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In vitro biological screening and evaluation of free radical scavenging activities of medicinal plants from the Brazilian Cerrado

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A total of 23 extracts derived from 14 medicinal plant species from Brazilian savanna ("Cerrado"), selected by ethnopharmacological information, was screened for leishmanicidal, antibacterial, antifungal and radical scavenging activities and toxicity to brine shrimp *(Artemia salina*) larvae. Crude extracts from 9 of these species showed potent activity in one or more of these assays.

Key words: Antileishmanial, antibacterial activity, antifungal activity, *Artemia salina*, diphenylpicrylhydrazyl (DPPH), Cerrado.

INTRODUCTION

Since ancient times, medicinal plants have contributed significantly to primary healthcare, and currently, about 25 to 30% of all drugs available as therapeutic agents are derived from natural products (plants, microbes and animals) (Newman and Cragg, 2006). Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in developing countries due to the relative unavailability of medicines and the emergence of widespread drug resistance (Cos et al., 2006).

Brazil shows an extensive territorial area, comprising five different biomes with great floristic and cultural diversities, which are a rich source of potentially bioactive compounds. One of these biomes, the Brazilian savanna, known as "Cerrado", comprises a very rich and characteristic flora that covers more than 2 million square kilometers of Brazilian inland (Burman, 1991). The aim of the present study was to screen for Cerrado medicinal plant extracts based on ethnopharmacological knowledge for their potential value for future development of new plant-derived drugs, by means of primary assays. In this paper, we present the results obtained from 14 Cerrado medicinal species belonging to 12 different families, which were selected for evaluation of their leishmanicidal, antibacterial, antifungal and free radical scavenging properties, as well as their toxicities against brine shrimp (*Artemia salina*) larvae.

MATERIALS AND METHODS

Plant

Plants (Table 1) were collected in Bonito, Mato Grosso do Sul,

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Table 1. Medicinal plants collected in the Cerrado of Mato Grosso do Sul, Brazil used in the essays.

Herbarium code	Plant species	Family
WG 264	*Alibertia edulis*	Rubiaceae
WG 265	*Anemopaegma arvense*	Bignoniaceae
WG 266	*Bowdichia virgilioides*	Leguminosae/Faboideae
WG 267	*Celtis pubescens*	Ulmaceae
WG 268	*Centratherum punctatum*	Asteraceae
WG 269	*Clavija nutans*	Teophrastaceae
WG 270	*Hymenaea stigonocarpa*	Leguminosae/Ceasalpinoideae
WG 271	*Luehea paniculata*	Tiliaceae
WG 272	*Macrosiphonia petraea*	Apocynaceae
WG 273	*Mamordica charantia*	Cucurbitaceae
WG 274	*Maprounea guianensis*	Euphorbiaceae
WG 275	*Melancium campestre*	Cucurbitaceae
WG 276	*Vernonia ferruginea*	Asteraceae
WG 277	*Palicourea rigida*	Rubiaceae

Brazil, between December and January, 2007 and were identified by Dr. Arnildo Pott, Vali Joana Pott and Ubirazilda Maria Rezende (Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil). Voucher specimens were deposited at the CGMS Herbarium of the Universidade Federal de Mato Grosso do Sul (UFMS).

Preparation of plant extracts

Air dried and powdered plant materials, approximately from 10 to 300 g, were extracted with ethanol 96° for 5 days at room temperature. After evaporation of the solvent under reduced pressure at 45°C, the respective ethanol extracts were obtained. All the extracts were kept in tightly stoppered bottles and stored under refrigeration until biological screenings were performed.

