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Leishmanicidal activity of the crude extract, fractions and major piperidine alkaloids from the flowers of *Senna spectabilis*

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ABSTRACT

Senna spectabilis (sin. *Cassia excelsa*, *C. spectabilis*) is an endemic tree of South America and Africa, very common in Brazil, where it is known as "canafistula-de-besouro" and "cassia-do-nordeste". In folk medicine, this plant is indicated for the treatment of constipation, insomnia, anxiety, epilepsy, malaria, dysentery and headache. Phytopharmacological studies have also confirmed anticonvulsive, sedative, anti-malarial, antimicrobial and cytotoxic properties of many parts of *S. spectabilis*. In this communication, we present a comparative study of the leishmanicidal activity of the crude ethanolic extract, its fractions and also the two major alkaloidal metabolites (−)-cassine/(−)-spectaline, trying to establish a relationship between the presence of piperidine alkaloidal constituents and leishmanicidal activity. The growth inhibitory effect of promastigote forms of *Leishmania major* was determined for the crude extract, fractions of the flowers of *S. spectabilis* and a mixture of (−)-cassine/(−)-spectaline in comparison to pentamidine used as standard drug. The cytotoxic effects were assessed on macrophage strain J774 by lactate dehydrogenase assay. Fractions dichloromethane (FL-DCM) and *n*-butanol (FL-Bu) and a mixture of (−)-cassine/(−)-spectaline (~7:3) exhibited significant activity against the parasite *Leishmania major* (IC_{50} values of 0.6 ± 0.1 μg/ml, 1.6 ± 0.9 μg/ml and 24.9 ± 1.4 μg/ml, respectively), without toxic effects on murine macrophages. Due to the promising results elicited, further studies *in vivo* need to be performed to confirm the therapeutic potential of *Senna spectabilis*.

Introduction

Senna is one of the biggest genus of Fabaceae family, with several species used in Traditional Medicine practices. *Senna* species have a diverse chemical profile, accumulating secondary metabolites of many chemical classes, including flavonoids, anthraquinones and estilbenoids. Some of these metabolites are reported for their pharmacological and biological properties as antimicrobial, laxative, anti-ulcerogenic, cytotoxic, antifungal, analgesic, anti-inflammatory, antioxidant and hepatoprotective (Samy et al. 1998). *S. spectabilis* (sin. *Cassia excelsa, Cassia spectabilis*) is an endemic tree of South America and Africa, very common in Brazil, Colombia, and subtropical and tropical African countries such as Angola, Burundi, Cameroon, Kenya, and South Africa (Bum et al. 2010; Lorenzi 1998; Nsonde-Ntandou et al. 2005; Pivatto et al. 2005).

In Brazil, this plant is known as "canafistula-de-besouro" and "cassia-do-nordeste" (Lorenzi 1998; Pivatto et al. 2005; Silva et al. 2010), where is used for ornamental purposes and the treatment

Abbreviations: CNS, central nervous system; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration; CEFL, crude fraction from the flowers of *Senna spectabilis*; FL-Hex, hexane fraction from the flowers of *Senna spectabilis*; FL-DCM, dichloromethane fraction from the flowers of *Senna spectabilis*; FL-Ha, hydroalcoholic fraction from the flowers of *Senna spectabilis*; FL-Bu, *n*-butanolic fraction from the flowers of *Senna spectabilis*; LDH, lactate dehydrogenase; IC_{50}, maximal inhibitory concentration; FBS, fetal Bovine Serum; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; TLC, thin layer chromatography TLC; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

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of constipation, insomnia, anxiety, epilepsy, malaria, dysentery and headache (Bum et al. 2010; Nsonde-Ntandou et al. 2005). Phytopharmacological studies have also confirmed anticonvulsive, sedative, antimalarial, antimicrobial and cytotoxic properties of many parts of *S. spectabilis* (Agarkar and Jadge 1999; Akah et al. 1998; Ayo et al. 2007; Bhakta et al. 1999; Ibrahim and Osman 1995; Jafri et al. 1997; Jain et al. 1997; Mascolo et al. 1998; Samy and Ignacimuthu 2000; Tona et al. 1999; Viegas et al. 2006). Góngora et al. (1996) described toxic effects of total alkaloids present in the bark extract, stimulating central nervous system (CNS) and behavioral changes in animals, with increase of alertness condition, irritability and convulsions. Alkaloids present in the flowers of this species showed cytotoxic activity against KB cells and *Artemia salina* (Sriphong et al. 2003), and selective cytotoxicity against mutant yeasts of *Saccharomyces cerevisiae* (Bolzani et al. 1995). Pivatto et al. (2005), Viegas et al. (2004, 2007) have also reported the isolation of several 3-hydroxy-2,6-dialkyl-piperidine alkaloids from the fruits, leaves and flowers of *S. spectabilis* with remarkable cytotoxic effects and inhibitory activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5-lipoperoxidase and cycloxigenase-1 and 2. In a recent study, the extract from the leaves of this plant have been investigated for antimicrobial properties against Gram positive (*Bacillus subilis* and *Staphylococcus aureus*), Gram negative (*Escherichia coli*, *Salomonella typhi* and *Pseudomonas aeroginosa*) bacteria and yeast (*Candida albicans*). Acetone and methanol crude extracts exhibited the best antimicrobial activity with minimal inhibitory concentration (MIC) of 0.625 to 2.5 mg/ml, and minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) values ranging from 1.25 to 5 mg/ml (Krishnan et al. 2010).

