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Trypanocidal activity of Brazilian plants against epimastigote forms from Y and Bolivia strains of *Trypanosoma cruzi*

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Abstract: Chagas disease is one of the main public health problems in Latin America. Since the available treatments for this disease are not effective in providing cure, the screening of potential antiprotozoal agents is essential, mainly of those obtained from natural sources. This study aimed to provide an evaluation of the trypanocidal activity of 92 ethanol extracts from species belonging to the families Annonaceae, Apiaceae, Cucurbitaceae, Lamiaceae, Lauraceae, Moraceae, Nyctaginaceae, and Verbenaceae against the Y and Bolivia strains of *Trypanosoma cruzi*. Additionally, cytotoxic activity on LLCMK2 fibroblasts was evaluated. Both the trypanocidal activity and cytotoxicity were evaluated using the MTT method, in the following concentrations: 500, 350, 250, and 100 µg/mL. Benznidazole was used for positive control. The best results among the 92 samples evaluated were obtained with ethanol extracts of *Ocotea paranapiacabensis* (Am93) and *Aegiphila lhotzkiana* (Am160). Am93 showed trypanocidal activity against epimastigote forms of the Bolivia strain and was moderately toxic to LLCMK2 cells, its Selectivity Index (SI) being 14.56, while Am160 showed moderate trypanocidal activity against the Bolivia strain and moderate toxicicity, its SI being equal to 1.15. The screening of Brazilian plants has indicated the potential effect of ethanol extracts obtained from *Ocotea paranapiacabensis* and *Aegiphila lhotzkiana* against Chagas disease.

Introduction

In Latin America, Chagas disease is an important cause of morbidity, affecting around 10 million people and representing a risk for 25 million from the South of the United States to the South of Argentina (WHO, 2010).

Since this disease affects mostly poor populations, the development of new therapeutic solutions is not an attractive business for the large pharmaceutical companies, and currently it can be said that this initiative is being extremely neglected, which is a very concerning fact on account of the needs of those people (Nwaka & Ridley, 2003). The two drugs available for the treatment of Chagas disease, nifurtimox and benznidazole, have potential toxic side effects and variable efficiency, both of them being ineffective in eradicating the infection during its chronic phase, which contributes to its low use rates (Coura, 2009). For this reason, the screening of potential new compounds is essential (Coura & Castro, 2002).

The difficulty to find a substance capable of fighting the parasite can be directly related to the morphological characteristics of the strain, mainly considering the presence of different populations, which present distinct tissue tropism. Therefore, different strain groups of *T. cruzi* should be considered in the evaluation of new drugs (Macedo et al., 2002).

This scenario clearly shows that it is necessary to develop therapies that stop the multiplication of *T. cruzi* without causing any severe side effect (Coura & Castro, 2002). Medicinal plants have been used in the treatment for parasitic diseases for a long time, and many works

sustain the therapeutic value of products from plant origin, also describing the trypanocidal activity of natural active compounds (Bastos et al., 1999; Saraiva et al., 2007; Batista Jr. et al., 2008).

Continuing our studies on the discovery of trypanocidal agents obtained from plants from both the Cerrado and the Atlantic Forest (Cotinguiba et al. 2009; Lopes et al., 2008; Regasini et al. 2009), 92 ethanol extracts of species belonging to the families Annonaceae, Apiaceae, Cucurbitaceae, Lamiaceae, Lauraceae, Moraceae, Nyctaginaceae, and Verbenaceae were tested against epimastigote forms of *Trypanosoma cruzi* (Y and Bolivia strains), and their cytotoxic activity on $LLCMK_2$ fibroblasts was evaluated. The emergency to find new antiprotozoal agents with trypanocidal activity and the evidence that some species of the aforementioned families have trypanocidal activity against parasitic forms of *T. cruzi* provided the motivation to carry out the screening of such extracts (Buainain et al., 1992; Fournet et al., 2007; Osorio et al., 2007; Cabral et al., 2010).