Antibacterial assay

Bacterial strains *Staphylococcus aureus*-ATCC 25923 and *Pseudomonas aeruginosa*-ATCC 27853 were used as test organisms. Stock solutions of all compounds were prepared by dissolving 20 mg of the compounds in 1000 µl of dimethyl sulfoxide (DMSO). The minimal inhibitory concentration of each extract was determined by using broth microdilution techniques as described by the Clinical and Laboratory Standards Institute for bacteria (M7-A6) (CLSI/NCCLS, 2003a) in flat-bottomed 96-well plastic tissue-cultured plates. The minimum inhibitory concentration (MIC) values were determined in Mueller-Hinton (Sigma) buffered to a pH 7.0 with 3-(N-morpholino)propanesulfonic acid (MOPS, Sigma). The microorganisms were cultured overnight at 30°C in Mueller-Hinton agar. For the assay, extracts stock solutions were two-fold diluted with Mueller-Hinton and serially diluted to concentrations between 1000, 500, 250 and 125 µg/ml and final DMSO concentration ≤1%. Then, 100 µl of these solutions were added onto microplates. Lastly, 5×10^4 colony forming units (CFU/ml) (according to McFarland turbidity standards) of standardized bacteria suspensions were inoculated onto microplates and the test was performed in a volume of 200 µl. Plates were incubated at 35°C for 16 to 20 h. The same tests were performed simultaneously for growth control (Mueller-Hinton + bacteria) and sterility control (Mueller-Hinton + extract). Chloramphenicol (1000 µg/ml) was used as reference compound with concentrations ranging from 32 to 0.25 µg/ml. All experiments were performed in duplicate and three replicate plates were used to determine the antibacterial activity. MIC value was determined as the highest dilution showing complete inhibition of tested strain. Extracts with MICs ≤ 1.000 µg/ml were considered active.

Antifungal assay

Fungal strains *Candida albicans*–ATCC 90028, *Candida krusei*–ATCC 6258 and *Cryptococcus neoformans* ATCC 32264 were used as test organisms. The MIC of each extract was determined by using microdilution technique as described by the Clinical and Laboratory Standards Institute for yeast (M27-A2) (CLSI/NCCLS, 2003b) in microtiters of 96 wells. The MIC were determined in RPMI 1640 (Sigma) buffered to a pH 7.0 with MOPS. The microorganisms were cultured for 48 h at 30°C in Sabouraud Dextrose Broth (SBD, Oxoid). The starting inocula were approximately 5.0×10^2 and 2.5×10^3 CFU/ml (according to McFarland turbidity standards). Microtiters trays were incubated at 35°C and MICs were recorded at 48 (*Candida* species) and 72 h, for *C. neoformans*. For the assay, extracts stock solutions were two-fold diluted with Roswell Park Memorial Institute (RPMI) and serially diluted to concentrations between 1000, 500, 250 and 125 µg/ml and a final DMSO concentration ≤ 1%. Then, 100 µl of these solutions were added onto microplates. A volume of 100 µl of inoculum suspension was added to each well. The same tests were performed simultaneously for growth control (RPMI 1640 + fungus) and sterility control (RPMI 1640 + extract). Amphotericin B (3600 µg/ml) was used as reference compound with concentrations ranging from 16 to 0.125 µg/ml. MIC was defined as the MIC of the extract which resulted in total inhibition of the fungal growth. All experiments were performed in duplicate and three replicate plates were used to determine the antifungal activity. Extracts with MICs ≤ 1.000 µg/ml were considered active.

Leishmanicidal assay

Antileishmanial activity was evaluated *in vitro* on a culture of

Leishmania amazonensis (MHOM/BR/77/LTB0016) promastigotes. This microorganism was maintained at 26°C in Schneider's insect medium (Sigma-Aldrich, St. Louis, USA), pH 6.9, supplemented with 10% heat-inactivated fetal calf serum and 100 mg/ml streptomycin and 100 U/ml penicillin. Parasites were maintained in culture until the tenth passage. Subsequently, new cultures were obtained from infected animals. The effect of the crude extract on the viability of *L. amazonensis* extracellular forms was determined by Thiazolyl Blue Tetrazolium Bromide (MTT) assay (Sigma-Aldrich, St. Louis, USA). Cells at exponential phase were counted in Neubauer chambers and adjusted to the concentration 1×10^6 promastigotes/ml and transferred to 96-well plates. The cells were incubated for 72 h in the presence of various concentrations in triplicate and maintained at 27°C. After incubation, 22 microliters of MTT solution (5 mg/ml) were added per well, and samples were incubated for 2 h longer. Then, 80 µl DMSO were added and the optical density was read at 570 nm on a µQuant reader (Bio-Tek Instruments, Winooski, USA). The sample extracts were dissolved in DMSO and added to each well up to 1% (v/v), after verifying that DMSO had no effect on parasite growth. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}). The starting concentration for screening was 50 µg/ml and pentamidine was the reference compound. Extracts with $IC_{50} \leq 50$ µg/ml were considered active (Torres-Santos et al., 2004).

