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Evaluation of *Syngonanthus nitens* (Bong.) Ruhl. extract as antifungal and in treatment of vulvovaginal candidiasis

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The purpose of this study was to evaluate the *in vitro* anticandidal activity of a methanolic extract of *Syngonanthus nitens* scapes against different *Candida* species and clinical isolates from patients with vulvovaginal candidiasis (VVC), and its effect *in vivo* in the treatment of vaginal infection. Chemical characterization of the extract was performed by HPLC-UV analyses and showed the presence of flavones derivatives. The extract was effective against several *Candida* strains from our collection and species recovered from VVC patients, and was able to inhibit the yeast-hyphal transition. No cytotoxic activity against human female reproductive tract epithelial cells and no hemolytic activity against human red blood cells were observed. In the *in vivo* model of VVC, we evaluated the efficacy of the intravaginal treatment with a cream containing the extract at doses of 0.5, 1.0 and 2.0%. The treatment eradicated the vaginal fungal burden in infected rats after 8 days of treatment. *S. nitens* extract could be considered as an effective and non-toxic natural antifungal agent in the treatment of vulvovaginal candidiasis.

Keywords *Syngonanthus nitens*, anticandidal activity, vulvovaginal candidiasis, yeast-hyphal transition

Introduction

Vulvovaginal candidiasis (VVC) is a significant problem affecting 75% of all women at least once during their lifetime [1]. *Candida albicans*, the causative agent in 85–90% of these infections, is a commensal dimorphic fungus routinely found in the gastrointestinal and genitourinary tracts. Exogenous factors associated with acute VVC include modulations or imbalances in reproductive hormones caused by oral contraceptive usage, pregnancy, and hormone replacement therapy, as well as antibiotic usage, and diabetes mellitus [2–4].

Despite advances in antifungal therapies, many problems remain to be solved for most antifungal drugs available. Genital isolates of *Candida* have been reported to be slightly susceptible to one or more of the commonly used azoles [5]. Mechanisms of resistance to azole drugs have been described for *C. albicans*, all based on altered gene expression and appearing to frequently accumulate in individual clinical isolates for stepwise development of high-level drug resistance [6,7].

The genus *Syngonanthus*, a member of the Eriocaulaceae, includes about 200 species found in Africa and America, with *Syngonanthus nitens* exclusively found in South America [8]. Some species are of great economic importance since they are exported as ornamental plants to various countries, mainly Germany and Japan. The scapes from *S. nitens* are used in the manufacture of ornamental products [8,9]. Literature reports the traditional use of Eriocaulaceae species to treat skin ulcers and bacterial infections [10], and some studies have described antioxidant [4],

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antimicrobial [11], cytotoxic, mutagenic [12], and antiulcerogenic activity [13]. Although little is known about the ethnopharmacological properties of the genus *Syngonanthus*, chemical studies have shown the presence of important flavones, predominantly luteolin *O*- and *C*-glucosides and apigenin *O*-glucosides [9]. Recently, our group identified 17 compounds among flavones and xanthones, including six new molecules in *S. nitens* extracts [14]. Due to the antimicrobial activity of the flavonoids reported, it led us to investigate the possible biological activities of the scapes of *S. nitens* and their antifungal properties against *Candida* species.

In the present report, we investigated the antifungal, cytotoxic, and hemolytic properties of methanolic extracts of scapes from *S. nitens* and the antifungal activity of a vaginal cream containing this extract in the therapeutic treatment in an immunosuppressed rat model of VVC.

Material and methods

Candida *isolates, growth conditions and inoculums*

The reference species of *Candida* used in this study were all obtained from the American Type Culture Collection (Rockville, MD, USA), i.e., *C. albicans* (ATCC 18804), *C. albicans* (NCPF 3153), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 750). The 14 clinical isolates of *C. albicans* were kindly provided by the Mycology division of Central Laboratory of Clinical Hospital from National University of Córdoba, Argentina. The clinical isolates were obtained from vaginal fluid and the species identified by conventional methods, including growth on chromogenic medium (CHROMagar Paris, France) according to the manufacturer's instructions. The isolates were cultivated on Sabouraud dextrose agar (SDA) (Britania, Argentina) for 48 h at 35–37°C. The inoculum of the isolates of *Candida* used in the experiments was standardized by counting 1.0×10^6 yeast/ml with Neubauer chamber (OD 0.08–0.1 at 625 nm) [15]. The number of viable yeast was checked on SDA [16]. *C. albicans* (NCPF 3153) was used for *in vivo* infection [17–19].

Plant material and extraction

Scapes of *S. nitens* (Bong.) Ruhl. were collected in December 2008 in 'Serra do Jalapão', Tocantins State, Brazil. A voucher specimen was authenticated by Paulo Takeo Sano and deposited in the Herbarium of the Institute of Biosciences of the University of São Paulo, under number SPF 189975. The plant material was dried in an oven at 45°C for 48 h. The dried material was powdered (410 g) and extracted successively with hexane, dichloromethane, and methanol by percolation. The methanolic extract (16 g) was used in this study.

