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Anti-*Trichomonas vaginalis* activity of marine-associated fungi from the South Brazilian Coast

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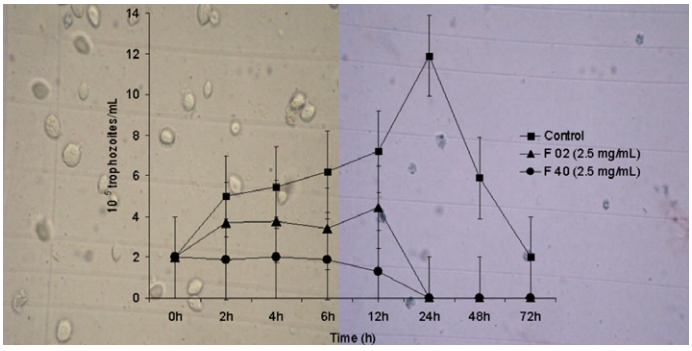
HIGHLIGHTS

- 42 marine-associated fungi species were selected to be screened against *T. vaginalis*.
- *Hypocrea lixii* and *Penicillium citrinum* were active against all isolates.
- The active filtrates also inhibited the metronidazole-resistant isolate growth.
- The *P. citrinum* F40 filtrate sample showed low cytotoxicity against Vero cells.
- Fungi from South Brazilian Coast are a potential source of antiparasitic molecules.

GRAPHICAL ABSTRACT



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ABSTRACT

Trichomonas vaginalis is the causative agent of trichomonosis, the most common non-viral sexually transmitted disease. Infection with this protozoan may have serious consequences, especially for women. Currently, 5-nitroimidazole drugs are the treatment of choice for trichomonosis, but the emergence of resistance has limited the effectiveness of this therapy. In this context, this study aimed to evaluate the anti-*T. vaginalis* activity of marine-associated fungi found in the South Brazilian Coast. A total of 42 marine-associated fungal species (126 filtrate samples) isolated from 39 different marine organisms, mainly sponges, were selected to be screened against *T. vaginalis*. Of these, two filtrate samples from *Hypocrea lixii* F02 and *Penicillium citrinum* F40 showed significant growth-inhibitory activity (up to 100%) against ATCC 30236 and fresh clinical isolates, including a metronidazole-resistant isolate. Minimum inhibitory concentration (MIC) values of *H. lixii* F02 and *P. citrinum* F40 samples for all isolates tested, including the metronidazole-resistant isolate, were 2.5 mg/mL. The kinetic growth curve showed that the filtrate samples were able to reduce the density of parasites to zero within 24 h of incubation, which was confirmed by microscopy. Both fungal filtrate samples exhibited no hemolytic activity, and the *P. citrinum* F40 filtrate sample showed low cytotoxicity against Vero cells. These data suggest that marine-associated fungi from the South Brazilian Coast may produce potential candidates for further investigation and possible use in the treatment of metronidazole-resistant trichomonosis.

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1. Introduction

Tricomonosis is a sexually transmitted disease (STD) caused by the protozoan parasite *Trichomonas vaginalis*. The spectrum of clinical presentation ranges from mildly symptomatic or totally asymptomatic, especially in men, to severe vaginitis with abundant vaginal discharge and cervicitis in women (Gilbert et al., 2000). Trichomonosis is the most common non-viral STD worldwide (WHO, 2001), and infection with this protozoan may lead to serious health complications, such as infertility (Cudmore and Garber, 2010), preterm delivery, low birth weight (Goldstein et al., 1993; Cotch et al., 1997), and cervical cancer (Viikki et al., 2000). Moreover, there is a strong association between human immunodeficiency virus (HIV) acquisition and *T. vaginalis* infection (van der Pol et al., 2008; Mayer et al., 2012).

