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Anti-inflammatory activity of *Vismia guianensis* (Aubl.) Pers. extracts and antifungal activity against *Sporothrix schenckii*

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ABSTRACT

Ethnopharmacological relevance: *Vismia guianensis* (Aubl.) Pers. is traditionally used in North and Northeast of Brazil for the treatment of dermatomycoses. Since the strategy associating immunomodulators with antifungal drugs seems to be promissory to improve the treatment efficacy in fungal infections, we aimed to investigate the antifungal activity of *V. guianensis* ethanolic extract of leaves (VGL) and bark (VGB) against *Sporothrix schenckii* ATCC 16345 and their antinflammatory activities.

Material and methods: The extracts were analyzed by HPLC-DAD-IT MS/MS for *in situ* identification of major compounds. Antifungal activity was evaluated *in vitro* (microdilution test) and *in vivo* using a murine model of *S. schenckii* infection. The production of TNF-α, IFN-γ, IL-4, IL-10 and IL-12 by measured by ELISA, as well as measured the production and inhibition of the NO after treatment with the plant extracts or itraconazole (ITR).

Results: Two O-glucosyl-flavonoids and 16 prenylated benzophenone derivatives already described for *Vismia* were detected. Both VGL and VGB showed significant antifungal activity either in *in vitro* assay of microdilution (MIC=3.9 μg/mL) and *in vivo* model of infection with reduction of *S. schenckii* load in spleen. It was also observed a predominance of reduction in the production of NO and the proinflammatory cytokines evaluated except TNFα, but with stimulation of IL-10, as evidence of a potential anti-inflammatory effect associated.

Conclusion: The results showed that both VGL and VGB have a significant antifungal against *S. schenckii* and an anti-inflammatory activity. These results can support the use of these extracts for alternative treatment of sporotrichosis.

1. Introduction

Besides being holder of the largest diversity, estimated at 20–22% of the world total (over 45,000 species), Brazil has a rich cultural diversity and ethnic resulting in considerable accumulation of knowledge and technology traditional, passed from generation to generation, highlighting the vast range of knowledge about the management and use of medicinal plants. However, the market for plant-derived drugs in this country (phytomedicines) is still very small, representing less than 5% of all marketed drugs. Therefore, studies approaching mechanistic aspects, safety, pharmacokinetics and clinical aspects are necessary to make it possible to develop new plant-derived drugs and herbal medicines in the future (Dutra et al., 2016).

Despite recent advances in antifungal therapy for opportunistic mycosis, there are still many unresolved aspects, regarding the long lasting therapy regimen, fungal resistance and toxicity, leading to sub-therapeutic antifungal drug concentrations and poorer clinical outcomes (Chau et al., 2014). The limited number of treatment options, joint to the increasing frequency of cross-resistance, makes necessary to develop new therapeutic strategies against fungal invasive infections. Some of these strategies are based in the improving of the antifungal effect and the stimulation of the immune response (Portuondo et al., 2015; Batista-Duharte et al., 2016). Many efforts have been made to evaluate the antifungal properties of different medicinal plants with the hope of discovering new and more efficient antifungal compounds as therapeutic alternative to classic chemotherapy. These studies have proven the effectiveness of certain plant extract and essential oil in treating fungal infections. (Cruz et al., 2007; Svetaz et al., 2010; Uma et al., 2016).

Vismia guianensis (Aubl.) Pers, popularly known as "lacre", pau-de-lacre, árvore-da-febre, caapiá e caopiá, belongs to the family *Clusiaceae* (also named *Guttiferae*). The plant is commonly found in

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Fig. 1. *Vismia guianensis* (Aubl.) Pers Aerial parts, leaves and stem bark.

countries and regions of Tropical America, such as: Colombia, Venezuela, Guyana and different areas of North and Northeast Brazil (Almeida-Cortez and Melo-de-Pinna, 2006). It is a shrub or small tree, with oval leaves, oblong, inflorescence in terminal panicles and fruits globose, fleshy indehiscent (Fig. 1). The latex (yellow-red resin called gum or gum-gutta seal) and the infusion of the leaves, are used in the treatment of dermatomycoses, and other applications such as purging. The decoction and infusion of its leaves and husks are also used for rheumatism, and as a tonic febrifuge. Several studies have indicated that *Vismia* species extracts can exhibit important antibacterial, antifungal, antiparasitic, insecticides, and antivirals properties that have been used by indigenous populations to treat diverse diseases. Regarding the antifungal properties, it was reported that the Wayãpi tribe living in French Guyana, use latex bark to treat oral fungal infections in children (Vizcaya et al., 2012). As support of this finding, Kuete et al., 2007, reported the *in vitro* antifungal effect of *Vismia laurentii* against *Candida albicans* y *Candida gabrata,* while Tamokou et al. (2009), showed the antifungal activities of the methanol crude extract and several isolated compounds of *V. rubescens* against *C. albicans, Candida tropicalis, Candida parapsilosis* and *Cryptococcus neoformans*. This finding supports the traditional use of this plant in the treatment of several infectious diseases (Hussain et al., 2012).

Sporotrichosis (also known as "rose gardener's disease") is a deep cutaneous mycotic infection caused by the dimorphic fungus *Sporothrix schenckii,* formed by a complex of clinically relevant species: *S. brasiliensis*, *S. globosa*, *S. mexicana* in addition to *S. schenckii* s. str. and *S. luriei* (Mora-Montes et al., 2015). Sporotrichosis has gained importance in recent years due to its worldwide prevalence, recognition of multiple cryptic species within the originally described species, and its distinctive ecology, distribution, and epidemiology across the globe. This disease is currently known emergent mycoses in several tropical and subtropical countries (Chakrabarti et al., 2015; Carlos and Batista-Duharte 2015). The fungal resistance to different environmental conditions and their adaptation to the host, are determinant for the dissemination and virulence of the different species (Téllez et al., 2014), while the host immunity determinate the clinical form of the disease (Gutierrez-Garrardo et al., 2015).