Material and methods

Parasites

In the assays both the Y and Bolivia strains were used, the former belonging to lineage I and the latter, to lineage II. The strains were kept in BALB/c mices and in LIT (Liver Infusion Tryptose) culture medium, in BOD incubator at 28 °C, at the Laboratory of Parasitology of the Faculty of Pharmaceutical Sciences of Araraquara-SP, Unesp.

Plant material and extraction

The plant material was collected by Maria Cláudia Marx Young in remaining areas of Atlantic Forest and Cerrado in the State of São Paulo, and it was identified by Inês Cordeiro, Institute of Botany, State Department of the Environment, São Paulo-SP. The voucher specimens were then deposited in the herbarium "Maria Eneyda P. Kaufmann" at the IBT-SMA. The codes of the extracts and voucher specimens can be found in Table 1.

After the collection, the botanical material was dried in the absence of light and then powdered using a cutting mill. A 30 g portion of the powder was extracted with ethanol (5x100 mL) during three weeks, at room temperature. After the filtration, the solvent was evaporated under reduced pressure, which resulted in the crude extracts.

Twenty-eight of the 92 ethanol extracts evaluated belong to the genera *Rollinia, Xylopia, Anaxagorea, Annona, Guatteria* and *Duguetia*, family Annonaceae; one to the genus *Hydrocotyle*, family Apiaceae; two to the genus *Cayaponia,* family Cucurbitaceae; two to the genera *Aegiphila*, family Lameaceae 36 to the genera *Nectandra* and *Ocotea*, family Lauraceae; one to the genus *Dorstenia*, family Moraceae; eight to the genera *Bougainvilleae, Pisonia* and *Guapira,* family Nyctaginaceae; and fourteen to the genera *Lantana, Starchytarpheta,* and *Lippia*, family Verbenaceae (Table 1).

In vitro *assay for trypanocidal activity*

Trypanocidal activity was evaluated by means of the MTT method, with changes (Muelas-Serrano et al., 2000).

The epimastigote forms (1.10^7 parasites/mL), obtained from culture in stationary phase, were cultured in plates with 96 wells in BOD incubator at 28 °C for 24 h, concentrations for the ethanol extracts being 500, 350, 250 and 100 µg/mL. After this period, the MTT (2.5 mg/mL) and PMS (0.22 mg/mL) solutions were added to each well, and the plate was incubated for 1 h. Then 100 µL of HCl (1M) and SDS (10%) were added to it. The plate was kept at room temperature for 30 min, and the reading was performed on a spectrophotometer at 595 nm. Benznidazole was used in the same concentrations for positive control.

The assays were in triplicate, and the results were expressed as IC50, calculated by the statistical method of sigmoid concentration-response curve using the GraphPad Prisma 4.0 software.

Cytotoxicity assay

Extracts with trypanocidal activity against epimastigote forms of *T. cruzi* were evaluated regarding their cytotoxicity on LLCMK2 fibroblasts by means of the MTT method, with changes (Muelas-Serrano et al., 2000).

$LLCMK_2$ cells (1.10^6 /mL) were cultured in plates with 96 wells and ethanol extracts in the following concentrations: 500, 350, 250 and 100 µg/mL. The plates were incubated in a CO_2 incubator at 5% and 37 °C for 24 h. After that period, 10 µL of MTT solution (5mg/mL) were added to each well, and the plates were incubated for 4 h. Then 100 µL of acid isopropyl were added, and the plate was kept at room temperature for 1 h. The reading was performed on a spectrophotometer at 595 nm. RPMI culture medium was used for positive control, whereas $LLCMK_2$ cells were used for negative control.

The assays were carried out in triplicate, and the results were expressed as CC50, calculated by the statistical method of sigmoid concentration-response curve using the GraphPad Prisma 4.0 software.

The cytotoxic activity (CC50) was related to the trypanocidal activity (IC50) in order to determine the correspondent Selectivity Index (IS=CC50/IC50).

Table 1. Ethanol extracts of plants from the Atlantic Forest and Cerrado.