Brine shrimp lethality (BSL) assay

A. salina L. encysted eggs (10 mg) were incubated in 100 ml of artificial seawater under artificial light at 28°C, pH 7 to 8. After incubation for 48 h, the larvae were extracted and counted using a Pasteur pipette. The extracts (triplicate) were dissolved in DMSO (20 mg/200 µl) and diluted serially to 1000, 500, 250 and 125 µg/ml in saline medium. Controls containing 50 µl of DMSO in artificial seawater were included in each experiment. Quinidine dissolved in DMSO was used as a positive control. Each vial containing 10 larvae of brine shrimp was filled to 5 ml total volume with saline medium. In each case, three replicates of each concentration were assayed. After 24 h, live larvae were counted and the LC_{50} values in µg/ml were calculated using Probit analysis (Meyer et al., 1982).

Radical scavenging activity by diphenylpicrylhydrazyl (DPPH)

Antioxidant potential of the extracts was determined by DPPH radical scavenging method. A 100 µl ethanolic solution of DPPH (0.04%) was mixed with 100 µl of a sample solution (200 µg/ml) in ethanol and after 30 min, the absorbance of the mixture was measured at 517 nm. Antioxidant activity of butylated hydroxytoluene (BHT) was employed as a positive control. Triplicate measurements were made and the antioxidant activity was calculated by the following equation (Dickson et al., 2007):

$$DPPH^{\cdot} \text{ scavenging activity } (\%) = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of the test extracts. A graph of percent inhibition against concentration was plotted and IC_{50} was determined using Excel programme. DPPH solution alone served as a negative control (A_0).

RESULTS AND DISCUSSION

A total of 23 extracts obtained from 14 different medicinal plant species collected in the Cerrado region of Mato Grosso do Sul, Brazil (Table 1) were evaluated for their leishmanicidal, antibacterial, antifungal and free radical scavenging activities, as well as brine shrimp toxicity and the results are depicted in Table 2.

In the assays for evaluation of antibacterial and antifungal activities, crude extracts showing MIC values below 1000 µg/ml were considered active (Rios et al., 1988). In the present work, four species (28.6%) inhibited the growth of *S. aureus* to some extent, showing MICs < 1000 µg/ml (*Bowdichia virgilioides*, *Hymenaea stigonocarpa*, *Luehea paniculata* and *Maprounea guianensis*), where extracts of both leaves and bark of *L. paniculata* and *M. guianensis* were found to be active. On the other hand, none of the extracts assayed displayed any significant activity against *P. aeruginosa*, and this resistance may be due to structural differences in the cell membranes of Gram-negative and Gram-positive bacteria (Koneman et al., 2001).

The screening for antifungal activity revealed that 9 species (64.2%) were active against at least 1 fungal strain. From these, the best antifungal potential was shown by *M. guianensis*, since both leaves and bark of this species were significantly active against all the fungal strains tested, with MIC values ranging from 15.6 to 125 µg/ml. *M. guianensis* is known by "capitão" and plants that have this popular name are commonly used as astringent and for treatment of cough, thrush, tumors, and colds (Pott and Pott, 1994). The genus *Maprounea* has attracted interest due to the potent inhibitory activity effect against human immunodeficiency virus (HIV)-1 reverse transcriptase exhibited by several triterpenoids and also to the antihyperglycemic activity shown by triterpenoids and daphnane diterpenoids isolated from the stems of *Maprounea africana* (Beutler et al., 1995; David et al., 2004). Only a single work describes the chemical study of *M. guianensis*, reporting the isolation of triterpenes and alkyl ferulates from stems (David et al., 2004).