Itraconazole (ITR) is currently considered the drug of choice to treat the vast majority of clinical cases of sporotrichosis, while amphotericin B is the first choice for the treatment of disseminated and severe cases. However, the antifungal therapy often is associated to toxicity and fungal resistant associated to long lasting therapy. (Batista-Duharte et al., 2015). Accordingly, the search of alternative methods for the sporotrichosis treatment is a matter of great current interest. Diverse formulations from various types of plants have been evaluated against *Sporothrix* species in different parts of the world. These studies evidenced the enormous potential of these natural preparations as alternative treatment against sporotrichosis (Johann et al., 2007; Apisariyakul et al., 1995; Stopiglia et al., 2011; Gaitán et al., 2011; Singh et al., 2008; Masoko et al., 2005, 2010; Waller et al., 2016). In addition, some recent evidences suggest that association of immunomodulation and the antifungal effect can improve the efficacy of the sporotrichosis treatment (Guterres et al., 2014; Batista-Duharte et al., 2016).

Taking in mind the reported antifungal effects of *Vismia* genus in *Candida* species and that until now there are no reports of the effect of *V. guianensis* in sporotrichosis, the aim of this study was to identify the bioactive constituents from the ethanol extract of leaves and bark from *V. guianensis* (Aubl.) Pers and investigate the antifungal and anti-inflammatory activities against *S. schenckii.*

2. Materials and methods

2.1. Collection and identification of plant materials

The plant materials composed of stem bark (VGB) and leaves (VGL) of *V. guianensis* (Aubl.) Pers. was collected in the urban area of the city of Manaus-AM, in the premises of the campus of the Federal University of Amazonas, from December to January 2009. Fertile representatives were collected for the production of voucher specimen has been identified and deposited in the Herbarium of the National Institute for Amazonian Research - INPA. A voucher specimen was registered under number 213 331.

2.2. Preparation of plant extracts

Either stem bark or leaf powder of *V. guianensis* (Aubl.) Pers. were differently soaked in ethanol: water as solvent solution at 10% (w/v). The mixture was agitated using an electric blender (to enhance proper mixing of the solvent with the powder), and then kept by 7 days in dark-coloured flasks with occasional stirring. The liquid phases were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents and renewal of liquid extractor. Combined supernatants were evaporated under vacuum at 40 °C using rotary evaporator and then lyophilized. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 °C.

2.3. Phytochemical screening

The VGB and VGL extracts were evaluated by phytochemical qualitative reactions for usual plant secondary metabolites. The screening was performed for anthraquinones, flavonoids, saponins, tannins, and alkaloids. The colour intensity or the precipitate formation was used as analytical responses to these tests (Pochapski et al., 2011).

2.4. Chromatographic analysis of plant extracts

Ten milligrams of freeze-dried VGL or VGB extracts were solubilized in a water/methanol solution (8: 2 v/v) for high performance liquid chromatography coupled with diode array detector and ion trap mass spectrometry with electrospray source (HPLC-DAD-ESI-IT MS/MS). Chromatography separation was performed using Shimadzu LC-20A with DAD CBM20A; Shimadzu coupled to the mass spectometer Amazon SL Bruker Daltonics. The mobile phase was MeOH (B) and H2O (A) containing 1.0% de acetic acid (v/v), in elution gradient: 0.01 min: 5.0% B, 45.0 min: 100.0% B, 50.0 min: 100.0% B, 55 min: 5.0% B, 60.0 min: 5.0% B. It was used a column Luna C-18 (4.6×250.0 mm, 5 µm, Phenomenex), flow of 1.0 mL/min, temperature: 35 °C, injection volume: 20 µL, pressure limits (A and B), 0 - 350 Kgf/cm2, collision gas N2 and collision energy amplitude 60%. The analyses were performed through Bruker DataAnalysis 4.2 software.

2.5. Microorganism and culture conditions

S. schenckii ATCC 16345, originally obtained from a human case of diffuse lung infection (Baltimore, MD) and kindly provided by the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), was used for all experiments. For mice infection, a piece of the fungal mycelium grown on Mycosel (BD Biosciences) agar tubes was transferred to an Erlenmeyer flask containing 100 mL of brain-heart infusion broth (Difco) and then cultured for 7 days at 37 °C with constant shaking at 150 rpm. After that, an aliquot containing 2×10^7 yeast cells was transferred to a new medium and cultured for 5 days more at the same conditions in order to achieve a virtually 100% mycelium-to-yeast conversion in a logarithmically growing culture.

2.6. In vitro antifungal activity

2.6.1. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was performed according to the procedures recommended for the fungal yeast forms. (Clinical and Laboratory Standards Institute 2008). The antifungal activity was detected by the Microplate Alamar Blue assay (MABA). It is a colorimetric method using Alamar Blue oxido-reducing substance as an indicator of microbial growth and it is useful for dimorphic fungi (de Paula e Silva et al., 2013), including *S. schenckii* (Batista-Duharte el at. 2016). Briefly, 100 µL of each extracts or ITR were added by well, separately (in triplicate) and the concentrations in RPMI 1640 ranged from 500 to 0.000875 µg/mL and 16 to 0.00003 µg/mL for ITR. After that 100 µL of the prepared fungal suspension (1×10^3 to 5×10^3 CFU/mL) was added to each well of 96-well microtiter plates containing. Growth and sterility controls were included for each isolate tested (growth control: RPMI medium with DMSO and organisms but with no drug added; sterility control: RPMI medium only, with no organisms or drug added). The MABA was used according the manufacturer's instructions (Invitrogen), adding 20 µL to the well of Alamar Blue at 72 h and the plates were incubated for an additional 24 h, totalling 4 days for the MIC final reading. The lowest antifungal agent concentration that substantially inhibited the growth of the organism was visually determined at the point at which there was no change in the original blue colour of the reagent. The fungal growing in each well was also monitored using an inverted microscopy.