Chemical characterization of the extract

For the chemical study, the extract was purified by solid phase extraction on silica cartridges C-18 (SPE-RP18, Phenomenex Co®, Torrance, CA, USA). The extract was dissolved in methanol and applied to the cartridge. Elution was performed according to Pacifico et al., 2011, using H2O/MeOH (58:42) as eluents and a flow rate of 2mL/min. Then the samples were dried, dissolved in methanol:water 5:95 v/v, filtered and analyzed by HPLC on a C-18 reverse-phase column (μ-Bondapak C-18, Waters®, Foster City, CA, USA, 250×4.60 mm i.d., particule size 5 μm), and detected with a photodiodes array detector with scanning range of 195–650 nm and a minimum interval of 1 nm, Model MD-20 140, and controlled by the software *Star Chromatography Workstation* 5.31 (Varian) e *EZChrom Elite Client/Server* 3.1.7 (Chromatec) for the processing of chromatographic data. Total flavonoids were estimated according to Georgetti *et al*. [20] and were calculated as luteolin equivalent from an analytical curve.

In vitro *antifungal activity*

The antifungal activity and MIC was performed according to a standard reference method [15]. The extract was dissolved in 25% methanol and water. The initial concentration of extract was 2000 mg/l and 0.1 ml of this concentration was added in a 96-well microtiter plate containing RPMI 1640 medium. The initial test concentration was serially diluted two-fold. Each well was inoculated with 0.02 ml of suspension containing 2.5×10^3 yeast/ml of yeast. The antifungal fluconazole (Lazar Laboratorios®, Buenos Aires, Argentina, initial concentration 65 mg/l) and 25% methanol and water were included in the assays as positive and negative controls. The microplates were incubated at 48 h at 37°C. The MIC of sample was detected following addition (0.05 ml) of 2.0% triphenyltetrazolium chloride (TTC, Sigma-Aldrich Brazil Ltda, São Paulo) [21]. The growth of yeast was visualized by changes to a red color. MIC was defined as the lowest extract concentration showing no visible fungal growth after incubation time. A sample from each well that showed antifungal activity was plated on SDA for to determination of minimal fungicidal concentration (MFC). In all assays, the samples were processed by triplicate, and each experiment was carried out in triplicate.

Inhibition of hyphal growth

Growth of *C. albicans* (NCPF 3153) from a 48 h stationary phase culture were transferred to microplate with RPMI 1640 medium supplemented with fetal bovine serum (FBS, PAA) to obtain a final concentration of

2.5×10^3 yeast/ml. Extract solution was added to the growth medium to obtain final concentrations of 125, 250 and 500 mg/l ($0.5 \times$ MIC, $1 \times$ MIC and $2 \times$ MIC), and the cultures were incubated for 12 and 24 h at 37°C, in 5% CO_2. The hyphal formation of *C. albicans* was observed through an inverted light microscope (Nikon TE 2000-U Eclipse) with a magnification of 400×. Amphotericin B (AMB, Fada Pharma®, Buenos Aires, Argentina, 5 mg/l) was used as a positive control [7].

MTT and LDH cell viability assay

Human epithelial cell line Hela cells (ATCC CCL-2) were cultured in a 96-well microtiter plate (Costar) and adjusted at cell density of 10^3 cells/ml in RPMI medium. The cells were treated with the extract (125, 250, 500 mg/l) for 24 h. Cell viability was determined using the conversion of MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) to formazan via mitochondrial oxidation [22]. MTT solution was added to each well at a final concentration of 10,000 mg/l and the plates were incubated at 37°C for another 4 h. After incubation, 200 µl of DMSO was added to each well to dissolve the dark-blue crystals of formazan and absorbance was measured at 570 nm using a BIO-RAD Microplate Reader, and data were expressed as viability index. Due the presence of flavonoids in the extract, the flavonoid luteolin (31 mg/l; Sigma) was included in the assays.

Cell injury was quantitatively assessed by the measurement of release of the cytoplasmic enzyme lactate dehydrogenase (LDH) after damage of the cells [23]. An aliquot of supernatant of Hela cells cultured in presence of extract (125, 250, 500 mg/l) or medium alone, were recovered for LDH assay. The data were expressed as LDH release and all experiments were performed in triplicate, as well as being repeated at least three times.

Hemolytic assay

Human erythrocytes from healthy individuals were collected and harvested by centrifugation for 10 min at 2000 rpm = 1200 × g (model Eppendorf 5810, Eppendorf®, São Paulo, Brazil) and washed three times by centrifugation in PBS. PBS was added to yield a 10% (v/v) erythrocytes/PBS suspension. This suspension was diluted 1:10 in PBS and 0.1 ml added to 0.1 ml samples from a two-fold dilution series of the extract in the same buffer, performed in triplicate, in 96-well microtiter plate. Total hemolysis was achieved with 1% Triton X-100. The microplate was incubated for 1 h at 37°C and then centrifuged for 10 min at 2000 rpm = 1200 × g. The absorbance of the supernatant was measured spectrophotometrically at 450 nm and the percentage of hemolysis was calculated [24]. The experiments were performed in triplicate and the data obtained were statistically analyzed using an analysis of variance (ANOVA) followed by Dunnett test (multiple comparisons with one control). A P value < 0.05 was considered statistically significant.