Extract/voucher sample	Species	Part of the plant	Extract/voucher sample	Species	Part of the plant
			Annonaceae		
M723	*Rollinea sericea*	Branches	Rm98	*Xylopia langsdorfiana*	Leaves
M1103	*Xylopia aromatica*	Fruits	Rm99	*Xylopia langsdorfiana*	Branches
M1143	*Anaxagorea dolichocarpa*	Leaves	Am03	*Guatteria elliptica*	Branches
M1144	*Anaxagorea dolichocarpa*	Branches	Am115	*Rollinea sericea*	Branches
R123	*Annona cacans*	Leaves	Am145	*Duguetia furfuracea*	Leaves
R124	*Annona cacans*	Branches	Am146	*Duguetia furfuracea*	Branches
R278	*Guatteria australis*	Leaves	Am223	*Annona coriacea*	Leaves
R279	*Guatteria australis*	Branches	Am224	*Annona coriacea*	Branches
R286	*Xylopia aromatica*	Leaves	Am338	*Guatteria nigrescens*	Leaves
R287	*Xylopia aromatica*	Branches	Am339	*Guatteria nigrescens*	Branches
R316	*Duguetia furfuracea*	Fruits	Am352	*Duguetia lanceolata*	Leaves
R404	*Annona cornifolia*	Leaves	Am379	*Duguetia lanceolata*	Branches
R405	*Annona cornifolia*	Branches	Am468	*Guatteria elliptica*	Leaves
Rm12	*Rollinea sericea*	Leaves	Am469	*Guatteria elliptica*	Branches
	Apiaceae			Lamiaceae	
M 861	*Hydrocotyle banariensis*	Leaves	Am158	*Aegiphila lhotzkiana*	Leaves
	Cucurbitaceae		Am159	*Aegiphila lhotzkiana*	Branches
Am 109	*Cayaponia tayiuya*	Fruits	Am160	*Aegiphila lhotzkiana*	Fruits
Am 110	*Cayaponia tayiuya*	Branches	R184	*Aegiphila sellowiana*	Leaves
Am 109	*Cayaponia tayiuya*	Fruits	R185	*Aegiphila sellowiana*	Branches
			Lauraceae		
M686	*Nectandra oppositifolia*	Leaves	R173	*Ocotea velutina*	Branches
M687	*Nectandra grandiflora*	Leaves	R188	*Ocotea silvestris*	Leaves
M698	*Nectandra grandiflora*	Branches	R189	*Ocotea silvestris*	Branches
M819	*Nectandra membracea*	Leaves	R388	*Ocotea megabotamica*	Leaves
R174	*Nectandra aspidata*	Leaves	R389	*Ocotea megabotamica*	Branches
R175	*Nectandra aspidata*	Branches	R429	*Ocotea pulchella*	Leaves
Rm128	*Nectandra membranaceae*	Leaves	R430	*Ocotea pulchella*	Branches
Am12	*Nectandra cissiflora*	Branches	Am71	*Ocotea laxa*	Leaves
Am46	*Nectandra membranaceae*	Branches	Am72	*Ocotea laxa*	Branches
Am257	*Nectandra cuspidata*	Leaves	Am73	*Ocotea elegans*	Leaves
Am258	*Nectandra cuspidata*	Branches	Am74	*Ocotea elegans*	Branches
M614	*Ocotea aciphylla*	Branches	Am92	*O. paranapiacabensis*	Leaves
M809	*Ocotea odorifera*	Branches	Am93	*O. paranapiacabensis*	Fruits
M823	*Ocotea velloziana*	Leaves	Am94	*O. paranapiacabensis*	Branches
M849	*Ocotea odorifera*	Leaves	Am245	*Ocotea corymbosa*	Leaves
R59	*Ocotea indecora*	Leaves	Am246	*Ocotea corymbosa*	Branches
R60	*Ocotea indecora*	Branches	Am447	*Ocotea teleiandra*	Leaves
R172	*Ocotea velutina*	Leaves	Am448	*Ocotea teleiandra*	Branches
			Moraceae		
Am29	*Dorstenia arifolia*	Branches			
			Nyctaginaceae		
R17	*Bougainvillea* sp.	Leaves	Am116	*Guapira opposita*	Leaves
R18	*Bougainvillea* sp.	Branches	Am117	*Guapira opposita*	Branches
R148	*Pisonia ambigua*	Leaves	Am202	*Guapira noxia*	Leaves
R149	*Pisonia ambigua*	Branches	Am203	*Guapira noxia*	Branches