2.7. Animals and experimental infection

Male Balb/c mice, 5–7 weeks old at the time of inoculation were obtained from the Central Bioterium of University of Campinas (UNICAMP, Brazil). For each independent experiment, 5 mice by groups were were used. Animals were intraperitoneally inoculated with 100 µL with 10^7 *S. schenckii* yeast cells in PBS or with an equal volume of PBS alone. 24 h after fungal inoculation the animals were treated with test substances (extract or antifungal). The groups and their dosages were as follows: VGB - Infected animals that received a daily dose of 10 mg/kg body weight of the stem bark extract of *V. guianensis*. VGL - Infected animals that received a daily dose of 10 mg/kg body weight of the extract of the leaves of *V. guianensis,* ITR - Infected animals that received a daily dose of 10 mg/kg weight of itraconazole. Infected - Infected animals receiving a daily dose of saline, serving as a placebo. Uninfected - Animals uninfected and untreated. All the treatment were administered by oral gavage. The dose of 10 mg/kg was chosen in this study taking into count that this is an effective therapeutic dose of ITR by oral way in human and feline sporotrichosis (Batista-Duharte et al., 2015; de Souza et al., 2016). Thus, we use this dose as reference to compare the potency *in vivo* of the plant extracts.

All procedures with laboratory animals were approved by the Institutional Ethics Committee and were in accordance with National Institutes of Health Animal Care Guidelines (Protocol N° 06/2014 CEUA/FCF/Car).

2.8. Obtaining peritoneal exudate cells

The animals were previously stimulated by intraperitoneal (i.p) inoculation of 3.0 mL of 3.0% sodium thioglycollate, three days before the collection of cells. After this period, the animals were euthanized by CO_2 inhalation chamber. These animals had their abdominal skin removed aseptically in a laminar flow chamber and exposed peritoneum. In the upper middle portion of the abdomen were injected 5.0 mL of phosphate buffered saline (PBS) at pH 7.2 and 4 °C with the aid of well sterile syringe and needle. A light hand massage was performed and peritoneal exudate cells were collected with the same syringe and dispensed in sterile conical tube for preparation of the cell suspension. The peritoneal exudate cells were washed three times with 5 mL of PBS (pH 7.2) and centrifuged at 400×*g* for 5 min in a centrifuge at room temperature. The pelleted cells were re-suspended in 1.0 mL of RPMI-1640 culture medium containing 2β 2×10–5 M-mercaptoethanol, penicillin 100 U/mL streptomycin, 100 U/mL, 2 mM L-glutamine and 5% serum fetal bovine, and the medium compound so designated RPMI-1640 complete medium (RPMI-1640-C). The number of cells was determined by counting in a Neubauer chamber hemocitométrica using 10 µL of the diluted cell suspension in 90 µL of liquid Lazarus. Cells were adjusted to the optimum concentration for each test in RPMI-1640-C.

2.9. Spleen removal and systemic fungal load study

Spleen from each mouse was aseptically removed and passed through a 100 µm cell strainer with the aid of a syringe plunger into a Petri dish containing 2 mL of PBS. The assessment of the systemic fungal load was performed by counting the CFU grown on Mycosel agar plates after the inoculation of a previously determined dilution of the spleen macerate, collected before the red cell lyses treatment.

2.10. Preparation of total splenocytes

Splenocytes were obtained as described above. For red cell lyses, the resulting suspension was added with 6 mL of a 0.17 M ammonium chloride solution and then incubated in ice for 5 min. Splenocytes were separated from the supernatant by centrifugation at 300×*g* for 5 min at 4 °C, washed once with 3 mL of RPMI complete medium and then