Preparation of vaginal cream

To prepare the vaginal cream at concentrations of 0.5, 1.0 and 2.0% of extract (w/w), we used a formula derived from an oil/water emulsion. Aqueous and oily phases were heated separately to 75°C for complete melting of the fatty material, pooled, and stirred until cool for incorporation of the extract, previously dissolved in propylene glycol. This cream was prepared without a preservative.

Experimental VVC

Female Wistar rats (body weight 100–150 g) were collectively housed in the experimental room for at least 7 days before experiments started. Animals were maintained at the Animal Resource Facility of the CIBICI-CONICET in accordance with the institutional guidelines. Protocols were approved by the Animal Experimentation Ethics Committee, Faculty of Chemical Science, National University of Córdoba (EUNC0045378/2010/RD939).

The rat model of vaginal infection was established based on modified models described previously [25–27] to obtain a chronic and homogeneous infection. Animals were immunosuppressed by administration of one dose of cyclophosphamide (CPA) (Sigma®, 20 mg/kg b.w.) and estrus was induced by subcutaneous administration of estradiol (Sigma®) at a dose of 0.2 mg/ml once daily for 4 days before infection. Rats were inoculated intravaginally (day 0) with 0.1 ml of *C. albicans* (NCPF 3153) (5.0×10^7 yeast/ml) [17], using a micropipette with disposable tips. On day 2, 6, and 10 after the infection, the vaginal load of *C. albicans* was evaluated through vaginal lavage with 0.1 ml of PBS, and determined by the CFU assay on SDA [28]. At day 10 the animals were euthanatized and vaginas were longitudinally removed. All vaginal sections were stained with Hematoxilin-Eosin (HE) and periodic acid-Schiff (PAS) and visualized by light microscopy.

Therapeutic treatment

For the evaluation of the clinical effects of extracts on *C. albicans* vaginal infections, the cream containing extract, the cream without extract (cream base) and miconazole cream (MCZ, Medley®, Campinas, São Paulo, Brazil) as control, were administrated topically to the infected animals. Wistar female ($n = 42$) were randomized equally into the following seven groups: Group 1: non-infected control; Group 2: infection control; Group 3: infected and treated

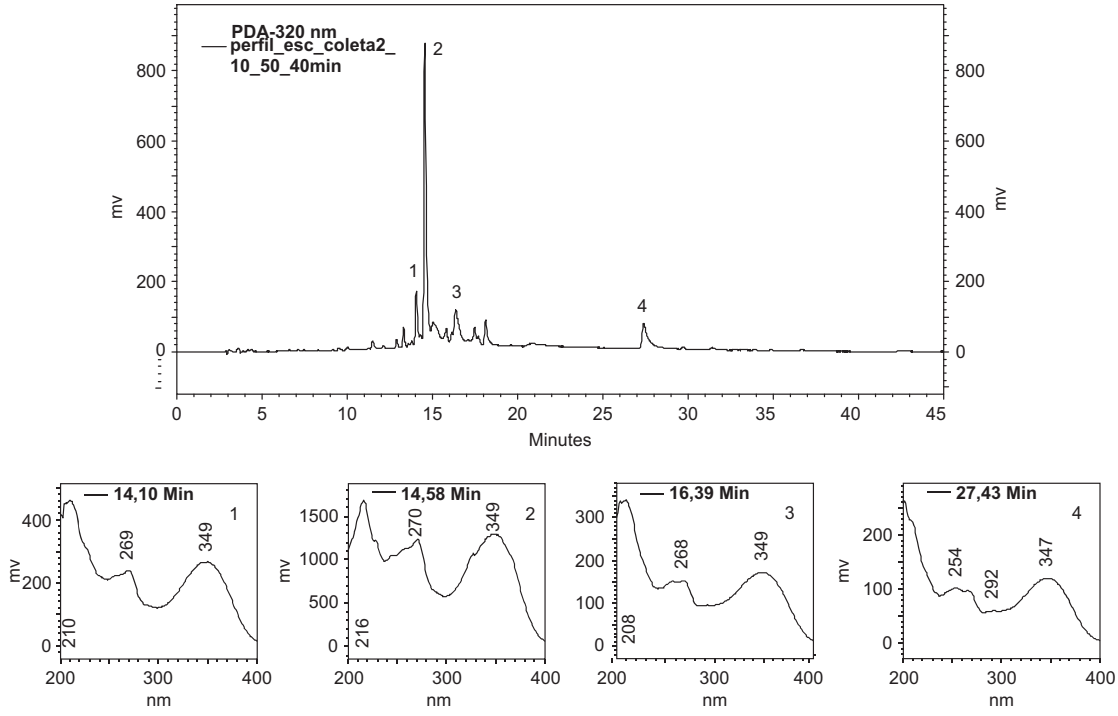

Fig. 1 Chromatogram representing the profile of the methanolic extracts of *Syngonanthus nitens* scapes (RP-18, 250 × 4.60 mm i.d. × 5 µm; Solvent A: H_2O + 0.05% TFA, solvent B: ACN + 0.05% TFA. Gradient 10–50% B in A in 40 min, flow 1.0 ml.min^{-1}, λ = 320 nm) and absorption spectra in the UV region of the peaks highlighted.

with MCZ cream (0.1 ml); Group 4: infected and treated with cream with 0.5% of extract (0.1 ml); Group 5: infected and treated with cream with 0.1% of extract (0.1 ml); Group 6: infected and treated with cream with 2.0% (0.1 ml); and Group 7: infected and treated with the cream without extract (vehicle control – 0.1 ml). Cream with extract, cream base alone or MCZ was administered to the infected animals two times per day for 7 consecutive days. *Candida* vaginal infections in the treated and control rats were monitored in each animal by means of enumerating the CFU/ml in the vaginal fluid on days 2, 6 and 10 of treatment [25–27]. Twenty-four hours after the administration of the last dose of antifungal agent (day 10), animals were euthanatized, and vaginas were removed and processed for histological studies.

