Brazilian flora extracts as source of novel antileishmanial and antifungal compounds

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Natural products have long been providing important drug leads for infectious diseases. Leishmaniasis is a protozoan parasitic disease found mainly in developing countries, and it has toxic therapies with few alternatives. Fungal infections have been the main cause of death in immunocompromised patients and new drugs are urgently needed. In this work, a total of 16 plant species belonging to 11 families, selected on an ethnopharmacological basis, were analyzed in vitro against Leishmania (L.) chagasi, Leishmania (L.) amazonensis, Candida krusei, *and C. parapsilosis. Of these plant species, seven showed antifungal activity against C. krusei, five showed antileishmanial activity against* L. chagasi *and four against* L. amazonensis, *among them species of genus* Plectranthus. *Our findings confirm the traditional therapeutic use of these plants in the treatment of infectious and inflammatory disorders and also offer insights into the isolation of active and novel drug prototypes, especially those used against neglected diseases as Leishmaniasis.*

Key words: *Leishmania* - *Candida* - plants - antimicrobial - drugs - therapy

Medicinal plants have been the basis for the treatment of various diseases in traditional methods. In Brazil, populations in rural areas rely on traditional medicines for the treatment of many infectious diseases. Some species are included in prescriptions for therapeutic purposes like the healing of wounds, inflammation due to microbial or parasitic infections, skin lesions, and ulcers (Lorenzi & Matos 2002). In some cases, the plant may be used for different purposes. For instance, the species of genus *Plectranthus* is traditionally used to treat a range of ailments including digestion, skin, infection, and respiratory problems (Lukhoba et al. 2006).

The Brazilian Atlantic forest (BAF) is one of the major biodiverse areas of the world, providing therapeutics for ancient diseases (Tempone et al. 2005). Plants used in folk medicine have offered natural products for the treatment of diseases caused by protozoan parasites. The discovery of artemisinin, a sesquiterpene lactone produced by *Artemisia annua*, as a pharmaceutical for the treatment of malaria promoted an interest in the discovery of new compounds from plants with antiprotozoal activity, especially those to treat against *Leishmania* parasites (Chan-Bacab & Peña-Rodrigues 2001). Leishmaniasis is a protozoan tropical disease that can manifest as a single cutaneous ulceration or can become a progressive and fatal disease (Singh & Sivakumar 2004). Besides the limited and toxic chemotherapeutic arsenal that is composed mainly of the pentavalent antimonials, amphotericin B (AmB) and pentamidine (PMD), the relapses due to resistant parasites demonstrate the urgent need for new drug candidates (Balaña-Fouce et al. 1998).

In recent decades, an increased incidence of fungal infections has been observed as a consequence of the growing number of immunocompromised patients, particularly those with AIDS or those taking anticancer drugs (Rahalinson et al. 1994). The expanding pool of high-risk patients susceptible to the opportunistic pathogen indicated that the yeast genus *Candida* is the most important invasive agent (Sobel 2006). Amb represents the main therapy for systemic fungal infections, but its use is highly limited as a result of renal toxicity and several other adverse effects (Zachino 2001). In some cases, azole-drugs, particularly fluconazole (FCZ), represent a less toxic alternative therapy agent although azole-resistant strains have been reported. Brazilian flora has been an astonishing source of biologically active compounds. Amazon forest plants have been showing promising results against *Candida* spp. (Carneiro et al. 2008). In this work, we have evaluated the antileishmanial and antifungal activities of 16 methanolic plant extracts from the BAF. Plants were selected from those that have traditional uses against cutaneous and infectious diseases such as ulcers, leishmaniasis, skin lesions, diarrhea, malaria, and inflammations, and those that have antimicrobial properties.

MATERIALS AND METHODS

Plant material - The plants were collected at Instituto Plantarum de Estudos da Flora, Brazil, in 2006. The vouchers of the species studied are deposited in the Herbarium Plantarum (HPL) and include the following:

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Plectranthus amboinicus (Lour.) Spreng. (voucher: H Lorenzi 3.411), *Aristolochia cymbifera* Mart. & Zucc. (voucher: H Lorenzi 2.115), *Plectranthus barbatus* Andrews (voucher: H Lorenzi 771), *Lippia alba* (Mill.) N.E. Br (voucher: H Lorenzi 1.713), *Hydrocotyle bonariensis* Lam. (voucher: H Lorenzi 3.516), *Plectranthus neochilus* Schlechter (voucher: H Lorenzi 1.441), *Justicia pectoralis* var. *stenophylla* Leon. (voucher: H Lorenzi 1.702), *Herreria salsaparilha* Mart. (voucher: H Lorenzi 3.391), *Mentha X piperita* L. (voucher: H Lorenzi 1.364), *Eleutherine bulbosa* (Mill.) Urb. (voucher: H Lorenzi 778), *Baccharis trimera* (Less.) DC. (voucher: H Lorenzi 432), *Calamintha adscendens* Willk. & Lang. (voucher: H Lorenzi 1701), *Albizia inundata* (Mart.) Barneby & J.W. Grimes (voucher: H Lorenzi 1692), *Bauhinia forficata* Link. (voucher: A Amaral Jr. 390), *Cymbopogon citratus* (DC.) Stapf. (voucher: H Lorenzi 3.462), and *Plectranthus grandis* (Cramer) R.H. Willemse (voucher: H Lorenzi 3.465).

Extract preparation - The dried (air incubator 55°C) and powered leaves (100 g) from plants were extracted with methanol (3 x 200 ml) through a maceration process for 48 h/ 25°C. The crude extracts were obtained after evaporation under reduced pressure at 45°C in a rotating evaporator. All extracts were kept at 4°C until used in biological assays.

Parasites- Leishmania (L.) chagasi (strain MHOM/BR/1972/LD) promastigotes were maintained in M-199 medium supplemented with 10% calf serum and 2% human male urine (Singh et al. 2000) at 24°C. *L. amazonensis* (strain PH8) was maintained in RPMI-1640 medium, supplemented with 20% calf serum at 24°C.

Determination of the 50% effective concentration (EC_{50}) - Promastigotes were used for all experiments, and the stationary phase was reached at 2-3 x 10^7 parasites/ml. The antileishmanial activity against promastigotes was determined using 96-well microplates (Sartorelli et al. 2007). Briefly, 1 x 10^6 promastigotes dissolved in methanol and diluted in M-199 medium (*L. chagasi*) or RPMI-1640 (*L. amazonensis*) medium were seeded with different extracts in the concentration range of 3.9 to 500 µg/ml. PMD was used as the standard drug. Plates were incubated at 24°C for 48 h and the viability of parasites was assayed colorimetrically by the mitochondrial oxidation of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl-tetrazolium bromide) (Tada et al. 1986). The optical density was detected at 570 nm with a MULTISKAN MS microplate reader and correlated with the number of living promastigotes, which were adequately standardized for each plate. Control wells without any sample were established as 100% viable. Methanol was incubated with parasites at 0.5%/well and used as an internal control.

Antifungal activity assay - In order to evaluate the susceptibility of the FCZ-resistant yeast *Candida krusei* (ATCC 6528) and the azole-susceptible *Candida parapsilosis* (ATCC 22019) to the extracts obtained, we developed an antimicrobial in vitro study. *Candida* spp. were maintained in RPMI 1640 medium (Sigma Chemicals, USA) without sodium bicarbonate, supplemented with L-glutamine (Gibco, Invitrogen) and 2% glucose at pH 7.0. Two-fold dilutions of extract in concentrations ranging from 3.90 to 500 µg/ml were dissolved in RPMI 1640 in 96-well microplates. Starting inoculum suspensions were obtained by the spectrophotometric method of inoculum preparation adjusted to 1-5 x 10^6cfu/ml and then diluted in test medium to 1-5 x 10^5cfu/ml. A 100 µl yeast inoculum was added to each well and the plates were incubated for 24 h and 48 h at 35°C in a humid atmosphere. After agitation, plates were read spectrophotometrically with an automatic plate reader (MULTISKAN FLOW, Sweden) at 492 nm. Amb was used as the standard drug. The MTT method was also used to assay the viability of yeasts in order to evaluate the fungicidal activity of *C. krusei* (Hjertstedt et al. 1998). Methanol was incubated with yeasts at 0.5%/well and was used as an internal control.

Statistical analysis - Data represent the mean ± SD of duplicate samples from two independent assays. The EC_{50} values were calculated using sigmoid dose-response curves in Graph Pad Prism 3.0 software, and the 95% confidence intervals were included in parentheses.