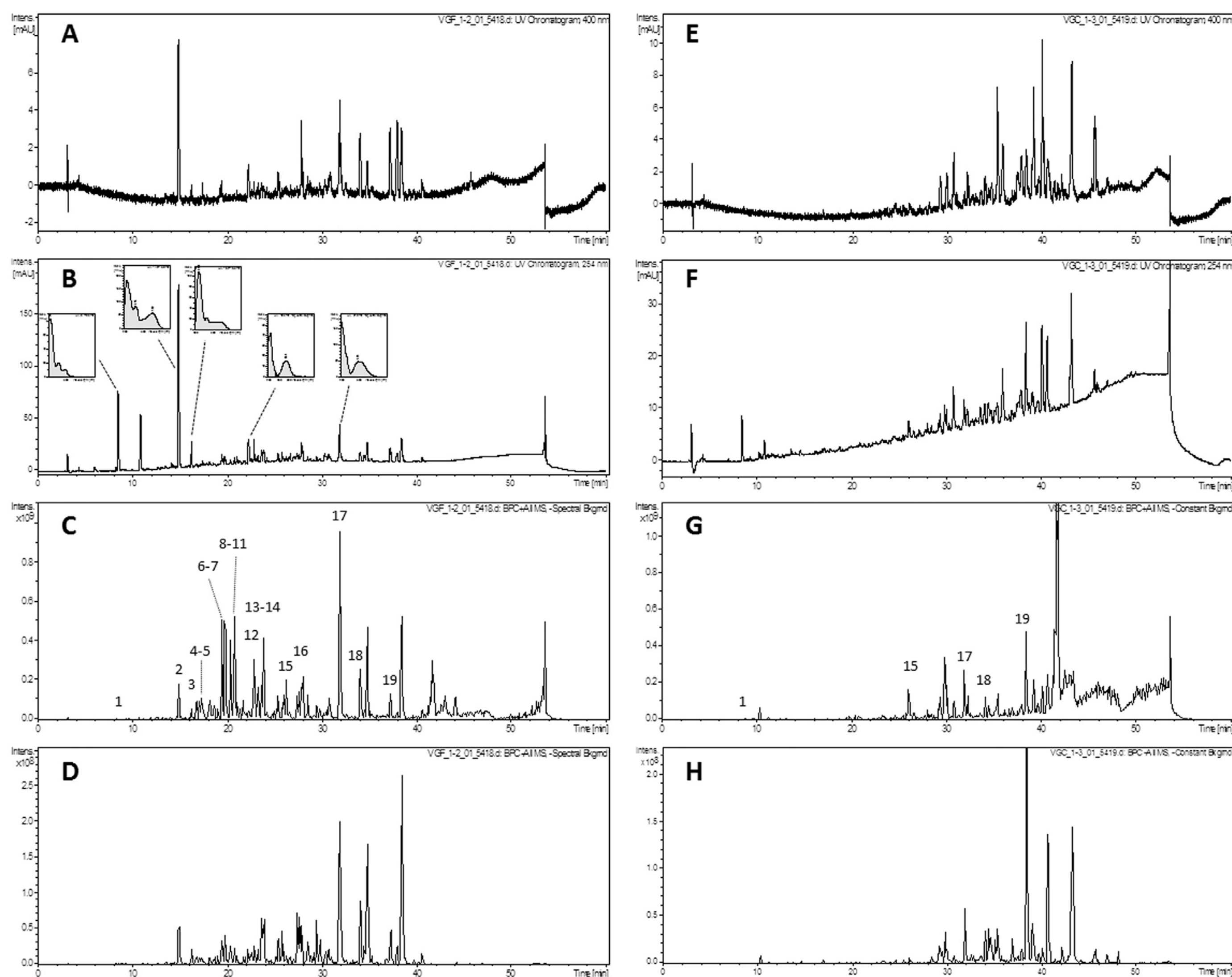

Fig. 2. HPLC-UV chromatograms of leaves of *V. guianensis*. A) at 400 nm, B) 254 nm – with UV spectra of main identified metabolites. C) BPC (base peak chromatogram) of *V. guianensis* leaves in ESI positive mode and D) negative mode. Numbes 1–19 represent the detected metabolites. E) HPLC-UV of *V. guianensis* barks at 400 nm, F) 254 nm, G) BPC-ESI positive and H) negative modes.

resuspended in 1 mL of the same medium. Cell concentration was determined by microscopy using the Trypan blue exclusion test and then adjusted to 5×10^6 cells/mL in RPMI complete medium.

2.11. Evaluation of cellular viability

The bromide 3- (4,5-dimethylthiazol-2)–2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used for the determination of cell viability of PEC. This assay is based on checking the activity and mitochondrial integrity interpreted as a measure of cell viability. The cell suspension were adjusted to a concentration of 5×10^6 cells/mL. 100 µL of the suspension was added to 96-well plates. The plates were incubated for 1 h with three concentrations (25, 50 or 100 µg/mL) of either VGL or VGB extracts at 37 °C and 5% CO_2. Only cells and culture medium RPMI-1640-C were used as a control, equivalent to 100% viability of macrophages. Measurements were taken using a spectrophotometer UV/Visible microplate at 540 nm with reference filter at 620 nm (Mosmann, 1983). Before the determination of nitric oxide and cytokine production by macrophages and lymphocytes from infected and non-infected mice the cell viability was also measured (Supplementary material).

2.12. Determination of nitric oxide production

Nitric oxide was quantitated by accumulation of nitrite in the culture medium is measured spectrophotometrically using the Griess reagent with $NaNO_2$ as standard (Green et al., 1982). The obtained cell suspension was adjusted to 5×10^6 cells/mL in RPMI-1640 medium. 100 µL were distributed this cell suspension in sterile 96 well plate. After this period, the supernatant was discarded. In some wells plate, 100 µL of the extracts were added at a concentration of 50 µg/mL. In others wells, 100 µL of LPS solution containing 10 µg/mL as a stimulant agent (positive control), and still other wells were added 100 µL of RPMI-1640 medium to the cell suspension as control cells (negative control). The plate was incubated for 24 h at 37 °C with CO_2 (5%). After incubation, 50 µL aliquots of each sample were withdrawn and passed to the plate and another additional 50 µL/well Griess reagent, consisting of 0.1% N-1-naphthyl-ethylenediamine, 0,1% sulfanilamide solution of 3% phosphoric acid. After 10 min in the dark at room temperature, the plates were read in a spectrophotometer UV/Visible microplate with 540 nm filter. Sodium nitrite concentrations were calculated from a standard curve previously established with known molar concentrations of sodium nitrite in RPMI-1640 medium. The tests were performed in triplicate and the values expressed in

Table 1
Metabolites detected by HPLC-DAD-IT MS/MS (ESI positive and negative modes) of *V. guianensis* extracts.