Data were expressed as means ± SD. Differences between group means were assessed using a one-way ANOVA followed by Tukey test. A P value < 0.05 was considered statistically significant.

Results

Chemical characterization

The analysis of some spectra obtained in the UV region indicated the presence of flavonoids, and flavones derivatives (Fig. 1). In the flavonoid UV spectrum it was possible to differentiate two absorption maxima, i.e., band II which was in a wavelength range of 240–285 nm and band I in the range of 300 and 550 nm. The nature of the flavonoid and its oxygenation pattern can be defined by the position and relative intensities of the absorption maxima. When there are changes in the A ring, band II is modified and revisions in rings B and C result in alterations of band I. The characteristic bands of flavones are located between 250 and 280 nm (band II) and 310–350 nm (band I) [29]. The total flavonoid content in the extract was 36.70% ± 0.07.

Antifungal susceptibility and inhibition of C. albicans *yeast-hyphal transition*

The results of the *in vitro* antifungal studies with different *Candida* species are shown in Table 1. MIC values for the

Table 1 Antifungal activity of the methanolic extract of scapes of *Syngonanthus nitens*.

Tested sample	*Candida albicans*[a]		*Candida albicans*[b]		*Candida krusei*		*Candida parapsilosis*		*Candida tropicalis*	
	MIC[c]	MFC[c]	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Extract	250	500	250	500	32.75	32.75	62.5	62.5	62.5	250

[a]ATCC 18804; [b]NCPF 3153; [c]Values given as mg/l.

Table 2 Antifungal activity of the methanolic extract of *Syngonanthus nitens* scapes against clinical isolates of *Candida albicans*.

Candida albicans	MIC	*Candida albicans*	MIC[a]
VF 1	125	VF 8	62.5
VF 2	62.5	VF 9	250
VF 3	125	VF 10	250
VF 4	125	VF 11	250
VF 5	125	VF 12	62.5
VF 6	62.5	VF 13	125
VF 7	125	VF 14	250

VF, vaginal fluid; [a]Values given as mg/l.

methanolic extract of scapes from *S. nitens* could be detected and higher concentrations of the tested extracts were required for growth inhibition of *C. albicans*. The MIC and fungicidal concentrations of the extract were the same for C. *krusei* and *C. parapsilosis*, but higher concentrations were required to achieve the same effects for *C. albicans* and *C tropicalis*. When the susceptibility of *C. albicans* clinical isolates recovered from CVV patients were tested, distinct growth inhibition endpoints were observed (Table 2). The MIC range of the extract was 250–62.5 mg/l, with six of 14 isolates were susceptible to 125 mg/l.

Hyphal growth is considered to be part of the virulence mechanism of *C. albicans*. Figure 2 illustrates the fungal growth and hyphal formation after 12 and 24 h of development in culture. After the exposure to different concentrations of the extract or AMB, the hyphal formation of *C. albicans*, as well as yeast growth was markedly inhibited.

Toxicity in human epithelial cells

To study whether the extract inhibited HeLa cell growth, we examined the cytotoxic effects using an MTT assay, after 24 h of treatment at different concentrations of extract. As shown in Figure 3, luteolin (positive control) significantly inhibited cell growth at the tested concentration ($P < 0.05$). Cells viability was not affected by the extract treatment when compared with untreated cells.

Cell injury was quantitatively assessed by the measurement of LDH release. After 24 h incubation, luteolin significantly increased cell cytotoxicity ($P < 0.05$) compared to the untreated control. No increased LDH release occurred with different extract concentrations compared to controls (Fig. 3).