Globally, the number of new trichomonosis cases is estimated to be 174 million per year (WHO, 2001), and 5-nitroimidazole drugs are recommended as the treatment of choice for this STD (Helms et al., 2008). However, treatment with these drugs is limited due to the increasing number of documented *T. vaginalis*-resistant isolates (Blaha et al., 2006; Upcroft et al., 2009; Krashin et al., 2010). Drug resistance poses major challenges to the management of infection, particularly with the paucity of new drugs with activity against this protozoan. This prompted us to investigate marine-associated fungi from the South Brazilian Coast as new alternatives to 5-nitroimidazole drugs currently used in the treatment of *T. vaginalis* infection.

Unlike investigations of well-known protozoan parasites, such as the causative agents of malaria, schistosomosis, Chagas disease, onchocercosis, leishmaniosis, and African trypanosomosis, research on natural products with antitrichomonal activity is rarely found in the literature. Anti-*Trichomonas* activity has been described in studies on marine organisms (Gehrig and Efferth, 2009; Watts et al., 2010), such as algae (Moo-Puc et al., 2008; Cantillo-Ciau et al., 2010; Machado et al., 2010), and on marine microorganisms, such as dinoflagellate *Amphidinium* sp. (Washida et al., 2006), but no study has specifically examined antitrichomonal activity in marine fungi.

Several biological activities of marine microorganisms have been described, such as antibiofilm, antimicrobial, antitumor, antiviral, antioxidant, and anti-inflammatory activities, as well as cell cycle and phosphatase/kinase inhibition (Mayer and Hamann, 2005; Blunt et al., 2007; Scopel et al., unpublished results). In addition, some metabolites from marine-derived fungi have been reported to have antiprotozoal activities against *Trypanosoma cruzi* and *T. brucei*, *Plasmodium falciparum* (Kasettrathat et al., 2008; Pontius et al., 2008; Watts et al., 2010), and other protozoa. However, to our knowledge, there is as yet no study examining antitrichomonal activity in marine-associated fungi from the South Brazilian Coast.

Given the need for new antiprotozoal therapies and the emerging importance of marine-associated fungi as a source of bioactive molecules, this study aimed to evaluate the anti-*T. vaginalis* activity of marine-associated fungal species isolated from different marine organisms, mainly sponges, found in the South Brazilian Coast.

2. Materials and methods

2.1. Fungal isolates and cultivation

Forty-two marine-associated fungi were isolated from 39 marine organisms, which were obtained from the Arvoredo Island in the Arvoredo Biological Marine Reserve (27°S 16′ 42.4″/48°W 22′ 30.8″/Santa Catarina state, Brazil) in November, 2007. All fungal strains were identified and the sequences were deposited in DDBJ/EMBL/GenBank under the accession numbers HE608773 to HE608809, as indicated in Table 1. The 42 isolates were grown on Sabouraud agar at 25 °C for 7 days. Three agar plugs (8 mm in diameter) of each strain were used to inoculate 250-mL flasks containing 50 mL of Sabouraud broth. Each strain was cultivated at 25 °C under static conditions for 7, 14, and 21 days. After each period, the strains were filtered under vacuum through filter paper on a Buchner funnel to separate the culture filtrate from the mycelium. The filtrate was filtered through a 0.22-μm sterile membrane and kept frozen until use.

2.2. T. vaginalis cultivation

The organisms used in this study included one isolate from the American Type Culture Collection (ATCC), 30236, and two fresh clinical isolates, TV-LACH1 and TV-LACM2 (metronidazole-sensitive and -resistant strains). The fresh clinical isolates were obtained from Laboratório de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, UFRGS, Brazil (project approved by UFRGS Research and Ethics Committee, protocol number 18923). Trichomonads were cultured axenically *in vitro* in a trypticase–yeast extract–maltose (TYM) medium (pH 6.0), supplemented with 10% heat-inactivated bovine serum (HIBS [v/v]), and incubated at 37 °C (±0.5) (Diamond, 1957). Organisms exhibiting motility and normal morphology during the logarithmic growth phase were harvested, centrifuged, washed three times with phosphate-buffered saline 1x (PBS) (pH 7.0), and resuspended in new TYM medium. All experiments were performed in triplicate with at least three independent cultures ($n = 3$).

