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Investigation of plant extracts in traditional medicine of the Brazilian Cerrado against protozoans and yeasts

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ABSTRACT

Aim of the study: To investigate the activities of the 217 plant extracts in traditional medicine of the Brazilian Cerrado against protozoans and yeasts.
Materials and methods: Plant extracts were prepared by the method of maceration using solvents of different polarities. The growth inhibition of chloroquine-resistant *Plasmodium falciparum* strain (FcB1) was determined by measuring the radioactivity of the tritiated hypoxanthine incorporated. Activity against *Leishmania* (*Leishmania*) *chagasi* and *Trypanosoma cruzi* was measured by the MTT colorimetric assay. The antifungal tests were carried out by using the CLSI method. The active extracts were tested also by cytotoxicity assay using NIH-3T3 cells of mammalian fibroblasts.
Results: Two hundred and seventeen extracts of plants were tested against *Plasmodium falciparum*. The eleven active extracts, belonging to eight plant species were evaluated against L. (L.) *chagasi*, *Trypanosoma cruzi*, yeasts and in NIH-3T3 cells. The results found in these biological models are consistent with the ethnopharmacological data of these plants. The ethyl acetate extract of *Diospyros hispida* root showed IC_{50} values of 1 μg/mL against *Plasmodium falciparum*. This extract demonstrated no toxicity against mammalian cells, resulting in a significant selectivity index (SI) of 435.8. The dichloromethane extract of *Calophyllum brasiliense* root wood was active against *Cryptococcus gattii* LMGO 01 with MIC of 1.95 μg/mL; and *Candida albicans* ATCC 10231 and *Candida krusei* LMGO 174, both with MIC of 7.81 μg/mL. The same extract was also active against *Plasmodium falciparum* and L. (L.) *chagasi* with IC_{50} of 6.7 and 27.6 μg/mL respectively. The ethyl acetate extract of *Spiranthera odoratissima* leaves was active against *Cryptococcus gattii* LMGO 01 with MIC of 31.25 μg/mL, and against *Plasmodium falciparum* with IC_{50} of 9.2 μg/mL and *Trypanosoma cruzi* with IC_{50} of 56.3 μg/mL.
Conclusion: The active extracts for protozoans and human pathogenic yeasts are considered promising to continue the search for the identification and development of leading compounds.

1. Introduction

Parasitic diseases kill thousands of people every year, especially in places where access to hospitals and medication is precarious. According to WHO, in 2006 there were 247 million cases of malaria worldwide, and 1 million deaths, mostly children under 5 years old. In 2008, malaria was considered endemic in 109 countries (World Malaria Report, 2008), being potentially transmitted in Brazil in 60% of its territory, mainly in areas where the socioeconomic and environmental conditions favor the spread mosquito vector – *Anopheles* sp. (de Mesquita et al., 2007).

Chagas disease – American trypanosomiasis – affects 16–18 million people in Central and South America, and is responsible for 14,000 deaths per year (Senior, 2007). In Brazil, the cases reached the 4 million people infected (Dias and Vinhaes, 2000). Leishmaniasis occurs predominantly in tropical and subtropical regions, and is considered endemic in 88 countries, where it is estimated that there are 12 million people infected and 200 million are in high-risk areas of contamination (WHO, 2009).

Another alarming problem is the number of fungal infections or mycosis in general, which has been increasing dramatically, particularly among immunocompromised patients (Traboulsi et al., 2008), being the yeasts of the genus *Candida* and *Cryptococcus* often

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isolated in these patients (Davis et al., 2007). The ARTEMIS DISK Surveillance Program – 1997–2003 – in several identified 140.767 species of fungi. Of these, 134.715 were from the genus *Candida*, therefore, corresponding, to 95.7% of clinical isolates (Pfaller et al., 2005). Patients affected by these diseases are exposed to different risk factors, such as infectious disease of the bloodstream, known as candidemia or hematogenous candidiasis. *Cryptococcus neoformans* is a ubiquous and opportunistic yeast causing meningoencephalitis in immunocompromised patients. In AIDS patients, according the Ministry of Health, 60% of 215810 patients presented cryptococcosis from 1980 to 2002 (Ministério da Saúde, 2002).

The increasing resistance of parasites and fungi to available drugs and resistance of vector insects to insecticides justify the search for new leading compounds (Dondorp et al., 2009). The resistance of strains of *Leishmania* to antimonials drugs led to the formulation of liposomal amphotericin B (Sundar et al., 2010), but with limits on use and cost, leading to the urgent need for new therapeutic options. Drug resistance was also observed in strains of *Plasmodium falciparum* and *Candida* (Dondorp et al., 2010).

Species found in the Cerrado biome have been used by traditional local communities to treat various diseases, and the potential to find out promising molecules to several application has already been demonstrated in diverse biological models (Espindola et al., 2004; de Mesquita et al., 2005a, 2005b; Rodrigues et al., 2005; de Mesquita et al., 2007; de Mesquita et al., 2009; Melo e Silva et al., 2009). This study investigated the activities of the plants extract in traditional medicine of the Brazilian Cerrado against *Plasmodium falciparum*. The selected active extracts were analyzed against *Leishmania* (*Leishmania*) *chagasi*, *Trypanosoma cruzi*, species of fungi of the genus *Candida* e *Cryptococcus*, and the toxicity determined in NIH-3T3 cells of mammalian fibroblasts.