Also worthy of mention is the strongest activity against *C. neoformans* exhibited by the leaves of *B. virgilioides* (MIC = 31.25 µg/ml). This species, popularly known as "sucupira-do-cerrado" or "sucupira-preta", is known for the presence of alkaloids (Torrenegra et al., 1989), flavonoids (Velozo et al., 1999), benzofuranoids and triterpenoids (Melo et al., 2001) and its bark is traditionally used for healing wounds and as an anti-ulcer and anti-diabetic agent (Arriaga et al., 2000), while the seeds are used for the treatment of rheumatism, arthritis, and skin diseases (Barbosa-Filho et al., 2004).

Four extracts (17.4%) among the 23 plant extracts screened in the present study were active *in vitro* against promastigotes stages of *L. amazonensis*, with IC_{50} values ≤ 25 µg/ml, namely *B. virgilioides*, *Centratherum punctatum*, *Momordica charantia* and *Vernonia ferruginea*. The strongest leishmanicidal activity was shown by the aerial parts of *M. charantia* (IC_{50} values 6.25 µg/ml). Recently, a new compound, momordicatine, isolated from the green

Table 2. Activity of plant extracts of medicinal plants collected in the Cerrado of Mato Grosso do Sul, Brazil against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans, Candida krusei, Cryptococcus neoformans, Leishmania amazonensis, Artemia salina* and antioxidant potential of the extracts.

Plant species	Part[a]	Assays							
		Bacteria MIC (µg/ml)		Fungi MIC (µg/ml)			Lsh IC_{50} (µg/ml)	BSL[h] IC_{50} (µg/ml)	DPPH IC_{50} (µg/ml)
		Sa[b]	Pa[c]	Ca[d]	Ck[e]	Crp[f]	L.a.[g]	-	-
Alibertia edulis	br	I	1000	I	1000	I	I	I	I
	l	I	1000	500	1000	I	I	I	I
Anemopaegma arvense	ap	I	1000	I	I	250	I	I	I
	r	I	I	1000	I	I	I	I	I
Bowdichia virgilioides	l	250	1000	500	500	31.25	I	I	I
	sd	1000	1000	I	I	1000	20	3.53	I
	st	I	1000	1000	1000	1000	I	610.1	I
Celtis pubescens	l	I	I	I	I	I	I	I	I
Centratherum punctatum	ap	1000	I	I	1000	I	25	352.9	I
Clavija nutans	l	I	I	500	1000	I	I	129.8	I
Hymenaea stigonocarpa	bk	250	1000	1000	250	250	I	I	10.11
	l	1000	1000	1000	250	1000	I	885.4	I
Luehea paniculata	bk	250	1000	I	125	1000	I	I	10.09
	l	500	1000	I	125	1000	I	I	12.88
Macrosiphonia petraea	ap	I	I	500	250	250	I	I	I
	r	I	I	500	I	1000	I	16.62	I
Mamordica charantia	ap	1000	1000	1000	1000	1000	6,25	114.6	I
Maprounea guianensis	bk	500	1000	125	15.6	62.5	I	I	9.62
	l	250	1000	125	15.6	62.5	I	I	7.54
Melancium campestre	ap	1000	I	I	1000	1000	50	I	I
Vernonia ferruginea	br	I	I	1000	500	500	45	I	I
	l	I	I	I	1000	1000	18	I	I
Palicourea rigida	l	1000	1000	I	1000	I	I	I	16.85
Controls[i]		0.5	8,0	1.0	0.25	0.25	1,6	33.80	104.9

a: ap – aerial part; bk – bark; br – branch; l- leaves; r – roots; sd – seed; st – stem. b: *Staphylococcus aureus;* c:*Pseudomonas aeruginosa;* d:*Candida albicans*; e:*Candida krusei*; f:*Cryptococcus neoformans*; g: *Leishmania amazonensis*; h: brine shrimp lethality assay; i: positive control for bacteria was chloramphenicol; positive control for fungi was amphotericin B; positive control for Lsh (*Leishmania*) was pentamidine; positive control for BSL was quinidine sulfate and for DPPH was BHT.