2.3. Anti-T. vaginalis screening

A total of 126 filtrates from marine-associated fungi were screened against *T. vaginalis* trophozoites (ATCC 30236). The assay was performed using 96-well microtiter plates with TYM medium, filtrate at a concentration of 2.5 mg/mL, and an initial inoculum of 2.5×10^5 trophozoite per mL, giving a final volume of 200 μL. After 24 h of incubation at 37 °C (±0.5), the number of viable trophozoites was measured using the quantitative resazurin method as previously described by Duarte et al. (2009). The interpretation of motility and normal morphology was confirmed by manual microscopy, and the viability of trophozoites was characterized using trypan blue dye exclusion (0.2% [v/v]). In control cultures, fungal filtrate samples were replaced with water.

2.4. Minimum inhibitory concentration (MIC)

First, the MIC value against *T. vaginalis* (ATCC 30236) was determined using only the fungal filtrate samples that reduced under 50% the parasite viability in the initial screening. MIC was established in 96-well microtiter plates containing TYM medium and an initial inoculum of 2.5×10^5 trophozoites per mL. The concentration of filtrate samples was obtained by serial dilution within the range of 20–0.156 mg/mL (Frasson et al., 2011). After 24 h of incubation at 37 °C (±0.5), trophozoite viability was evaluated using the quantitative resazurin method (Duarte et al., 2009). Then, only the fungal filtrate samples that produced the lowest MIC values, which were considered active filtrate samples, were tested against fresh clinical isolates of *T. vaginalis*. These active fungal filtrate samples were used in the subsequent experiments.

Table 1
Species from the marine associated fungi screened against *T. vaginalis* ATCC 30236; (%) viability represents the percentage of living organisms compared to control parasites, considering the amount of resazurin reduced by viable *T. vaginalis*.

Supernatant code	Fungal strain (BLAST similarity/assecion number)	Culture days		
		7 days Viability (%)	14 days Viability (%)	21 days Viability (%)
Control		100	100	100
SB[a]		95.25	96.05	161.56
F01	*Westerdykella purpurea* (HE608773)	88.46	112.02	141.85
F02	*Hypocrea lixii* (HE608774)	97.91	102.42	0
F03	*Calonectria canadense* (HE608775)	184.17	91.66	163.60
F04	*Penicillium citrinum* (HE608776)	73.86	102.08	132.11
F05	Ascomycete class (HE608777)	84.24	117.01	104.83
F06	*Penicillium* sp. (HE608778)	105.43	112.02	133.24
F07	Unknown	31.48	102.42	124.62
F08	*Dothideomycete class* (HE608779)	200.15	92.90	N.D.
F10	*Cladosporium* sp. (HE608780)	83.80	104.51	124.54
F11	*Cladosporium cladosporioides* (HE608781)	135.78	138.51	130.98
F12	*Polyporus* sp. (HE608782)	134.74	159.46	61.31
F13	*Penicillium corylophilum* (HE608783)	67.26	144.78	132.92
F14	*Cladosporium* sp. (HE608784)	137.67	97.57	142.73
F15	*Ramichloridium apiculatum* (HE608785)	121.73	126.70	95.65
F16	*Ascomycete class* (HE608786)	83.00	94.11	106.91
F17	*Cladosporium cladosporioides* (HE608787)	126.76	106.39	88.94
F18	*Westerdykella* sp. (HE608788)	105.30	79.21	97.67
F19	*Penicillium daleae* (HE608789)	89.11	88.32	77.16
F20	*Eutypella leprosa* (HE608790)	188.20	105.57	91.45
F21	*Simplicillium lanosoniveum* (HE608791)	352.84	226.93	44.43
F22	Sordariales order (HE608792)	185.88	170.14	83.00
F23	Unknown	57.81	90.54	35.96
F24	*Cladosporium cladosporioides.* (HE608793)	67.03	148.40	59.64
F25	*Phoma* sp. (HE608794)	72.39	N.D.	92.71
F26	*Phoma* sp. (HE608795)	53.40	283.60	84.19
F27	*Rhinocladiella* sp. (HE608796)	51.65	N.D.	147.65
F28	*Pestalosphaeria hansenii* (HE608797)	82.43	339.99	103.40
F29	*Alternaria alternata.* (HE608798)	133.68	196.12	182.04
F30	*Westerdykella purpurea* (HE608799)	173.91	347.25	184.10
F32	Unknown	132.89	257.84	43.43
F33	Xylariales order (HE608800)	95.56	170.85	132.18
F34	Unknown	92.40	258.55	128.20
F35	*Phoma* sp. (HE608801)	140.07	198.11	N.D.
F36	*Aspergillus versicolor.* (HE608802)	89.61	N.D.	121.52
F37	*Penicillium* sp. (HE608803)	165.79	187.36	57.71
F38	*Nigrospora oryzae* (HE608804)	105.95	156.29	N.D.
F39	Unknown	141.84	46.53	53.48
F40	*Penicillium citrinum* (HE608805)	166.16	0	53.01
F41	Xylariales order (HE608806)	107.20	N.D.	100.86
F42	*Aspergillus versicolor* (HE608808)	104.39	32.00	235.52
F43	*Aspergillus oryzae* (HE608808)	139.13	16.07	131.37
F44	*Penicillium* sp. (HE608809)	117.01	43.09	218.75