RESULTS AND DISCUSSION

In our search for plants with antileishmanial and antifungal activities, we tested methanolic crude extracts of native plants from the BAF. Our methodology was focused on plants traditionally used in Brazil and other countries for their anti-inflammatory and antimicrobial properties, as well as their specific antileishmanial and antifungal activities. Table I describes the 16 selected plants and their use in folk medicine. Many nonspecific symptoms such as fever, intestinal colic, and skin illness could be frequently described as microbial or parasitic in origin. Thus, many traditionally used plants may have an effect on a nonspecific target, thus contributing only as an anti-inflammatory. Laboratory in vitro testing models using cultured parasites, bacteria, or fungi, could be a useful and very important tool to confirm a specific activity, as well as an ethnopharmacological use.

As show in Fig. 1, five crude extracts (*P. amboinicus*, *A. cymbifera*, *P. barbatus*, *L. alba* and *H. bonariensis*) killed 100% of *L. chagasi* promastigotes at their highest concentration (500 µg/ml). *L. chagasi* is the etiologic agent of Visceral Leishmaniasis in Brazil, a systemic disease that kills 100% of untreated patients (Rath et al. 2003). From these five active extracts, four presented significant EC_{50} values in the concentration range of 45 to 89 µg/ml (Table II), showing very promising possibilities for future fractionation. These data suggest that very active compounds might be present in the crude extracts, and these may be a pool of a dozen independent secondary metabolites. Further isolation of the active compound could result in an individual prototype substance probably with a small EC_{50} value and better parasite selectivity. Based on 95% confidence intervals, *P. barbatus* and *L. alba* presented similar EC_{50} values against parasites, as did *P. amboinicus* and *P. barbatus*.

TABLE I

Plants, their traditional uses and activity described in the literature

Species (Family)	Traditional use and activity described	References
Albizia inundata (Fabaceae - Mimosoideae)	Species of this genus are used in African traditional Medicine to treat microbial infection	Runyoro et al. (2006)
Aristolochia cymbifera (Aristolochiaceae) *Cipó-mil-homens*[a]	Used in medicinal popular to treat fever, itching and eczema	Lorenzi and Matos (2002)
Baccharis trimera (Asteraceae) *carqueja*[a]	Used as anti-inflammatory and analgesic Used against intestinal verminosis and malaria	Gene et al. (1996) Lorenzi and Matos (2002)
Bauhinia forficata (Fabaceae-Cercideae) *pata-de-vaca*[a]	Used in medicinal popular as anti-diabetic, against intestinal parasitosis and anti-inflammatory	Duarte-Almeida et al. (2004), Lorenzi and Matos (2002)
Calamintha adscendens (Lamiaceae)	Species of this genus are used as antimicrobial agent	Panizzi et al. (1993)
Cymbopogon citratus (Poaceae) *capim-limão*[a]	Used in popular medicine to treat diarrhea, fever, cold Antimicrobial properties	Di Stasi et al. (2002) Betoni et al. (2006)
Eleutherine bulbosa (Iridaceae) *marupari*[a]	Used as wound-healing agents on superficial and internal wounds (gastric ulcers) Used against intestinal verminosis and diarrhea	Villegas et al. (1997) Lorenzi and Matos (2002)
Herreria salsaparilha (Agavaceae) *Salsaparilha*[a]	Used to treat inflammations, fever, skin infections and psoriases	De Carvalho (2001), Lorenzi and Matos (2002)
Hydrocotyle bonariensis (Apiaceae) *acariçoba*[a]	Used to remove freckle and spots Species of this genus is used to treat psoriases	Lorenzi and Matos (2002) Natarajan and Paily (1973)
Justicia pectoralis var. *stenophylla* (Acanthaceae) *chambá*[a]	Used in folk medicine for respiratory tract diseases, lung infections and rheumatism	Leal et al. (2000) Chariandy et al. (1999)
Lippia alba (Verbenaceae) *erva-cidreira, salvia*[a]	Used against bacterial respiratory infections Effective to relief intestinal and uterus colic	Caceres et al. (1991) Matos (1996)
Mentha X piperita (Lamiaceae) *hortelã-pimenta*[a]	Used as bactericidal, antifungal, anti-ulcer, anti-inflammatory	McKay and Blumberg (2006)
Plectranthus amboinicus (Lamiaceae) *alfavaca-grossa*[a]	Used in the treatment of cutaneous leishmaniasis due to *Leishmania* species	Franca et al. (1996), Lorenzi and Matos (2002)
Plectranthus barbatus (Lamiaceae) *boldo-brasileiro*[a]	Used in treatment of ailments of infectious and/or inflammatory nature	Matu and Van Staden (2003), Lukhoba et al. (2006)
Plectranthus grandis (Lamiaceae)	Species of this genus are used to treat digestive, skin, infective and respiratory problems Antimicrobial properties in this genus	Wellsow et al. (2006) Lukhoba et al. (2006)
Plectranhus neochilus (Lamiaceae)	Species of this genus are used to treat digestive, skin, infective and respiratory problems Antimicrobial properties in this genus.	Lukhoba et al. (2006) Wellsow et al. (2006)

a: common name in Brazil.