Due to these wide spectrum of biological and pharmacological properties described for *S. spectabilis*, and mainly for antimicrobial activities reported earlier, we decided to include this plant in an ongoing bioprospection project searching for new natural sources and secondary metabolites with leishmanicidal activity as goal. In this paper we present the results of the leishmanicidal effects of crude ethanolic (CEFL), hexane (FL-Hex), dichloromethane (FL-DCM), hydroalcoholic (FL-Ha) and *n*-butanolic (FL-Bu) fractions from the flowers of *S. spectabilis* and also for a mixture of (−)-cassine/(−)-spectaline, the major alkaloidal constituents in this plant.

Materials and methods

General procedures and techniques

Chemicals and standard drugs used in this study were pentamidine (Sigma), Schneider' medium (Sigma–Aldrich), Fetal Bovine Serum (FBS, Sigma), Dulbecco's Modified Eagle's medium (DMEM, Sigma). The work solutions of CEFL, FL-Ha, FL-Bu, FL-DCM, FL-Hex, (−)-cassine/(−)-spectaline and pentamidine were prepared in dimethyl sulfoxide (DMSO, vehicle, Sigma). Al_2O_3 grade I, type WN-3 and silica gel (200–400 mesh) were used in column chromatography and thin layer chromatography (TLC). Visualization of TLC plates was made by spraying with iodochloroplatinate (Merck) and Dragendorff's reagents.

Plant material

Flowers of *S. spectabilis* were collected in January 2000 in Araraquara-São Paulo, Brazil. A voucher (SP 370917) was deposited at the herbarium of São Paulo Botanic Garden, São Paulo-SP, Brazil.

Preparation of extracts and isolation

Plant material (5 kg) was dried at 60 °C, ground and extracted with EtOH (5 × 5 l), furnishing 200 g of a crude extract (CEFL), after solvent evaporation. Then, an aliquot of CEFL (40 g) was suspended in MeOH/H_2O 4:1 and the insoluble residue (10.5 g) removed by filtration. The remaining hydroalcoholic solution was partitioned successively with hexanes, CH_2Cl_2, and *n*-BuOH to furnish the organic fractions FL-Hex (2.0 g), FL-DCM (7.9 g), FL-Bu (2.7 g), respectively, and 5.2 g of the residual hydroalcoholic fraction (FL-Ha). Analysis of the organic fractions by TLC revealed that most of the alkaloidal constituents were concentrated in the FL-DCM fraction. An aliquot of this fraction (3 g) was then fractionated by neutral alumina CC as described earlier (Viegas et al. 2004) to afford 1.4 g of a mixture of (−)-cassine/(−)-spectaline (7:3, high-performance liquid chromatography (HPLC)) (Pivatto et al. 2005), that was characterized by nuclear magnetic resonance (NMR) analysis and compared to literature data (Bolzani et al. 1995; Viegas et al. 2004, 2007).

Cells and parasites

Murine macrophages J774 were obtained from the Cell Bank of Federal University of Rio de Janeiro (Brazil). These adherent-phenotype macrophage line was cultured in DMEM supplemented with 10% FBS at 37 °C with 95% humidity and 5% CO_2. The promastigote forms of *L. major* IOC/L0581 (MHOM/SU/1973/5-ASKH) were obtained from *Leishmania* collection of the Oswaldo Cruz Institute (Brazil). The parasites were maintained *in vitro* in Schneider's medium, supplemented with 10% FBS and 2% human urine.

Cytotoxity assay

The deleterious effect of crude extract, fractions and alkaloids isolate from the flowers of *S. spectabilis* were determined by assessing their cytotoxicity on murine macrophages (J774 cell line). Briefly, cell suspensions containing 7.5×10^5 cell/ml were placed in a 96-well plate in triplicate an incubated at 37 °C for 1 h. Once this time had elapsed, CEFL, FL-Ha, FL-Bu, FL-DCM and FL-Hex were added at six serial concentrations starting at 100 μg/ml [Final volume: 200 μl; concentrations of 100, 30, 10, 1 and 0.3 μg/ml] and cell growth media free from aqueous extractive solutions (basal growth control). Pentamidine was used as standard drug at concentrations of 34, 10.2, 3.4, 0.1, 0.3 and 0.1 μg/ml, corresponding to 100, 30, 10, 3, 1 and 0.3 μM, respectively. The mixture of (−)-cassine/(−)-spectaline was added at six serial concentrations (100, 30, 10, 3, 1 and 0.3 μM, corresponding to 30.4, 9.1, 3, 0.9, 0.3 and 0.1 μg/ml, respectively). The cells were also cultured in a medium free from compounds and fractions or vehicle (basal growth control) and in a medium with DMSO (0.1, 0.03, 0.01, 0.003, 0.001 and 0.0003%) as a vehicle control. After a period of 48 h at 37 °C and 5% CO_2, the assay was performed in an absorbance microplate reader, by the measurement of LDH (Koski et al. 1983).

Antileishmanial assay

The cytotoxicity effect of CEFL, FL-Ha, FL-Bu, FL-DCM, FL-Hex, (−)-cassine/(−)-spectaline and pentamidine (standard drug) against promastigote forms were determined. The stationary phase of promastigotes of *L. major* were plated in 96-well vessels (Nunc) at 1×10^5 cells per well, in Schneider's medium, supplemented with 10% FBS and 2% human urine. Solutions of the crude extract and its fractions were added at serial concentrations (100, 30, 10, 3, 1 and 0.3 μg/ml). Pentamidine and (−)-cassine/(−)-spectaline were also added at serial concentrations (100, 30, 10, 3, 1 and 0.3 μM). The cells were cultured in a medium free from compounds or vehicle (basal growth control) and in a media with DMSO 0.1% (vehicle control). After 48 h, extracellular load of *L. major* promastigotes was