[a] SB, sabouraud broth without fungi growth; N.D., not determined.

2.5. Kinetic growth assay

Kinetic growth experiments were performed using the ATCC 30236 isolate and fungal filtrate samples at MIC treated and not treated by heating (autoclaving at 121 °C for 20 min). For these experiments, an initial inoculum of 2.5×10^5 trophozoites per mL was grown in TYM medium (1.5 mL final volume), using microtubes. Parasites were counted using a hemocytometer during 72 h (at 0, 2, 4, 6, 12, 24, 48, and 72 h). Trophozoites were characterized considering motility, normal morphology, and trypan blue dye exclusion (0.2% [v/v]). The results were expressed as the number of living organisms compared to that of untreated parasites.

2.6. Hemolytic assay

This assay was performed according to Gauthier et al. (2009), with modifications. Fresh human blood was obtained from healthy voluntary donors, collected in Alsever's solution (1:1 [v/v]). The erythrocytes were washed three times with PBS 1x and resuspended to obtain an erythrocyte suspension (1.0% [v/v]). The erythrocytes were incubated with the fungal filtrate samples at MIC on a shaker at 37 °C for 60 min. Filtrate absorbance was measured at 540 nm. The percentage of hemolysis induced by each sample was calculated using commercial fractions containing *Quillaja saponaria* saponins (S5) as a reference for 100% hemolysis (positive control) (Sun et al., 2008). Erythrocytes incubated with PBS were used as negative controls. The hemolytic assay was performed in triplicate on three independent experiments ($n = 3$).

2.7. Cytotoxicity against Vero cells

Vero cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. For this assay, 1.5×10^4 cells per well were seeded in 96-well microtiter plates for 24 h. After this period, the medium was replaced with fresh medium containing or not (control condition) fungal filtrate samples at MIC. A solution containing 1% Triton was added as a positive control. The plates were incubated for 24 h, and then, after one wash with PBS, a

Table 2
Minimum inhibitory concentration (MIC) of marine associated fungi filtrate for different *T. vaginalis* isolates.