To our knowledge, this data corroborates, for the first time, the ethnopharmacological use of *P. amboinicus* for Leishmaniasis, having an EC_{50} value of 45.14 µg/ml. This species has been used in the treatment of skin ulcerations caused by *Leishmania* spp. among the rural population of the cocoa-producing coastal area in the state of Bahia, Brazil (Franca et al. 1996,) and has also been used to treat inflammatory disorders (Lorenzi & Matos 2002).

A. cymbifera, a member of the Aristolochiaceae family, has been widely used to treat fevers, which are a very common and unspecific symptom (Lorenzi & Matos 2002). In our assays, it has presented a high antileishmanial activity with an EC_{50} of 89.17 µg/ml (Table II), and it has a fungicidal activity with an EC_{50} of 119.7 µg/ml (Table III). To date, only antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* by *A. cymbifera* has been described in the literature (De Barros Machado et al. 2005). Our results expand the scientific knowledge by demonstrating promising activity against human pathogenic fungi and a protozoan parasite. The traditional uses of *P. barbatus* in Kenya for the treatment of infectious and inflammatory diseases (Matu & van Staden 2003) have led our research to the finding of an antileishmanial activity with a very promising EC_{50} value of 54.46 µg/ml against *L. chagasi* (Table II). Despite the cellular similarity among *Leishmania* species, no activity was found against *L. amazonensis*, one of the etiologic agents of Cutaneous Leishmaniasis (CL) in Brazil. CL affects most poor communities all over the world. In some circumstances, the parasites migrate to mucosal areas, dramatically destroying the local tissue and contributing to a progressive and disfiguring inflammatory skin disease.

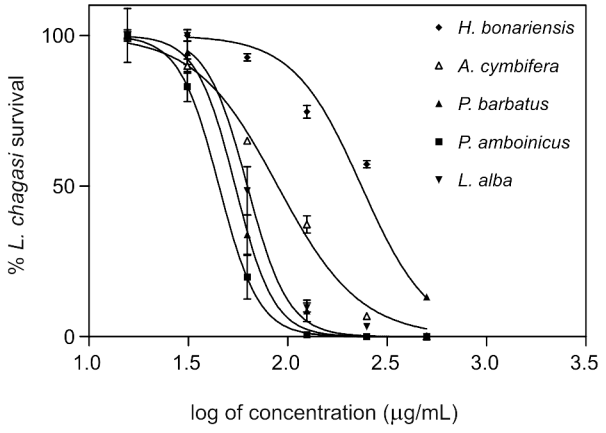

Fig. 1: dose-response curve for the determination of the 50% Effective Concentration (µg/ml) of extracts of five plants against *Leishmania (L.) chagasi* promastigotes. Viability of parasites was determined at 570 nm using MTT.

From the 16 plant extracts studied against Leishmaniasis, five presented activity against *L. chagasi*, and three distinct extracts showed an activity against *L. amazonensis*. *P. grandis* showed a marked antileishmanial activity (EC_{50} of 188.0 µg/ml) (Fig. 2), corroborating the ethnophamacological use of this genus against skin illness, infection, and respiratory problems (Lukhoba et al. 2006). C. *citratus* has been traditionally used to treat diarrhea and fever and it led our research to the discovery of an antiparasitic activity with an EC_{50} of 113.1 µg/ml (Table II). The medicinal use of *B. trimera* against the worldwide protozoan parasitic disease, malaria, as well as intestinal worms (Lorenzi & Matos 2002), resulted in our finding of an antileishmanial activity with an EC_{50} of 374.3 µg/ml. We also determined fungicidal activity against the FCZ-resistant *C. krusei* with an EC_{50} of 133.1 µg/ml (Fig. 3).