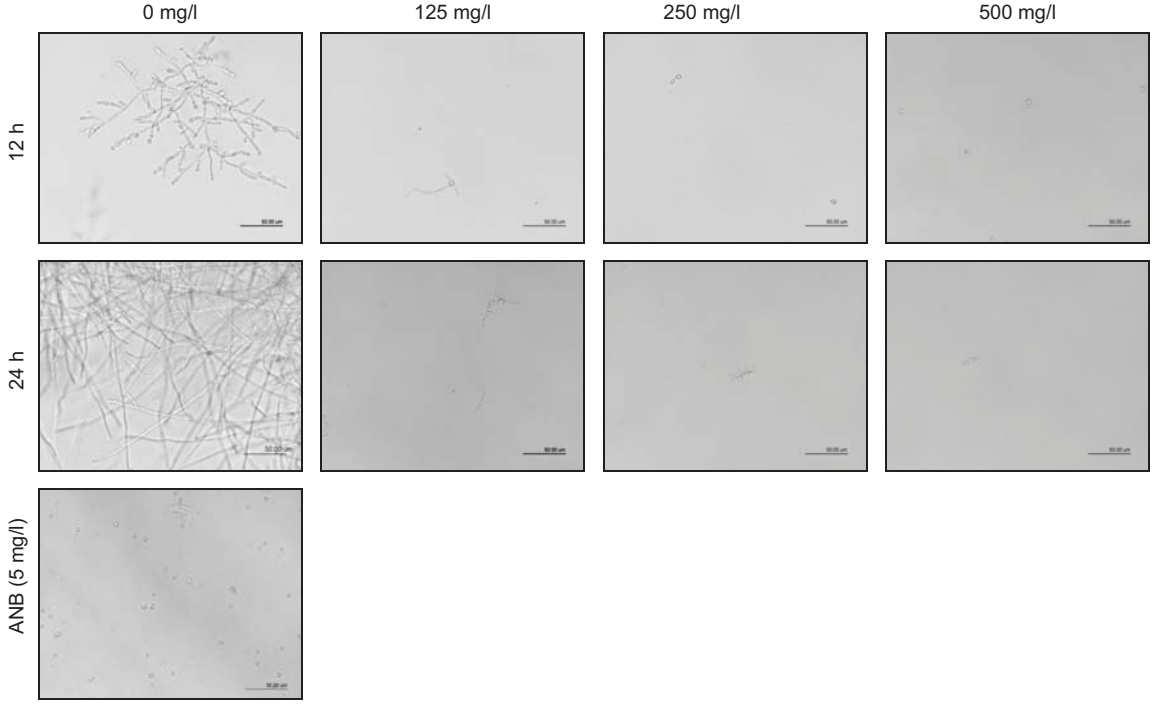

Fig. 2 Hyphal formation of *Candida albicans* NCPF 3153. *Candida albicans* were cultured with different concentration of methanolic extract of *Syngonanthus nitens* (125, 250 and 500 mg/l) during 12 and 24 h at 37°C, 5% CO_2. Amphotericin B (AMB, 5 mg/l) was used as a positive control. The experiments were performed in duplicate and repeated three times. Representative microphotographs are showed. The black bar represents a length of 50 μm (magnification of 400×).

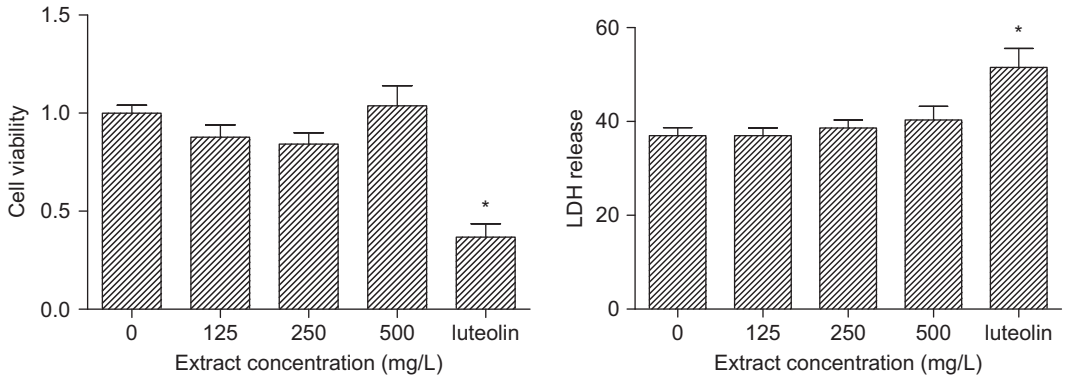

Fig. 3 Anti-proliferative effect of the *Syngonanthus nitens* extract on HeLa cells. Cells were treated with different concentrations of extract. Luteolina (31 mg/l) was used as positive control. Viability was determined using the MTT assay and LDH leakage assay. The results are the means ± SD of three independent experiments.* Significantly different from the basal conditions and treatments with the extract $P < 0.05$.

Hemolytic activity

The potential hemolytic activity of the extract was investigated by measuring the lysis of a 10% (v/v) human red blood cell suspension through the use of a spectrophotometric assay. In this experiment, Triton X-100 1% (positive control) induced 95.0 ± 3.1% of red blood cell lysis whereas the negative control (solvent) induced no lysis. The extract was tested at an initial concentration of 1000 mg/l and serially diluted to 78 mg/l but did not cause a significant red blood cell lysis ($P =$ NS) (Fig. 4).

Experimental VVC and therapeutic treatment

After establishing antifungal activity *in vitro*, we examined the activity of a vaginal cream containing the extract in an immunosuppressed and estrogen-dependent rat model in which the animals were infected with *C. albicans*. The rats were infected with *C. albicans* on day 0 and fungal burden was assessed after 2 days. At this time the infected animals were treated with different concentrations (0.5, 1.0 and 2.0% of extract) of the vaginal cream or MCZ. Figure 5a shows the results obtained in different groups. The treatment exerted a marked acceleration of clearance of the yeast, as demonstrated by a statistically significant decrease in CFU counts on the 4th day after the initiation of treatment (day 6), compared to the untreated and vehicle-treated controls. MCZ treatment, used as a positive control, showed a pattern of clearance comparable to that induced by the vaginal cream with different extract concentrations. Table 3 shows that vaginal cream caused a rapid clearance of the fungal burden from the vagina of the experimentally infected rats. The number of animals infected significantly decreased after the fourth day treatment with 2.0% vaginal cream and there was a statistically significant difference compared to the controls. Our results show that the control group of infected and untreated animals remained infected throughout the experiment.