Filtrate code	MIC (mg mL^{-1})		
	ATCC 30236	TV-LACH1 (fresh clinical)	TV-LACM2 (fresh clinical)[a]
F02	2.5	2.5	2.5
F07	>20	N.D.	N.D.
F21	>20	N.D.	N.D.
F23	>20	N.D.	N.D.
F32	>20	N.D.	N.D.
F39	>20	N.D.	N.D.
F40	2.5	2.5	2.5
F42	>20	N.D.	N.D.
F43	>20	N.D.	N.D.
F44	>20	N.D.	N.D.

N.D., not determined.
[a] Metronidazole-resistant isolate.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) was added to and kept in the wells for 1 h. The plates were washed twice with PBS, and the insoluble purple formazan was dissolved in dimethyl sulfoxide (DMSO). The amount of reduced MTT was measured at 570 nm.

3. Results

In the present study we screened the anti-*T. vaginalis* activity of 126 filtrate samples from 42 marine-associated fungi (Table 1). This initial screening revealed that 10 filtrate samples (F02- *Hypocrea lixii,* F07-Unknown, F21- *Simplicillium lanosoniveum*, F23-Unknown, F32-Unknown, F39-Unknown, F40- *Penicillium citrinum*, F42- *Aspergillus versicolor*, F43- *Aspergillus oryzae*, F44- *Penicillium* sp.) were effective in reducing parasite viability by at least 50% (Table 1). The 10 active samples were submitted to MIC assay, and only two were more effective with low values of MIC (2.5 mg/mL), while all others allowed trophozoite growth at concentrations higher than 20 mg/mL (Table 2). Therefore, the subsequent experimental procedures were performed using only these two filtrate samples: *H. lixii* F02 (21-day culture) and *P. citrinum* F40 (14-day culture). Two different fresh clinical isolates of *T. vaginalis* were tested, a metronidazole-sensitive (TV-LACH1) and a metronidazole-resistant (TV-LACM2) isolate. Both filtrate samples (*H. lixii* F02 and *P. citrinum* F40) were effective against these isolates and had the same MIC value of 2.5 mg/mL (Table 2).

Fig. 1 shows the kinetic growth curve of the activity of *H. lixii* F02 and *P. citrinum* F40 filtrate samples against *T. vaginalis* trophozoites. The filtrate samples were able to completely inhibit parasite growth after 24 h of incubation (Fig. 1). Conversely, as expected, trophozoite growth from the control culture increased over the incubation period, reaching the highest density in 24 h and decreasing only after 72 h of incubation (Fig. 1). Heating reverted the cytotoxic effect of *P. citrinum* F40 filtrate, and parasite growth was the same as that of the control. In contrast, the activity of *H. lixii* F02 filtrate sample was preserved after heating, although it was less potent than the untreated sample (Fig. 1).

Table 3
Hemolytic effect of F02 and F40 marine associated fungi filtrate.

Samples	% Hemolysis[a]
Positive control (*Q. saponaria*)	100 ± 0
Negative control	1.6 ± 0.44
F02 (2.5 mg mL^{-1})	9.3 ± 0.03
F40 (2.5 mg mL^{-1})	2.61 ± 0.33

[a] The values represent the mean ± standard deviation.

The hemolytic test, which allows predict the damage in cellular membranes, was used to examine the potential effect of *H. lixii* F02 and *P. citrinum* F40 filtrate samples upon mammalian cells. In Table 3 it can be observed that both filtrate samples exhibited no hemolytic activity. In addition, the viability of Vero cells was also evaluated to examine the potential cytotoxicity of these fungal filtrate samples. While *H. lixii* F02 filtrate sample showed high toxicity against mammalian cells, *P. citrinum* F40 sample showed low cytotoxicity against Vero cells, with 79% of cell viability (Fig. 2).