The traditional use of *L. alba* to treat bacterial respiratory infections (Caceres et al. 1991), as well as intestinal colic (Matos 1996), guided our research in the finding of new extracts against intracellular protozoan parasites and pathogenic yeasts. Our data confirmed the potent activity of *L. alba,* which was included among the four most active extracts against *L. chagasi* (EC_{50} of 62.67 µg/ml). Our work reports for the first time a fungicidal anti-*Candida* activity with an EC_{50} of 105.9 µg/ml. This data corroborates the anti-*Candida* activity of the

TABLE II

50% Effective Concentration (EC_{50}) of extracts against *Leishmania (L.) chagasi* and *Leishmania (L.) amazonensis* promastigotes. Viability of parasites was determined at 570 nm using MTT. Pentamidine EC_{50} – 0.16 µg/ml (95% C.I. - 0.11-0.23 µg/ml)

Plant extract	EC_{50} (µg/ml) *L. chagasi*	95% C.I. *L. chagasi*	EC_{50} (µg/ml) *L. amazonensis*	95% CI *L. amazonensis*
Plectranthus amboinicus	45.14	44.33 - 45.96	>500	–
Aristolochia cymbifera	89.17	77.51 - 102.6	>500	–
Plectranthus barbatus	54.46	41.50 - 71.47	>500	–
Lippia alba	62.67	55.10 - 71.28	>500	–
Hydrocotyle bonariensis	235.0	148.7 - 371.5	302.5	236.1 - 387.5
Baccharis trimera	>500	–	374.3	364.4 - 384.4
Cymbopogon citratus	>500	–	113.1	81.19 - 157.7
Plectranthus grandis	>500	–	188.0	187.0 - 188.9
Calamintha adscendens	>500	–	>500	–
Bauhinia forficata	>500	–	>500	–
Albizia inundata	>500	–	>500	–
Justicia pectoralis	>500	–	>500	–
Eleutherine bulbosa	>500	–	>500	–
Herreria salsaparilha	>500	–	>500	–
Mentha X *piperita*	>500	–	>500	–
Plectranthus neochilus	>500	–	>500	–

CI: confidence interval; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl-tetrazolium bromide method.

TABLE III

50% Effective Concentration of extracts against *Candida krusei*. Growth inhibition of yeasts was determined at 492 nm

Plant Extract	EC_{50} (µg/ml)	95% CI
Plectranthus amboinicus	>500	–
Aristolochia cymbifera	49.66	40.99-60.16
Plectranthus barbatus	>500	–
Lippia alba	165.20	136.90-199.40
Hydrocotyle bonariensis	137.70	94.16-201.30
Baccharis trimera	187.50	102.80-341.80
Cymbopogon citratus	>500	–
Plectranthus grandis	>500	–
Calamintha adscendens	112.20	98.57-127.80
Bauhinia forficata	>500	–
Albizia inundata	49.66	40.00-60.16
Justicia pectoralis	>500	–
Eleutherine bulbosa	>500	–
Herreria salsaparilha	>500	–
Mentha X *piperita*	>500	–
Plectranthus neochilus	49.84	32.98-75.34

CI: confidence interval.

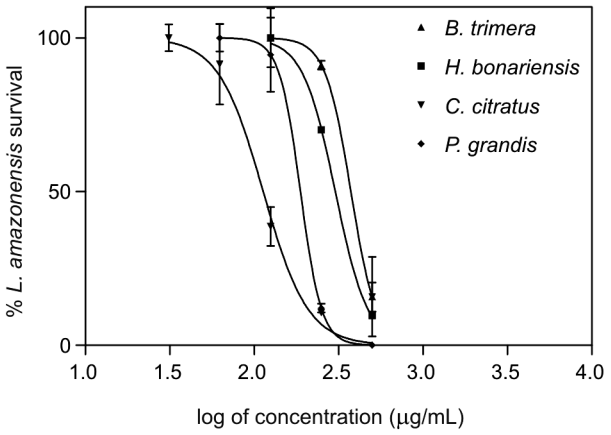

Fig. 2: dose-response curve for the determination of the 50% Effective Concentration (µg/ml) of extracts of four plants against *Leishmania (L.) amazonensis* promastigotes. Viability of parasites was determined at 570 nm using MTT.

