published by Langmann (1979) and Rücker et al. (1981), who evaluated two different samples of *A. triangularis*, and also did not detect the presence of aristolochic acid derivatives.

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AUTHOR CONTRIBUTIONS

Sérgio Augusto de Loreto Bordignon and Cesar Paulo Simionato were responsible for collecting and identifying the samples of *Aristolochia triangularis* Cham; Simone Quintana de Oliveira, Sapfo Dimitrakoudi and Argyro Vontzalidou performed the fractionation of the extracts, chromatographic analyses, isolation and structure elucidations; Danielle Tocantins Moura Costa, Tatiana da Rosa Guimarães, Jadel Müller Kratz, were responsible for planning and carrying out the biological assays; Vitor Clasen Chaves contributed with the HPLC analysis. Simone Quintana de Oliveira, Cláudia Maria Oliveira Simões, Flávio Henrique, Mário Steindel and Eloir Paulo Schenkel contributed to the design and implementation of the research, the analyses of the results and the manuscript writing/editing. All authors contributed to the final manuscript.

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