4. Discussion

Resistance of *T. vaginalis* to metronidazole has been reported to reach up to 9% (Schwebke and Barrientes, 2006). Therefore, and worryingly, in the absence of a non-nitroimidazole alternative to treatment, cure can only be achieved by increasing doses of metronidazole (Cudmore et al., 2004). However, higher doses lead to adverse events severe enough to cause cessation of treatment (Cudmore et al., 2004; Schwebke and Barrientes, 2006). Thus, our efforts are now focused on exploring Brazil's natural potential resources to expand sources of novel therapeutic agents potentially useful for treatment of *T. vaginalis* infection. Marine microorganisms which colonize sponges are responsible for the stabilization of the sponge skeleton and participate in the host's chemical defense system against predators, among other functions (Lee et al., 2001). This chemical defense is mediated by the production of secondary metabolites, which gives to these microorganisms different abilities, such as antimicrobial, antifungal, antitumor,

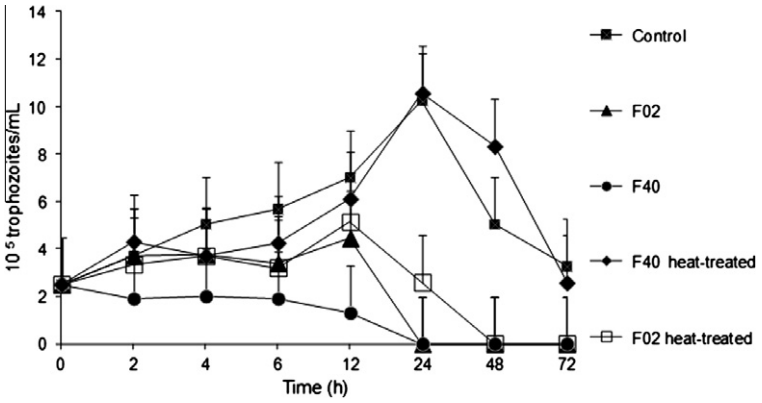

Fig. 1. Effect of *H. lixii* F02 and *P. citrinum* F40 filtrate samples on the *T. vaginalis* (ATCC 30236 isolate) kinetic growth curve (heat-treated and untreated samples). Parasite growth was completely inhibited by both filtrates within the first hours of incubation, and heat treatment affected only the activity of *P. citrinum* F40. Data represent mean ± standard deviation of at least three experiments.

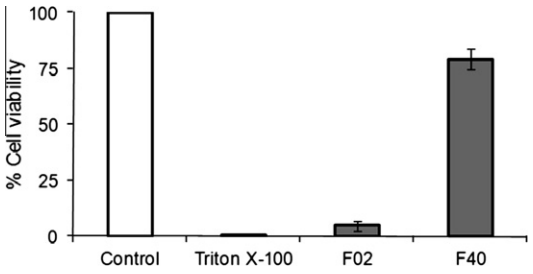

Fig. 2. Effect of *H. lixii* F02 and *P. citrinum* F40 filtrate samples on the viability of Vero cells. While *H. lixii* F02 was highly cytotoxic, *P. citrinum* F40 was able to sustain cell viability. Control represents Vero cells in DMEM medium, without samples. Data represent mean ± standard deviation of at least three experiments.

and antifeeding action, among others (Saravanakumar et al., 2011), suggesting the property anti-*T. vaginalis*.

The initial screening revealed that most fungal filtrates produced an increase in the number of trophozoites. This may occur due to high glucose availability from Sabouraud broth, which has 20% of glucose in its composition. Kuile (1996) stated that the enzymatic route of trichomonads involved in glucose metabolism is stimulated in the presence of high glucose availability.

Among all filtrate samples, only *H. lixii* F02, isolated from the sponge *Axinella corrugata* and *P. citrinum* F40, isolated from the sponge *Stoeba* sp., were effective in eliminating *T. vaginalis* trophozoites at a low concentration (Table 2). Importantly, the filtrates of these two fungal species were effective against both ATCC 30236 and fresh clinical isolates, including the metronidazole-resistant TV-LACM2. Moreover, the effect of *H. lixii* F02 and *P. citrinum* F40 filtrates on the kinetic growth of *T. vaginalis* trophozoites could be observed in the first hours of incubation and within 24 h no viable parasites could be detected (Fig. 1). The samples exhibited different behaviors when treated by heating. *P. citrinum* F40 lost its activity after heat treatment, probably because of the instability of chemical constituents, while *H. lixii* F02 appeared to have thermostable active compounds. These results indicate that there are two possible different molecular entities capable of acting upon *T. vaginalis*.