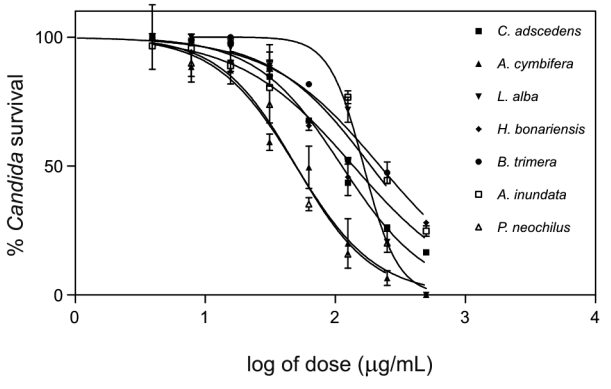

Fig. 3: activity of extracts of seven plant against *Candida krusei* at 492 nm. 50% Effective Concentration (µg/ml) curves were determined using sigmoid dose-response at Graph Pad Prism 3.0 software.

essential oil obtained from leaves of *L. alba* (Duarte et al. 2005). In the search for plants used to treat skin illness, we studied the methanolic extract of *H. bonariensis*, which has been used in folk medicine to treat psoriasis, an inflammatory skin disorder (Natarajan & Paily 1973). We found activity against *L. amazonensis* (EC_{50} of 302.5 µg/ml), as well as *L. chagasi* promastigotes (EC_{50} of 235.0 µg/ml) in which 100% of parasites were killed at the highest concentration (500 µg/ml) (Fig. 1). Despite the elevated EC_{50} values when compared to other active plant extracts, *H. bonariensis* also presented a marked antifungal activity (EC_{50} of 31.63 µg/ml) (Table III) against the FCZ-resistant strain, *Candida krusei*. *H. bonariensis* has a fungicidal, rather than fungistatic activity, as demonstrated by the lack the oxidative mitochondrial activity assayed with MTT (data not shown). Candidiasis are fungal infections that commonly affect the human skin. Recently, due to HIV and other immunocompromised patients, candidiasis have been largely reported as a challenging invasive systemic infection. Many plants have been used in folk medicine to treat fungal infections (Lorenzi & Matos 2002). Besides the use of *H. bonariensis* in traditional medicine against skin illness, which could be frequently caused by pathogenic fungi, our data extend this knowledge to novel antiparasitic and antifungal activities.

Some other extracts showed a marked inhibition of the FCZ-resistant yeast, *C. krusei*. No antifungal activity of any tested extracts was found against the azole-susceptible *C. parapsilosis*. Besides the investigation of the growth inhibition at 492 nm (Clancy & Nguyen 1997), we extended our assays in order to demonstrate the potential killing effect of these plants, as most of the current drugs used in clinical therapy are fungistatic, rather than fungicidal, compounds. This characteristic limits the use of such agents by HIV-patients, because its prophylactic use should be limited to selected high-risk patients to limit the risk of the emergence of azole-resistant strains (Charlier et al. 2006).

Among the most effective tested extracts, *P. neochilus* demonstrated, for the first time in the literature, a marked a marked EC_{50} value of 20.51 µg/ml, killing 100% of yeast (*C. krusei*) at the highest concentration (Fig. 3). *P. neochilus* have also been traditionally used to treat skin and infective problems (Wellsow et al. 2006). Species of genus *Albizia* are used in African traditional medicine to treat microbial infection (Runyoro et al. 2006). In addition, species of *Calamintha* are traditionally used as antimicrobial agents in popular medicine (Panizzi et al. 1993). Despite the lack of antileishmanial activity detected for *A. inundata* and *C. adscendens*, our results show an antifungal activity against *Candida albicans* and a fungicidal activity with an EC_{50} value of 148.5 µg/ml at 492 nm and 196.3 µg/ml by MTT- 570 nm (data not shown) for *A. Inundata*. The anti-*Candida* activity of *A. inundata* has been recently described and our results corroborate those found in literature for the genus *Albizia* (Runyoro et al. 2006).

Despite the broad traditional use of *J. pectoralis* var. *s. stenophylla* (Chariandy et al. 1999, Leal et al. 2000), *H. salsaparilha* (Rodrigues & De Carvalho 2001), *Men-*

tha X piperita (McKay & Blumberg 2006), and *E. bulbosa* (Villegas et al. 1997) in closely related diseases, such as skin illnesses, our assays demonstrated neither antileishmanial nor anti-Candida activities. Other pathogenic species from the same genus could be susceptible to these same extracts. Continuous investigations must be done in order to find novel potent molecules against these important human diseases. Furthermore, Leishmaniasis is a tropical neglected disease and investments for the search of novel drugs are urgent. The use of ethnopharmacological information could significantly slow the time and cost of the research, allowing the study of new drug prototypes for drug design studies.

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