Vaginal sections of all the animals were also studied by light microscopy and representative images are presented in Figure 5. The histological findings for infected and untreated animals showed extensive presence of the

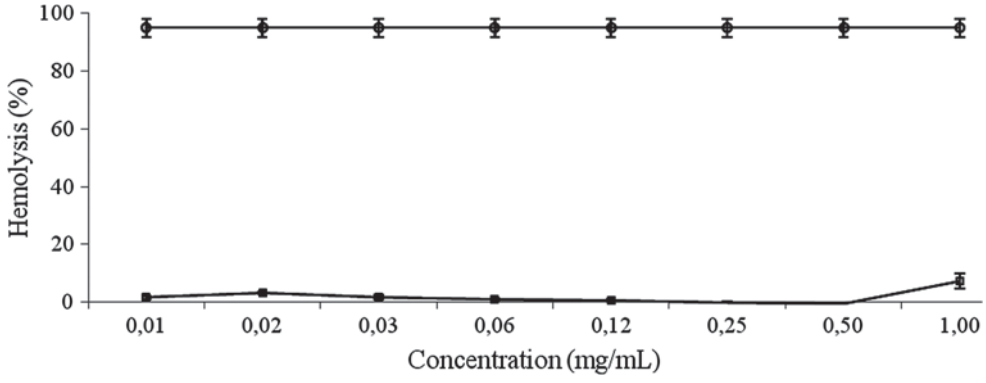

Fig. 4 Hemolytic activity of extract of scapes from *Syngonanthus nitens*. The hemolytic activity of different concentration of extract was tested against human erythrocytes (squares). Triton X-100 was employed as positive control (circle). Each curve represents the mean ± SD of three independent experiments ($P =$ NS).

Table 3 Number of infected animals and quantification of fungal burden observed for animals treated with vaginal cream containing different concentrations of methanolic extracts of scapes of *Syngonanthus nitens* and their respective controls.

	Day 2		Day 6		Day 10	
Treatment Group	Infected animals (%)	Log CFU ± SD	Infected animals (%)	Log CFU ± SD	Infected animals (%)	Log CFU ± SD
Infected control	6/6 (100)	3.48 ± 0.85	6/6 (100)	2.18 ± 0.66	6/6 (100)	2.94 ± 0.96
Positive control (MCZ)	6/6 (100)	2.76 ± 0.53	0/6 (0)	0 ± 0*	0/6 (0)	0 ± 0*
0.5% vaginal cream	6/6 (100)	2.86 ± 0.09	3/6 (50)	1.09 ± 0.31*	0/6 (0)	0 ± 0*
1.0% vaginal cream	6/6 (100)	3.19 ± 0.15	2/6 (33.3)	1.04 ± 0.07*	0/6 (0)	0 ± 0*
2.0% vaginal cream	6/6 (100)	3.23 ± 0.65	2/6 (33.3)	0.46 ± 0.30*	0/6 (0)	0 ± 0*
Vehicle	6/6 (100)	3.04 ± 0.62	6/6 (100)	2.86 ± 0.37	6/6 (100)	3.29 ± 0.37

*$P < 0.05$ compared to treatment controls.