fruits of *M. charantia* showed leishmanicidal activity (Gupta et al., 2010). However, in this work, the fruits of this plant were not assayed.

In the BSL assay for evaluation of the toxicity of plant extracts, those presenting LC_{50} values lower than 1000 µg/ml were considered active (Meyer et al., 1982). Seven of the 23 extracts assayed in this work (30.4%) were active in the BSL assay, 6 of them being also active in other assays. The extracts from the seeds of *B. virgilioides* and from the roots of *Macrosiphonia petraea* were the most active, with LC_{50} values of 3.53 and 16.62 µg/ml, respectively. The seeds of *B. virgilioides* have not

been previously chemically analyzed, but its essential oil showed antibacterial activity (Rodrigues et al., 2009). Native people from Brazil use the crude oil to treat rheumatism (Pott and Pott, 1994) and the roasted seeds are used as depurative and to treat fever. Similarly, *M. petraea*, known as “velame” and “velame-branco”, is traditionally used for the treatment of colds, fevers, syphilis, peptic ulcer and rheumatism (Rodrigues and Carvalho, 2001) and also widely marketed by healers, but there are no studies on its chemical composition or its biological properties.

Six species among the 14 analyzed (42.8%) showed the strongest antioxidant activities by the DPPH method (IC_{50} values ranging from 7.54 to 16.85 µg/ml) and extracts from the leaves and barks were found to be the most active ones. Antioxidant properties are mainly associated with the presence of phenolic compounds.

H. stigonocarpa with edible fruits is a typical species of Cerrado and its astringent bark is used to treat bronchitis and inflammation (Rodrigues and Carvalho, 2001). In a previous phytochemical screening, Santana et al. (2010) identified flavonoids, steroids, triterpenoids and tannins in the heartwood of this species. The extract of its bark showed moderate activity against *S aureus*, *C. krusei* and *C. neoformans*, and a significant radical scavenging property. These activities, however, are probably associated with the presence of tannins.

The seeds of *B. virgilioides* stand out by their significant leishmanicidal and cytotoxic activities. On the other hand, the leaves of this plant, for which there are no reports of use, showed antibacterial and antifungal activities, while its stems were toxic to *A. salina* larvae.

It is also worthy of mention, the great number of active extracts (30.4%) in the BST test and also the leishmanicidal activity of 6 extracts. Although, *M. guianensis* was shown to be active in most of the assays, some of these activities, namely the highest antioxidant potential displayed by the extracts of leaves and bark (IC_{50} values of 7.54 and 9.62, respectively), might be due to the presence of tannins. In spite of the traditional medicinal use of the plants investigated in the present work, studies on their biological properties and/or chemical composition have not been previously reported for majority of them. In this research, several plant species showed potent antifungal, antileishmanial, cytotoxic and/or antioxidant activities.

In conclusion, the evaluation of 23 extracts from plants of the Cerrado used by the local traditional medicine allowed the selection of several active extracts with different biological properties. Overall, extracts from 85.7% of the species collected were active in at least one of the bioassays adopted in this screening. If we consider only the most active extracts (bacteria ≤ 50 µg/ml; fungi ≤ 50 µg/ml; Lsh ≤ 20 µg/ml; BSL ≤ 50 µg/ml and DPPH ≤ 20 µg/ml), this percentage was found to be of 57.1%, which can be considered a very good hit rate, reinforcing the importance of the ethnomedical information in the search of bioactive extracts.

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