n°	Rt (min)	UV max (nm)	Identification*	[M-H]⁻	Fragmentation	[M+H]⁺	Fragmentation	Ref
1	8.2	330	chlorogenic acid	353.2	MS_2[353] 191; 179; 135	–	–	–
2	14.8	348, 255	quercetin-*O*-deoxyhexose	447.2	MS_2[447] 301; 269; 257; 229; 191; 179; 151	449.2	MS_2[449] 303; 257; 219; 129	Vikic and Guttman, 2010
3	16.2	345, 265	kaempferol-*O*-deoxyhexose	431.1	MS_2[431] 285; 255; 179; 163	433.1	MS_2[433]287; 259; 219; 153; 147; 129	Vikic and Guttman, 2010
4	17.4	287, 370	hydroxyl-vismiaguianone D or E	443.2	MS_2[443] 425; 413; 385; 367; 343; 331; 313; 299; 291; 271; 255; 187; 161	445.2	MS_2[445]427; 409; 387; 373; 355	Seo et al., 2000
5	17.5	288	benzophenone derivative	413.2	MS_2[413]395; 355; 341; 319; 293; 283; 269; 225; 177; 137	415.2	MS_2[415]397; 379; 343; 325; 303; 267; 249; 175; 133	Fuller et al., 1999
6	19.4	287, 370	hydroxyl-vismiaguianone D or E	443.2	MS_2[443] 425; 411; 393; 385; 349; 323; 317; 299; 287; 247; 165; 137	445.2	MS_2[445]427; 413; 373; 355; 341; 297; 267; 185	Seo et al., 2000
7	19.9	284	vismiaguianone derivative	457.2	MS_2[457]425; 401; 367; 331; 303; 255; 229; 201; 185; 163; 147	459.3	MS_2[459] 427; 409; 387; 355; 247; 237	Seo et al., 2000
8	20.4	289	vismiaguianone D or E	427.2	MS_2[427]412; 395; 383; 369; 333; 307; 275; 257; 243; 137	429.2	MS_2[429] 411; 357; 339; 309; 281; 263	Seo et al., 2000
9	22.7	263, 310	vismiaguianone derivative	445.3	MS_2[445] 427; 413; 375; 351; 319; 293; 275; 251; 237; 205; 161; 137	447.2	MS_2[447] 429; 411; 393; 375; 357; 339; 303; 259; 227; 203;	Seo et al., 2000
10	22.7	292	benzophenone derivative	413.2	MS_2[413]395; 383; 369; 355; 339; 355; 325; 319; 293; 283; 231; 223; 141	415.2	MS_2[415]397; 379; 343; 321; 303; 267; 249; 215; 121	Fuller et al., 1999
11	22.9	293	benzophenone derivative	367.1	MS_2[367]353; 336; 318; 309; 291; 265; 252; 247; 216	369.1	MS_2[369] 351; 297; 275; 259; 231; 203; 121	Fuller et al., 1999
12	23.6	285, 337 (sh)	vismiaguianone D or E	427.2	MS_2[427] 395; 377; 357; 333; 325; 313; 307; 237; 137	429.2	MS_2[429] 411; 393; 339; 264	Seo et al., 2000
13	25.8	289, 335 (sh)	sesamin	353.1	MS_2[353] 336; 323; 293; 281; 259; 133	355.2	MS_2[355] 337; 331; 293; 279; 261; 231;	Yan et al., 2007
14	25.8	289, 335 (sh)	unknown	371.2	MS_2[371] 339; 321; 277; 245; 233; 205	373.2	MS_2[373] 355; 313; 301; 279; 261; 195	–
15	27.5	285, 337 (sh)	vismiaguianone derivative	427.2	MS_2[427] 409; 395; 357; 341; 333; 301; 263; 247; 231; 207; 187; 161; 149	429.2	MS_2[429] 411; 393; 357; 339; 313; 299; 275; 263; 255; 225; 219; 189; 121	Seo et al., 2000
16	28.5	289	vismiaphenonederivative	397.2	MS_2[397] 379; 339; 325; 303; 231; 163; 149	399.2	MS_2[399] 381; 343; 327; 305; 267; 233; 205; 199	Fuller et al., 1999
17	31.9	310	vismiaphenone G	411.2	MS_2[411] 379; 367; 327; 319; 291; 259; 221	413.2	MS_2[413] 395; 357; 341; 319; 302; 263; 247; 191 MS_2[357] 339; 281; 263; 245; 221; 193; 169; 119	Fuller et al., 1999
18	34.5	290	unknown	355.2	MS_2[355] 341; 301; 261; 249; 235; 233; 219	357.2	MS_2[357] 339; 325; 301; 263; 207; 191; 175	–
19	36.9	298	vismiaphenone D	395.3	MS_2[395] 380; 337; 325; 301; 275; 257; 233	397.2	MS_2[397] 379; 341; 325; 285; 231; 203; 121	Fuller et al., 1999

Table 2
In vitro direct antifungal activity of VGL, VGB AND ITR after 48 h.

Evaluated substances	MICs	MABA (μg/mL)	MIC range (**(μg/mL) ITR**[a]
VGL	3.9	3.9	–
VGB	3.9	3.9	–
ITR	0.97	0.97	0.25–1.0

MIC: minimum inhibitory concentration.
MABA: microplate AlamarBlue assay.
[a] *Itraconazole(ITR) MIC range determined in 19 S. schenckii strains (Alvarado-Ramírez and Torres-Rodríguez 2007).*

μmols NO/5×10^6 cells.

2.13. Determination of the inhibitory activity of V. guianensis extracts in peritoneal cell cultures of mice for the production of NO

Peritoneal exudate cells were used at a concentration of 5×10^6 cells/mL. Cells were incubated in the concomitant presence of 100 μL of LPS solution (10 mg/mL) and 100 mL of extracts at a concentration of 100 μg/mL. Incubation was performed for 24 h at 37 °C with CO_2 (5%). NO production was measured spectrophotometrically at nitrite accumulation in the culture medium using the Griess reagent in the same way as in NO production test.