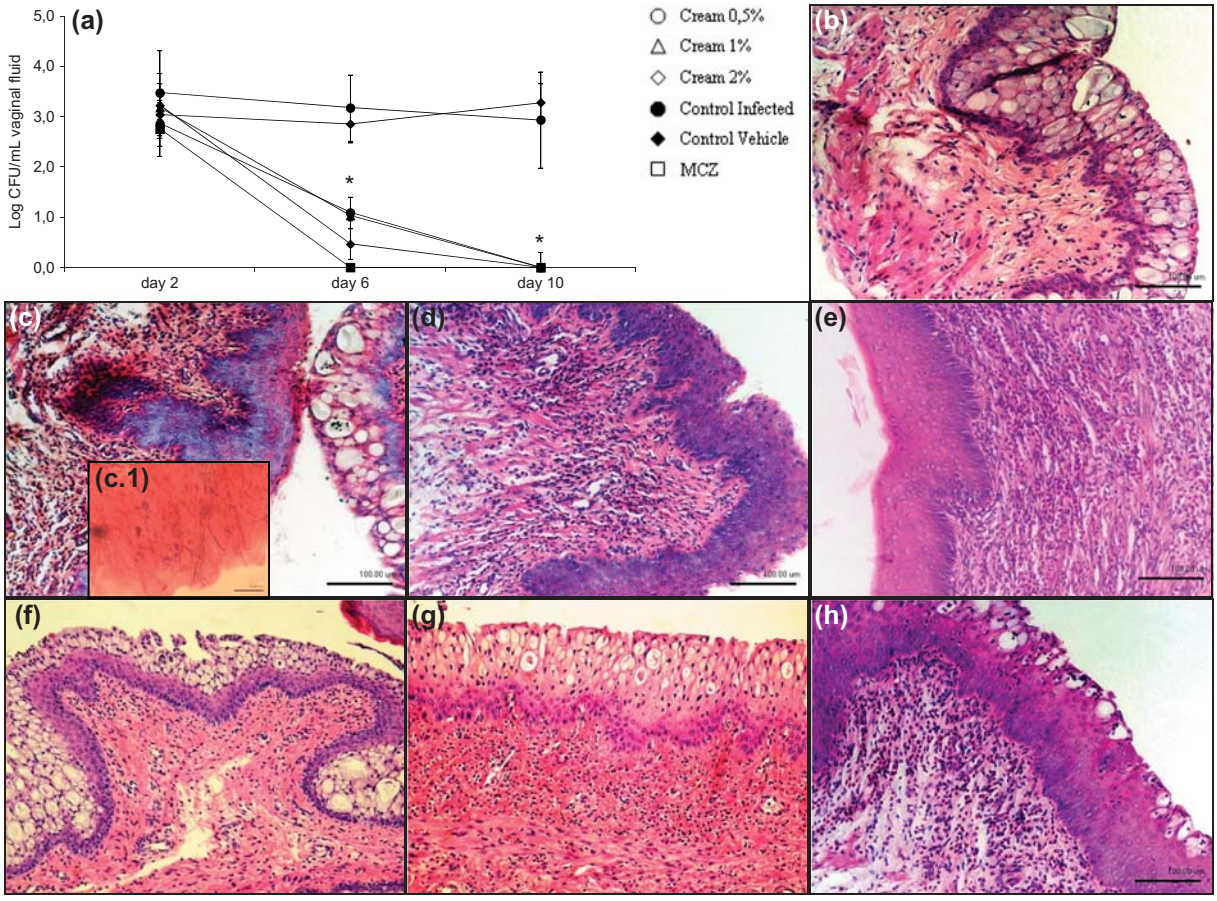

Fig. 5 Effect of vaginal cream containing extracts of *Syngonanthus nintes* on VVC. (a) Effect of vaginal cream containing 0.5% (open circle), 1.0% (open triangle) and 2.0% (open diamond) of methanolic extracts of scapes of *S. nitens* on vaginal fungal burden of rats infected with *C. albicans* and their respective controls: infected (filled circle), vehicle (filled diamond) and MCZ (open square). Data are expressed as mean ± SD of two independent experiments (rats per group $n = 6$ for each treatment).*Significantly different from the vehicle and infected non-treated animals ($P < 0.05$). *Significantly different from the vehicle and infected non-treated animals. Histological evaluation of vaginal sections after different treatments: (b) Non-infected, immunosuppressed, estrogen-dependent animals (negative control); (c) Infected immunosuppressed, estrogen-dependent non-treated animals. Inset c.1 showing extensive colonization by numerous hyphae (Magnification 1000×, black bar represents a length of 20 µm); (d) Infected, immunosuppressed, estrogen-dependent, treated with MCZ (positive control). No evidence of *Candida albicans* infection was seen; (e) Infected animals treated with cream base (vehicle control); (f) Infected animals treated with 0.5% vaginal cream; (g) Infected animals treated with 1.0% vaginal cream; (h) Infected animals treated with 2.0% vaginal cream. Representative photomicrographs obtained 10 days post infection. HE and PAS stain. Magnification = 100×. The black bar represents a length of 100 µm. **"**This Figure is reproduced in color in the online version of *Medical Mycology*."

invasive fungus morphotype, numerous hyphae involving the stratum corneum and the luminal keratin debris was accompanied by a inflammatory cell response with characteristic leukocyte microabscesses (Fig. 5c, 5d); as was expected, the non-infected untreated control group did not show the fungus present or histopathological alteration (Fig. 5b). Therapeutic treatment with vaginal cream at different concentrations completely eradicated vaginal *C. albicans* and only a residual inflammatory infiltrate and ulcerations indicated that a previous infectious process was affecting the local mucosa (Fig. 5f, g and h). Regarding the positive control group, *C. albicans* was not found in the vaginal lumina, and regenerative changes associated with the restoration of the vaginal mucosa to its normal status following MCZ treatment could also be seen (Fig. 5d).

Discussion

The ethnopharmacological properties of *Syngonanthus nitens*, a member of the Eriocaulaceae family which is exclusively found in South America, have not been elucidated. However, it is known that other members of this family have been used for treatment of different pathologies, as well as exhibiting antimicrobial and antifungal activities [4,10–13]. In the present study, we investigated the antifungal properties of a methanolic extract of scapes from *S. nitens*. The extract exhibited antifungal activity against the five species of *Candida* included in the tests and fungistatic activity was only noted with *C. albicans*, *C. parasilopsis* and *C. tropicalis* at higher concentrations. Pathogenic strains of *C. albicans* recovered from patients with VVC also were sensitive to the antifungal effect of the extract. Chemical analysis of Eriocaulaceae species revealed the presence of flavonoids [9,14] which have been isolated and identified in compounds with antimicrobial activity [30]. The results presented in this study concerning the chemical composition of the extract analyzed by HPLC-UV confirmed the presence of flavones as major compounds. Our results are in agreement with those reported by Pacifico *et al*. [14], which showed the presence of flavonoid and xanthone compounds in *S. nitens* scapes and flowerheads, and 17 compounds were isolated, including six new molecules.