To our knowledge, this is the first study to report an anti-*T. vaginalis* activity of *H. lixii* F02 and *P. citrinum* F40, although some studies evaluating their biological activities have already been described. Strains of the marine fungus *P. citrinum* have been shown to produce a hemiacetal compound with cytotoxic activity against the human promyelocytic leukemia (HL-60) cell line (Ni et al., 2011). Tricitrinols also isolated from this specie have shown cytotoxicity and inhibited human topoisomerase IIα in different experimental models (Du et al., 2011). *P. citrinum* may also be found in soil samples, and antioxidant activity as well as antibacterial properties against *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Enterococcus durans* have been observed in *in vitro* assays (Amagata et al., 2003; Castillo-Machalskis et al., 2007; Arora and Chandra, 2011). Conversely, only a few studies have examined the biological activities of *H. lixii* (*Trichoderma harzianum*). Isolated metabolites of this fungus have been able to inhibit the soilborne pathogens *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Fusarium oxysporum* (Choudary et al., 2007), and the molecule 6-n-pentyl-pyrone has been reported to have antifungal and antibacterial activity (Tarus et al., 2003). Recently, the marine strain of *H. lixii*, included in a screening study against methicillin-resistant *Staphylococcus aureus* (MRSA), has exhibited strong anti-MRSA and anticancer activities for Hep2 and MCF7 (Zhang et al., 2011; Bhimba et al., 2012).

The hemolytic assay evaluated possible cytotoxic mechanisms involving membrane damage. Our results showed that both *H. lixii* F02 and *P. citrinum* F40 filtrate samples had no hemolytic effect, suggesting that there were no toxic effects towards plasma membranes, and both fungal filtrate samples were found to be compatible with red blood cells. Surprisingly, and in contrast to the results of the hemolytic assay, the *H. lixii* filtrate sample showed strong cytotoxicity against Vero cells. A similar observation has already been described for three metabolites, trichodenones A–C, produced by the fungus *H. lixii* (*Trichoderma harzianum*), which exhibited significant cytotoxicity against cultured P388 cells (Amagata et al., 2003). However, *P. citrinum* F40 filtrate sample allowed the growth and viability of cells, demonstrating low cytotoxicity against mammalian cells.

In a comparison with studies on *Plasmodium*, xanthones isolated from the marine-derived fungus *Chaetomium* sp. have shown antiprotozoal activity, although with pronounced cytotoxicity, and the analysis of the mode of action has indicated that these molecules are able to inhibit heme polymerization causing the death of parasitic cells (Pontius et al., 2008). Another mode of action against *Plasmodium* has been observed with the fungal metabolite apicidin by the inhibition of histone deacetylase (HDAC) (Darkin-Rattray et al., 1996).

In conclusion, our findings on *H. lixii* F02 and *P. citrinum* F40 indicate these metabolites as extremely promising alternatives against *T. vaginalis* due to their high cytotoxicity verified in ATCC and fresh clinical isolates. It is worth emphasizing that these two fungal samples were also effective against a metronidazole-resistant isolate and that *P. citrinum* F40 filtrate had no hemolytic activity, exerting a low cytotoxic effect on mammalian cells. In this sense, our findings warrant further studies with *H. lixii* F02 and mainly *P. citrinum* F40 filtrates, which appear to be promising targets in the field of antiprotozoal metabolites for the development of novel antitrichomonal drugs. Accordingly, our current research efforts focus on the purification and elucidation of active molecules from these fungi for further testing against host vaginal epithelial cells.

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