2.14. Determination of TNF-α, IFN-γ, IL-4, IL-10 and IL-12

The cytokines TNF-α and IL-12, were quantified in the supernatant obtained from the culture of macrophages, while IL-4, IL-10 and IFN-γ in splenocytes culture, using ELISA immunoenzymatic assay, (Pharmingen) according to the instructions manufacturer. Polystyrene 96 well microplates were adsorbed with a capture antibody (mouse serum) anti-IL-4, anti-IL-10, anti-IL-12, anti-TNF-α purified mouse or the 4 μg/mL (100 μL/well) and incubated in PBS buffer "overnight" at room temperature. The plates were washed 3 times with phosphate buffered saline, pH 7.2 (PBS) containing 0.05% Tween-20 (PBS-T). After washing, 300 μL were blocked with 1% BSA in PBS (PBS / BSA, 5% sucrose and 0.5% sodium azide) at room temperature for 60 min and washed 3 times with PBS-T. It was added to the plate 100 μL of each pattern of cytokine or cell culture supernatants. The plates were incubated at room temperature for 120 min, and washed 4 times with PBS-T. Then was added 100 μL/well of monoclonal antibody goat anti-IL-4, anti-IL-10, anti-IL-12, anti-mouse TNF-α labeled with biotin at a concentration of 400 ng/mL reagent diluent (1% BSA, 0.05% Tween 20 in Tris-NaCl buffer). The plates were incubated at

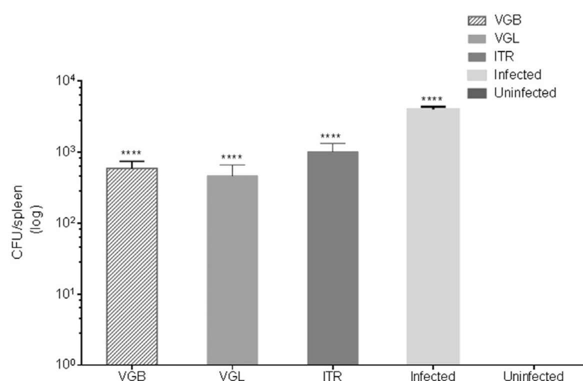

Fig. 3. Fungal loads in the spleen of a Balb/c model of infection with *S. schenckii* ATCC 16345. The mice were organized into 5 groups one of which was not infected with *S. schenckii*. Infected animals were inoculated i.p with 10^6 yeast *S. schenckii*. These were divided into 4 groups: VGC (infected and treated animals with the stem bark extract of *V. guianensis*); VGF (infected and treated animals with the extract of *V. guianensis* leaves); ITR (animals infected and treated with itraconazole) and only infected and untreated animals. Statistical significance was determined by 2-way ANOVA for multiple comparisons using the Sidak test and confidence interval *(p < 0.05), **(p < 0.01), ***(p < 0.001) and ****(p < 0.0001) compared with the control group at each point corresponding unless otherwise indicated. Results are presented as mean ± standard deviation of 3 animals.

room temperature for 120 min and washed 3 times with PBS-T, and then added 100 µL streptavidin-peroxidase conjugate diluted in PBS/BSA and further incubated at room temperature for 30 min. After this process, the plates were washed 3 times with PBS-T and 100 µL of substrate (10 mM citrate-phosphate buffer containing 0.4 mM tetramethylbenzidine [Sigma] and 1.2 mM H_2O_2) were added to each well. The reaction was stopped by adding 50 µL of 2N H_2SO_4. The absorbance was read at 450 nm in a spectrophotometer UV/vis microplate and cytokine concentrations were quantified using a previously established standard curve with known amounts of IL-4, IL-10, IL-12, IFN-γ or TNF-α, standard. The tests were performed in triplicate and the results expressed as pg/mL.

2.15. Statistical analysis

The data obtained were analysed by the Prism program Version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) and expressed as the means ± SD of each experimental group. To determine statistically significant differences between groups an ANOVA for multiple comparisons using the Sidak test and confidence interval *(p < 0.05), **(p < 0.01), ***(p < 0.001) and ****(p < 0.0001).

3. Results and discussion

According to World Health Organization, medicinal plants would be the best source of a variety of drug. Unfortunately, traditional medicines based on plant products remains empirical regarding, doses, durations of treatment or interactions with other drugs are not clearly defined. Therefore there are urgent necessities for chemical and pharmacological studies that may help achieve the standardization of such treatment (Uma et al., 2016).

Sporotrichosis is an opportunistic infection where the immunocompetence is determinant in the pathogenesis (Gutierrez-Gallardo et al., 2015). Studies developed in our lab evidenced that different immune mechanisms of the innate and adaptative immune response, are involved in the defence against *S. schenckii* (Gonçalves et al., 2015; Ferreira et al., 2015; Maia et al., 2016; Negrini et al., 2013; Sassá et al., 2012; Portuondo et al., 2016). On the other hand, it was observed that an excessive inflammatory reaction induced by the infection could be deleterious, causing tissular damages and even hindering the efficient fungal elimination (Maia et al., 2016). In this way, it was described that the exogenous supply of antioxidants, as natural compounds that scavenge free radicals, might represent an important additional tool for the treatment of opportunistic fungal infections during HIV infection (Otang et al., 2012). These findings suggest that the strategy associating immunomodulator/antinflammatory compounds with antifungal drugs seems to be promissory to improve the treatment efficacy in infections by *S. schenckii* (Guterres et al., 2014; Batista-Duharte et al., 2015, 2016).

Several etnomedical and experimental studies showed that plants of the *Vismia* genus, have significant antifungal effect against *Candida* sp. and *C. neoformans* (Kuete et al., 2007; Tamokou et al., 2009; Vizcaya et al., 2012) but so far there is not studies of the antifungal effect of this genus against *S. schenckii*. In the present study, the anti-inflammatory activity of *V. guianensis* extracts and antifungal activity against *S. schenckii* were investigated for the first time.

Firstly, a phytochemical screening was performed in order to identify the main classes of compounds present in the species *V. guianensis*. The results evidenced the presence of polyphenol compounds as anthraquinones, flavonoids and tanines. Furthermore, *in situ* identification based on LC-DAD-IT MS/MS were performed by on-flow data-dependent acquisition mode (autoMSMS) in alternated positive and negative electrospray ionization modes for comprehensive data collection with the measurement of CID fragment spectra from all detectable metabolites of the sample after isolation of the corresponding parent ions by the trap (Broecker et al., 2011).

