Antifungal Activity of Silver Salts of Keggin-Type Heteropolyacids Against Sporothrix spp.

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Introduction

Sporothrix spp. are dimorphic, saprophytic and geophilic fungi of the Sporothrix complex and responsible for sporotrichosis, a chronic and subacute mycosis causing epidemiological outbreaks involving sick cats and humans in southeastern Brazil. The systemic disease prevails in cats and in humans, with the symptoms restricted to the skin of immunocompetent individuals. Under these conditions, the prolonged treatment of animals and cases of recurrence justify the discovery of new treatments for sporotrichosis. This work addresses the antifungal activity of silver salts of Keggin-type heteropolyacid salts (Ag-HPA salts) such as Ag₃[PW₁₂O₄₀]₄, Ag₆[SiW₁₀V₂O₄₀]₄, Ag₃[SiW₁₀O₃₈]₄ and Ag₃[PMo₁₂O₄₀]₄ and interactions with the antifungal drugs itraconazole (ITC), terbinafine (TBF) and amphotericin B (AMB) on the yeast and mycelia forms of Sporothrix spp. Sporothrix spp. yeast cells were susceptible to Ag-HPA salts at minimum inhibitory concentration (MIC) values ranging from 8 to 128 μg/ml. Interactions between Ag₃[PW₁₂O₄₀]₄ and Ag₃[PMo₁₂O₄₀]₄ with itraconazole and amphotericin B resulted in higher antifungal activity with a reduction in growth and melanization. Treated cells showed changes in cell membrane integrity, vacuolization, cytoplasm disorder, and membrane detachment. Promising antifungal activity for treating sporotrichosis was observed for the Ag-HPA salts Ag₃[PMo₁₂O₄₀]₄ and Ag₃[PW₁₂O₄₀]₄, which have a low cost, high yield and activity at low concentrations. However, further evaluation of in vivo tests is still required.

Keywords: Pathogenic fungus, zoonosis, silver, polyoxometalate, antifungal effect
coordination, and usually silicon (Si) or phosphorus (P) are the preferred choice [13]. Several Keggin-type HPA salts containing the cations Na+, Fe3+, K+, Ce3+, and Ba2+ showed activity against viruses and bacteria [15]. Silver ion metal (Ag+) and silver nanoparticles (AgNPs) have been known for centuries as highly toxic to microorganisms, showing strong antimicrobial effects against fungi and bacteria [16–18]. The positive charge of silver ions is crucial for their antimicrobial activity through electrostatic attraction toward the negatively charged cell membrane of microorganisms, causing cell lysis [19]. In Candida albicans, AgNPs break down the membrane permeability barrier by perturbing membrane lipid bilayers, causing leakage of ions and other materials as well as forming pores and dissipating the electrical potential of the membrane [18]. The antimicrobial effects of Keggin-type HPAs on many bacteria and viruses have been investigated, but no information concerning the morphology and ultrastructure of fungal cells is available. The aim of this study was to evaluate the antifungal activity of Keggin-type Ag-HPA salts against Sporothrix spp. strains by analyzing the inhibition of growth and interference with melanin production and observing their effects on cell ultrastructure and membrane permeabilization.

Materials and Methods

Synthesis of HPA Salts

The method was conducted according to previous work with minor modifications [20]. The FTIR spectra of the Ag-HPA salts were obtained from samples dispersed in KBr disks in the range of 4,000–400 cm⁻¹ in a spectrophotometer model Prestige-21 (Shimadzu, Japan).

X-Ray Diffraction

Ag-HPA salts (Ag₃[PW₁₂O₄₀], Ag₃[PMo₁₂O₄₀], Ag₆[SiW₁₀V₂O₄₀], and Ag₄[SiW₁₂O₄₀]) were analyzed by X-ray diffraction using an X-ray diffractometer URD 65 model-SEIFFERT/GE and an X-ray tube with a copper filament. Samples were treated previously at 90°C for 30 min, sieved through 200-mesh netting and maintained in a desiccator.

Fungal Strains and Growth Conditions

Some selected strains present different levels of melanization, which, according to the literature, could have some influence on drug response mechanisms [21]. The strains used were Sporothrix spp. (CMDB/IOC 01980599, CMDB/IOC 01990699, CMDB/IOC 02050799, CMDB/IOC 02020699, and ATCC 32285) obtained from Instituto Oswaldo Cruz (IOC), Rio de Janeiro, Brazil. Our clinical strains were isolated from infected cats (LSASs01, LSASs02, LSASs03) by the clinical practitioner of the Veterinary Hospital of Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil. Further, all samples were stored in dry desiccators.

Reagents and Antifungals

The Ag-HPA salts were suspended in sterile distilled water to a ratio of 51.2 mg/ml. The antifungal drugs miconazole nitrate (Sigma-Aldrich), itraconazole and terbinafine (Sigma-Aldrich) were diluted in dimethyl sulfoxide (Sigma-Aldrich), and amphotericin B was diluted in sterile distilled water to obtain stock solutions of 12.8 mg/ml, which were maintained at −70°C. All reagents used as controls in this step were purchased from Sigma-Aldrich, USA.