		Verbenaceae			
M872	*Lantana undulata*	Leaves	Am270	*Lippia velutina*	Leaves
M873	*Lantana undulata*	Branches	Am271	*Lippia velutina*	Branches
M943	*Starchytarpheta cayenensis*	Leaves	Am371	*Lippia lupulina*	Leaves
M944	*Starchytarpheta cayenensis*	Branches	Am372	*Lippia lupulina*	Branches
R297	*Lippia salviaefolia*	Leaves	Am373	*Lippia lupulina*	Flowers
R298	*Lippia salviaefolia*	Branches			

Table 2. Trypanocidal activity and cytotoxicity of families of the Brazilian flora against epimastigote forms of the Y strain of *Trypanosoma cruzi* and $LLCMK_2$ fibroblasts, respectively.

N. Extract	Species	Family/part of the plant	IC50 µg/mL	CC50 µg/mL	SI	Trypanocidal activity	Cytotoxicity
Am93	*Ocotea paranapiacabensis*	Lauraceae/Fruits	179.8	392.2	2.18	Inactive	Moderately toxic
R60	*Ocotea indecora*	Lauraceae/Branches	214.8	498.2	2.32	Inactive	Moderately toxic
Am160	*Aegiphila lhotzkiana*	Lamiaceae/Fruits	126.0	104.1	0.83	Inactive	Moderately toxic
Am116	*Guapira opposita*	Nyctaginaceae/Leaves	386.4	115.9	0.30	Inactive	Moderately toxic
Am379	*Duguetia lanceolata*	Annonaceae/Branches	250.2	52.23	0.21	Inactive	Toxic
Am03	*Guatteria elliptica*	Annonaceae/Branches	345.1	103.3	0.30	Inactive	Moderately toxic
Am352	*Duguetia lanceolata*	Annonaceae/Leaves	157.9	332.4	2.11	Inactive	Moderately toxic
M1103	*Xylopia aromatica*	Annonaceae/Fruits	253.1	98.40	0.39	Inactive	Toxic
Benznidazole**:** IC50 11.77 µg/mL							

Table 3. Trypanocidal activity and cytotoxicity of families of the Brazilian flora against epimastigote forms of the Bolivia strain of *Trypanosoma cruzi* and $LLCMK_2$ fibroblasts, respectively.

N. Extract	Species	Family/part of the plant	IC50 µg/mL	CC50 µg/mL	SI	Trypanocidal activity	Cytotoxicity
Am93	*Ocotea paranapiacabensis*	Lauraceae/Fruits	26.93	392.2	14.56	Active	Moderately toxic
Am73	*Ocotea elegans*	Lauraceae/Leaves	350.8	140.2	0.400	Inactive	Moderately toxic
Am160	*Aegiphila lhotzkiana*	Lamiaceae/Fruits	90.89	104.1	1.150	Moderately active	Moderately toxic
Benznidazole**:** IC50 0.99 µg/mL							

Results and Discussion

Ninety-two ethanol extracts of different species of Brazilian flora were tested. The trypanocidal activity of the samples was classified according to criteria set by Osorio et al. (2007). The extracts were classified as highly active (IC50<10 µg/mL), active (IC50>10<50 µg/mL), moderately active (IC50>50<100 µg/mL) and inactive (IC50>100 µg/mL). With regard to their cytotoxicity, the samples were classified as highly toxic (CC50<10 µg/mL), toxic (CC50>10<100 µg/mL), moderately toxic (CC50>100<1000 µg/mL) and potentially non-toxic (CC50>1000 µg/mL).