After data acquisition, the MS/MS spectra were cross referenced against database (DPN, Metlin, Massbank) considering all data reported in the literature for *Vismia* taxon (Hussain et al., 2012; Carnevale-Neto et al., 2013). The putative molecular detections were justified based on measured *m/z*, UVmax and MS/MS fragmentation. The integrated LC-DAD-MSMS approach, depicted in Fig. 2, led to the detection of 19 known metabolites (Table 1), classified in flavonoids *O*-glycosides and prenylated benzoquinone as vismiaphenone and vismiaguianone derivatives previously described for *Vismia* (Fuller et al., 1999; Seo et al., 2000; Hussain et al., 2012).

Flavonoids are phenolic compounds found in much higher plants and are characterized by their antioxidant action (Vukics and Guttman, 2010; Carnevale-Neto et al., 2013). Polyisoprenylated benzophenones were mainly present in the fruits, twigs, pericarps, stems, leaves, stem bark, fruit hulls and pulp of the genus *Vismia* and important chemomarkers in Clusiaceae. These molecules are known for antifungal activity, usually related to pyran moiety (Rubio et al., 1999; Kumar et al., 2013). Wabo and colleagues 2007 demonstrated moderate activity of xanthones, isolated from *V. laurentii* against Gram-positive (Wabo et al., 2007). In another study, Suffredini and coworkers 2006 determined that the extracts of *V. guianensis* have not significant antimicrobial activity against the bacteria *E. coli, P. aeruginosa, S. aureus and E. faecalis*, since the MIC was greater than 200 µg/mL (Suffredini et al., 2006). However, in this study a potent antifungal activity for both leaves and bark was evidenced, even in comparison with the reference drug ITR. In the microdilution assays, extract concentrations ranged from 500 to 0.000875 µg/mL for the plant extracts and 16 to 0.00003 µg/mL for ITR. It was observed that the Minimum Inhibitory Concentration (MIC) of the VGL and VGB extract were 3.9 µg/mL, while itraconazole (0.0078 < 16 µg/mL) was able to inhibit fungal growth until 0.97 µg/mL tested at 48 h of incubation (Table 2).

The antifungal *in vitro* study matched with the *in vivo* study where a significant reduction of the fungal load in the spleen, as reference target organ, after treatment with both extracts was observed. Surprisingly in this study it was detected that after 7 days of treatment in the infected animals group, VGL extract reduced the fungal infection in 53.9% (1.6×10^4 CFU), whereas ITR, reduced infection over 46.4% (1.9×10^4 CFU) compared to untreated animals (3.6×10^4 CFU) (Fig. 3). Although more studies are necessary to elucidate the mechanisms of this effect, we think that the immunomodulator effects of the plant can