This approach was used to optimize the screening process taking advantage of the former results with the *Plasmodium* model, applying the active extracts to other protozoa and fungi. It is widely recognized that some antifungal agents such as amphotericin B and itraconazole have effect against *Leishmania* species (Sundar et al., 2010). *Leishmania* (L.) *chagasi* was chosen mainly because the most lethal form of leishmaniasis is the visceral disease for which that parasite is responsible in the Americas (Romero and Boelaert, 2010).

2. Materials and methods

2.1. Plant extracts

Plant species were collected in 2006 and 2007, in the Cerrado biome, Federal District of Brazil and identified by botanist Prof. José Elias de Paula. The voucher numbers were kept in the Herbarium of the University of Brasilia (UB/UnB). The plant organs were separated, dried, stabilized, and sprayed through the process of extraction by maceration with solvents of different polarities. The extractive solution was concentrated in rotaevaporator, providing different crude extracts, which were stored at −20 °C.

2.2. In vitro antiplasmodial activity

Extracts were tested against the chloroquine-resistant *Plasmodium falciparum* FcB1/Colombia strain. *Plasmodium falciparum* was maintained continuously *in vitro* in human erythrocytes according to Trager and Jensen (1976). The antiplasmodial activity was determined according to Desjardins et al. (1979). The extracts were dissolved in dimethylsulfoxide (DMSO) and tested at a concentration of 10 μg/mL. The extracts showing significant inhibition rates were submitted to serial dilutions with culture medium before being added to asynchronous parasite cultures (1% parasitemia and 1% final hematocrit) in 96-well microplates for 24 h at 37 °C. [3H] hypoxanthine at a concentration of 0.5 μCi was then added to each well, and parasites were maintained for an additional 24 h. The growth inhibition for each extract concentration was determined by comparing the radioactivity incorporated in the treated culture with that in the control culture maintained on the same plate. The concentrations causing 50% inhibition of parasite growth (IC_{50}) were calculated from the drug concentration–response curves. Assays were performed in triplicate.

2.3. In vitro antileishmanial activity

The extracts were tested against promastigotes of *Leishmania* (*Leishmania*) *chagasi* (MCER/BR/79/M6445) maintained in culture medium containing NNN, Schneider (Sigma®) and 10% heat-inactivated fetal calf serum, at 22 °C. The antileishmanial activity was determined according to Tempone et al. (2001) with minor modifications. The extracts were dissolved in DMSO and diluted in Schneider's medium (Sigma®). The final concentration of DMSO did not exceed 1% and for each experiment, there was a growth control with and without DMSO. The experiments were performed in 96-well plates with extracts at concentrations of 100 μg/mL (de Mesquita et al., 2005a). The inoculum consisted of 10^6 parasites per well in logarithmic phase. The cells were incubated at 37 °C for 48 h. Miltefosine was used as the reference drug. Negative controls were performed with 10^6 parasites in Schneider's and culture medium alone. The viability of the promastigotes was based on the cellular conversion of the soluble tetrazolium salt MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide – Sigma®) into the insoluble formazan by mitochondrial enzymes. MTT (10 mg/mL) was dissolved in PBS, 20 μL/well, for 4 h at 37 °C. Formazan was dissolved using 10% SDS (100 μL/well). The number of living promastigotes was determined spectrophotometrically at 570 nm. Assays were performed in triplicate.

2.4. In vitro antitrypanosomal activity

The antitrypanosomal activity was determined according to Tempone et al. (2005) with minor modifications. The extracts were dissolved in DMSO and diluted in LIT medium (Sigma®). The final concentration of DMSO did not exceed 1% and for each experiment, there was a growth control with and without DMSO. The experiments were performed in 96-well plates with extracts at concentrations of 100 μg/mL. The inoculum consisted of 10^6 parasites per well in stationary phase. The cells were incubated at 28 °C for 72 h. Benznidazole (*N*-benzyl-2-nitro-1-imidazolacetamide; Roche®) was used as the reference drug. Negative controls were performed with 10^6 parasites in culture medium alone. The viability of the epimastigotes was based on the cellular conversion of MTT into the insoluble formazan by mitochondrial enzymes. The number of living epimastigotes was determined spectrophotometrically at 570 nm. Assays were performed in triplicate.

2.5. Antifungal activity method

The minimal inhibitory concentration (MIC) of each extract was determined for *Cryptococcus neoformans* LMGO 02, *Cryptococcus gattii* LMGO 01, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida krusei* LMGO 174, and *Candida glabrata* LMGO 44 by using broth microdilution techniques as described by the Clinical and Laboratory Standards Institute. LMGO (Laboratório de Micologia de Goiás) strains are clinic isolates from patients at the Federal University of Goiás. The MIC values were determined