According to this classification, all the 92 ethanol extracts tested against epimastigote forms of the Y strain of *T. cruzi* are inactive (Table 2).

Regarding the Bolivia strain, the fruit extract of *Ocotea paranapiacabensis* (Lauraceae) (Am93) is considered active, whereas the fruit extract of *Aegiphila lhotzkiana* (Lamiaceae) (Am160) and the leaf extract of *Ocotea elegans* (Am73) were respectively classified as moderately active and inactive against the same parasitic forms (Table 3).

The IC50 values for benznidazole against epimastigote forms of the Y and Bolivia strains were 0.99 and 11.77, respectively (Tables 2 and 3).

Regarding the cytotoxicity analysis, the extracts of *Duguetia lanceolata* (Am379) and *Xylopia aromatica* (M1103) were classified as toxic to $LLCMK_2$ cells, whereas the extracts of *Ocotea paranapiacabensis* (Am93), *Ocotea elegans* (Am73), *Ocotea indecora* (R60), *Aegiphila lhotzkiana* (Am160), *Guapira opposita* (Am116), *Guatteria elliptica* (Am03), and *Duguetia lanceolata* (Am352) were classified as moderately toxic (Tables 2 and 3).

The most promising samples were those that proved to be more active against epimastigote forms of *T. cruzi* and less toxic to $LLCMK_2$ cells.

According to this classification, the most promising extracts for chemical and pharmacological investment were the fruit of *Ocotea paranapiacabensis*, Lauraceae (Am93), which proved to be active against epimastigote forms of the Bolivia strain and moderately toxic to LLCMK2 cells, its SI being equal to 14.56, and the fruit extract of *Aegiphila lhotzkiana*, Lamiaceae (Am160), which was also tested against the Bolivia strain and showed moderate activity regarding the parasites and the LLCMK2 cells.

By comparing the trypanocidal activity of the extracts against the Y strain and the Bolivia strain, a clear difference could be noted. The material tested against the Y strain did not show a satisfactory activity. On the other hand, two extracts (Am93 and Am160), which were tested against the Bolivia strain, were found to be, respectively, active and moderately active against such parasitic forms. This difference in sensitivity between the strains can be explained by the fact that *T. cruzi* populations show large intraspecific variability, as it can be noted by differences in their morphology, virulence, pathogenicity, evasion ability in case of an immune response from the host, antigenic composition and biochemical properties (Fernandes et al., 1998; Tibayrenc & Ayala, 2002).

The trypanocidal activity of the ethanol extract of *Ocotea paranapiacabensis* (Lauraceae) against epimastigote forms of the Bolivia strain is reported for the first time in this work. Data from the literature report the activity of isolated alkaloids of *Ocotea odorifera* against promastigote forms of *Leishmania braziliensis, L. donovan* and *L. amazonensis* and trypomastigote forms of *T. cruzi* (Fournet et al., 2007). Extracts of branches and roots of the same species were found to be active against *Plasmodium falciparum*. Popular medicine recommends the use of these plants in the treatment for dermatoses, rheumatism, fever and syphilis (Botsaris, 2007).

Aegiphila lhotzkiana, which showed trypanocidal activity against the Bolivia strain, is widely distributed in Northeastern Brazil, where it is popularly known as *pau-de-sebo*. The oil obtained from its fruit is used in popular medicine for treating pediculosis and scabies, and its extract is used as an antidote to snakebite (Costa-Lotufo et al., 2004). The activity of this crude extract was unknown until this research was carried out, because there are no reports in the literature on the trypanocidal activity of this species, not even on the genus it belongs to.

The screening of Brazilian plants has indicated the potential effect of ethanol extracts obtained from fruits of *Ocotea paranapiacabensis* (Lauraceae) and *Aegiphila lhotzkiana* (Lamiaceae) against Chagas disease, considering the epimastigote forms of the Bolivia strain of *T. cruzi.*

These data reinforce the importance of the efforts to promote the sustainable use of Brazilian biodiversity, focusing on the search for new therapeutic agents for the treatment of some neglected diseases that affect millions of people in Brazil and other countries.

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