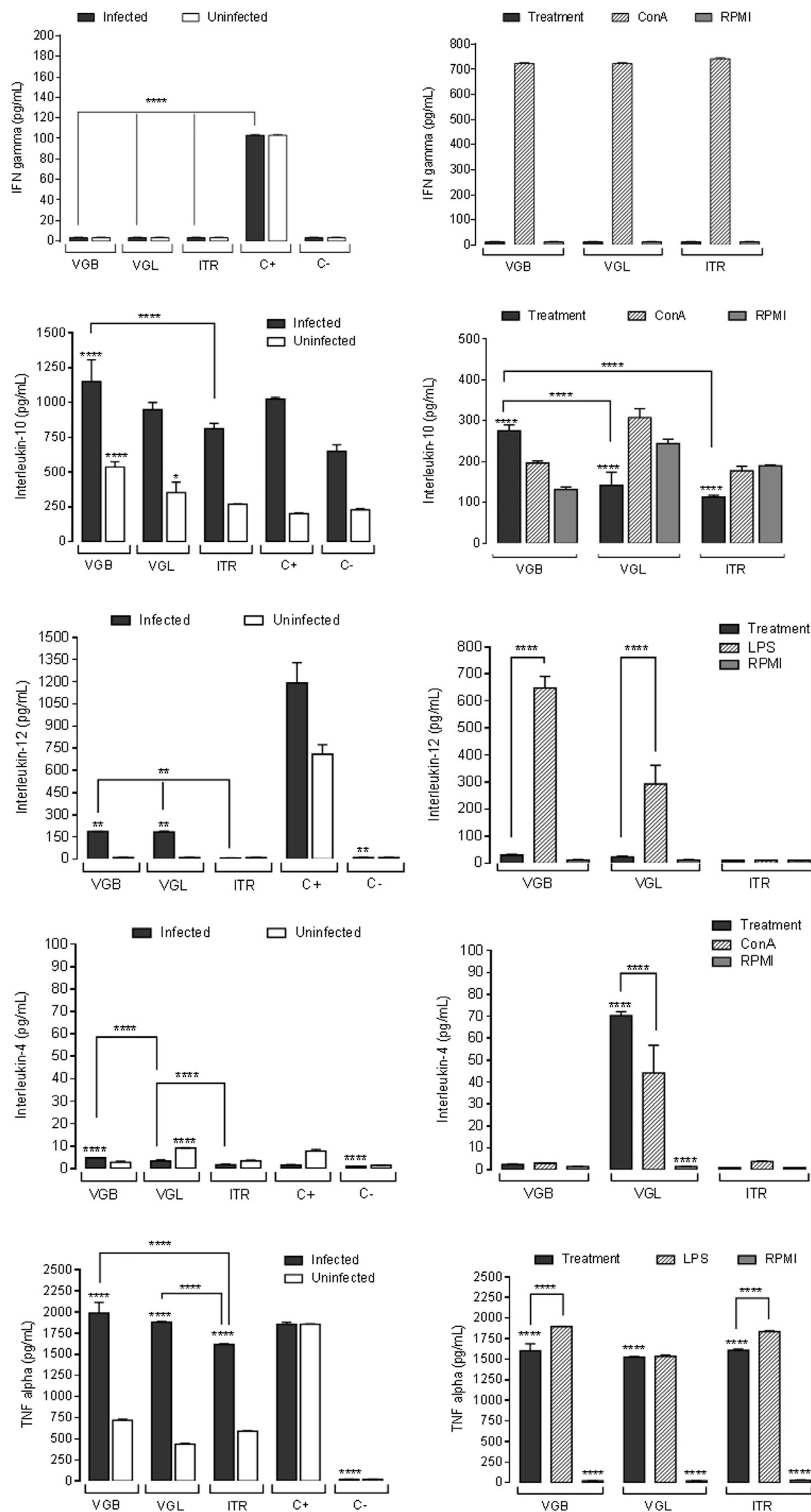

Fig. 4. Ex-vivo cytokine release. The cells (peritoneal macrophages or splenocytes) were cultured for 24 h in the presence of the test substancesThe cytokines TNF-α and IL-12, were quantified in the supernatant obtained from the culture of macrophages, while IL-4, IL-10 and IFN-γ in splenocytes culture, by ELISA. Left graphics depicts the cytokine releasing from infected or non-infected mice whose cells (either macrophages or splenocytes) where stimulated *in vitro* by the referred stimulus. Right graphics depicts the cytokine releasing treated with *V. guianensis* extracts ou itraconazol. VGL extract of *V. guianensis* stem bark; VGF extract of *V. guianensis* leaves, ITR: itraconazole, C +: LPS / Concanavalin A, C-: RPMI-1640 medium. Statistical significance was determined by 2-way ANOVA for multiple comparisons using the Sidak test and confidence interval *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) and ****($p < 0.0001$) compared with the control group at each point corresponding unless otherwise indicated. Results are presented as mean ± standard deviation of 3 animals.

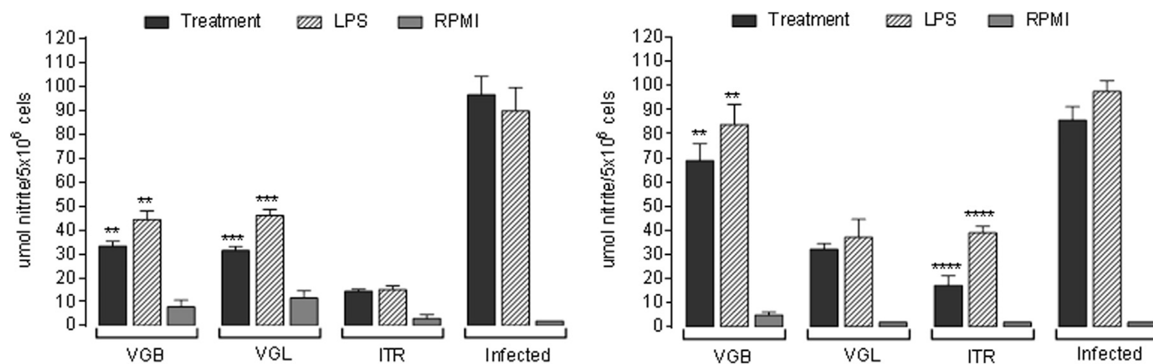

Fig. 5. Assay of nitric oxide production/inhibition: A) Nitric oxide production and B) inhibitory activity of the hydroethanol extract of bark and leaves extract of *V. guianensis* in peritoneal cell cultures of mice for the production of NO. Statistical significance was determined by 2-way ANOVA for multiple comparisons using the Sidak test and confidence interval **($p < 0.01$), and ****($p < 0.0001$) compared with the control group.

be involved in the more effective fungal clearance in comparison with ITR. A recent study suggested that an early immune response could be sufficient to overcome the fungal infection without antifungal drugs in this model (Ferreira et al., 2015).

The *ex vivo* cytokine release demonstrated that animals treated with extract from either leaves and barks are able of induce the production of cytokines, such as IL-10 and TNF-alpha and in lower proportion IL-4, IL-12, mainly in infected mice, while IFN-γ, other important cytokine participating in the immune response, is not stimulated (Fig. 4). These results suggest that the stimulation observed in the infected animals were consequence of the fungal infection, and the treatment can favours a profile anti-inflammatory. This pattern with reduction of pro-inflammatory cytokines and elevated production of IL-10 is associated with anti-inflammatory effect in plants (Wang et al., 2016). In addition, the observed result regarding the reduction of NO production, associated to the treatment with the extracts also support our hypothesis about the anti-inflammatory activity of the plant (Fig. 5).

The inflammatory response triggered by certain infections is frequently the cause of tissue damage and death (Garcia et al., 2010). In this way, this favourable anti-inflammatory profile induced by the evaluated extracts can be potentially important for reducing the tissular damage associated to the infectious process, helping for a faster recovery.

This study is the first to show the antifungal effect of *V. guianensis* against *S. schenckii in vitro* and *in vivo* as well as an antinflammatory effect that can act synergically to reduce the deleterious effect of the fungal infection and a faster relieve. These pharmacological effects observed seem to be associated to the balanced compositions of the extracts. Under this point of view, *V. guianensis* might be investigated as a promising candidate for antifungal treatment against sporotrichosis, specially with *in situ* detection of several prenylated benzoquinone with known antifungal effect. Further studies are being conducted to elucidate the antifungal and immunomodulatory mechanism of action of these extracts and the role of each component in the described effects.

Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2016.11.030.

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