The array of virulence attributes displayed by *C. albicans* makes this opportunistic yeast a successful commensal of human mucosal surfaces and contributes to its pathogenic behavior in patients with innate or acquired immune defects [31–33]. The ability of *C. albicans* to exhibit different morphotypes is undoubtedly one of the most important strategies of aggression and evasion by this pathogen. As a pseudohyphal form, *C. albicans* has the ability to invade host tissues, avoid phagocytic cell ingestion and survive intracellular control mechanisms [7,33,34]. In this study, we provide evidence that the extract we prepared was able to inhibit the transition from yeast to hyphal form which further suggests the use of extracts of *S. nitens* in the treatment of fungal infections.

In vitro cytotoxicity assays can be used to predict human toxicity by a general screening of chemicals. The LDH leakage and the MTT assays are the most commonly used for the detection of cytotoxicity or cell viability following exposure to toxic substances. We performed these assays using cultures of a human female reproductive tract epithelial cell line to evaluate the *S. nitens* extract toxicity. Since luteolin and their derivatives are the major constituents present in *Syngonanthus* species [9], it was included in our studies. While the extract was harmless to epithelial cells at all concentrations evaluated, luteolin induced HeLa cell damage, which is in agreement with its known anti-proliferative activity [35]. Interestingly, despite the chemical composition of *S. nitens* extract, the toxic activity of flavonoid could be inhibited by the presence of other compounds present in this natural product. Preparations obtained from plants provide a complex mixture of different chemicals, which can act synergistically or antagonistically in the induction of cytotoxicity and other biological activities [36]. Human red blood cells can also be used as tools in the toxicity studies of the compounds, because their membrane properties are well known, and their lysis can be easily monitored by measuring the release of hemoglobin [24]. Although many antifungal drugs, such as amphotericin B, have hemolytic activity which induces a fall in production of red blood cells and are highly nephrotoxic [25,26], our results indicate that extract do not have hemolytic activity. Taken together, the MTT, LDH, and hemolytic results indicate that extract was not harmful for the human cells tested.

A number of clinically relevant models of mucosal candidiasis have been established. However, the development of mucosal infection models generally requires the use of immunosuppressive agents, antibiotic, estrogen treatment, or the use of germ-free animals [2,3,27,31]. Local rather than systemic immunity is critical for anti-*Candida* defense in the vaginal mucosa. Estrogen transforms the columnar epithelium into thicker stratified squamous epithelium and increases the glycogen content, pH, and growth substrates, all of which facilitate *C. albicans* infection. Estrogen may also inhibit innate or adaptive immune defenses, thus facilitating tissue evasion [4,37], but in the absence of pseudoestrus, vaginal infections are short-lived [3,38]. The use of immunosuppressive is a valid method of quickly inducing fungal colonization. In rodents, CPA inhibits the production of antibodies, and is active in cells with high mitotic activity inhibiting cellular and humoral immune responses [37,38]. Studies have shown

that immunosuppression is necessary to the success of VVC model [25,26,38].

The yeast concentration, 5×10^7 CFU/ml, for the inocula used to induce primary vaginal infection was based on a previously standardized study [27] as a single intravaginal dose 5 days after hormonal induction in CPA-treated rats. The infection was considered sufficient if a mean count for the vaginal lavage cultures from each rat was at least 10^3 CFU/ml. Dhawan *et al*. [26] evaluated the efficacy of a compound obtained from *Mentha* spp. that was found to possess promising antimicrobial property in an *in vivo* model immunosuppressed with CPA. Using a similar model, Mondello *et al*. [39] investigated the *in vivo* anti-*Candida* activity of essential oil from *Melaleuca alternifolia* and its bioactive component, terpinen-4-ol, respectively, showing that both compounds were active against different species of *C. albicans*. Zhang *et al*. [7] evaluated the anti-*Candida* activity of a saponin isolated from *Tribulus terrestris*, finding significant antifungal activity after 14 days of treatment. We examined the therapeutic activity of methanol extracts of scapes from *S. nitens* incorporated into a vaginal cream formulation in an experimental model of immunosuppressed and estrogen-dependent VVC. After the 4 days of treatment with the cream containing the extract at 0.5, 1.0 or 2.0%, the fungal burden was reduced significantly and, on the 8th day, the reduction was 100%. The histological study of groups treated with vaginal cream showed residual inflammatory infiltrates, epithelial desquamation and hyperkeratosis as signs of tissue reaction associated with the infection. Despite therapeutic treatment with extract completely eradicated the vaginal fungal burden from infected rats, the extract was not able to decrease the signals of inflammation. Histologically, the epithelium of the vagina of non-infected group was no different from animals treated with MCZ.

In conclusion, our results demonstrate that the methanolic extract of scapes from *S. nitens* is a natural product with antifungal properties against several species of *Candida* in our collection and those species recovered from VVC patients. Additionally, advantages of the extract were its ability to inhibit a key step of fungal-host invasion and its innocuous effects on human cells. Finally, we also provide robust evidence about the *in vivo* effects of *S. nitens* extract in the treatment of VVC in an animal model. The results of this study indicate that *S. nitens* represents a potential source of antimicrobial compounds in the treatment of this mycotic disease.

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