Growth Inhibition on Sporothrix spp.

The activity of the Ag-HPA salts on fungal growth was tested against three strains of Sporothrix spp. (ATCC 32285, CMDB/IOC 01980599 and LSASs01). To obtain yeast cell cultures, from each stock culture, a small fragment of agar containing colonies was transferred and spread onto Petri dishes containing Brain Heart Infusion Agar and allowed to grow at 37°C for 5 days. To access yeast forms, the isolates were maintained by transferring the cultures into the same medium weekly. A suspension of inoculum was obtained from yeast cells in a tube with 10 ml of sterile 0.85% saline, corresponding to 0.5 McFarland suspension of 5 × 10⁶ CFU/ml carried out in a photometer OD₅₅₀ (Densimat, France), as established by the manufacturer. To assay the efficacy of each Ag-HPA salt (32 μg/ml) on yeast growth, 0.1 ml of fresh inoculum and 0.1 ml of each Ag-HPA salt were added to 1.8 ml BHI broth Infusion.
Broth (BHI) and incubated at 37°C. Optical density readings at 550 nm were taken at zero hour, and then readings were conducted every 24 h for the following 14 consecutive days. For cell growth, itraconazole (500 μg/ml), and medium plus HPA acid, and HCl (32 μg/ml) were used as positive and negative controls, respectively. The latter was used to evaluate the effect of H⁺ on fungal growth. The optical densities were plotted as a function of the incubation time for each fraction. Experiments were performed in triplicate and repeated two times. The inhibition rate was defined as the median value.

**Determination of MICs**

Minimum inhibitory concentrations (MIC) for the Ag-HPA salts were determined using the broth microdilution technique, according to Clinical Laboratory Standards Institute M27-A3 recommendations [22]. The compound suspensions were twofold serially diluted in RPMI 1640 medium (India) buffered with 0.16 M MOPS (3-[N-morpholino] propane sulfonic acid) (Sigma-Aldrich) in 96-well tissue culture plates at concentrations varying from 512-0.125 μg/ml. Antifungals such as itraconazole, miconazole nitrate, terbinafine and amphotericin B (Sigma-Aldrich) were used as positive controls. An inoculum of yeast cells was obtained in saline solution (0.85%), corresponding to 0.5 McFarland suspension of 5 × 10⁵ CFU/ml, which was determined with a densitometer at OD550 and diluted 1:1000 in RPMI 1640 medium. Each well was filled with 0.1 ml of Sporothrix spp. inoculum and 0.1 ml of compound suspensions to obtain a final Ag-HPA salt concentration of 0.0625-256 μg/ml. All microtiter plates were incubated in a humid chamber at 37°C for 96 h. The MIC values were defined as the lowest concentration that imposed complete inhibition of Sporothrix spp. growth, which was revealed by adding 30 μl/well resazurin (100 μg/ml) reagent (Sigma-Aldrich). The tests were performed in triplicate on two different days.

**Interaction HPA-Antifungal Assay**

To test for the interaction effect among HPA and antifungal drugs and further examine the synergistic-like effect of HPA and antifungal drugs on microorganisms, the Ag-HPA salts were tested together with the disks of antifungal agents such as itraconazole (ITC, 10 μg), amphotericin B (AMB, 100 μg), ketoconazole (KTC, 50 μg), fluconazole (FLC, 25 μg) and miconazole nitrate (MCZ, 50 μg). Ag-HPA salts were mixed with Brain Heart Infusion agar to obtain final concentrations of 128 and 256 μg/ml. To each Petri dish, 0.1 ml of yeast (CMDB/IOC 01980599 and clinical strain LSASs01) suspension, corresponding to 0.5 McFarland suspension of 5 × 10⁵ CFU/ml, which was determined with a densitometer at OD550 and diluted 1:1000 in RPMI 1640 medium. Each well was filled with 0.1 ml of Sporothrix spp. inoculum and 0.1 ml of compound suspensions to obtain a final Ag-HPA salt concentration of 0.0625-256 μg/ml. All microtiter plates were incubated in a humid chamber at 37°C for 96 h. The MIC values were defined as the lowest concentration that imposed complete inhibition of Sporothrix spp. growth, which was revealed by adding 30 μl/well resazurin (100 μg/ml) reagent (Sigma-Aldrich). The tests were performed in triplicate on two different days.

**Membrane Permeabilization Assay**

To investigate membrane permeabilization in fungi, a qualitative assay was used [24], with minor modifications, based on the uptake of SYTOX Green (Molecular Probes, Invitrogen, USA), an organic compound that fluoresces after interacting with nucleic acids in cells with compromised plasma membranes. The yeast cells (CMDB/IOC 01980599) were allowed to grow for 96 h in BHI broth in the presence or absence of 32 μg/ml Ag-HPA salts, were fixed at RT for 1 h in 2.5% glutaraldehyde (Sigma-Aldrich) and postfixed using 1% osmium tetroxide (Sigma-Aldrich), diluted in potassium cacodylate buffer (Sigma-Aldrich) (0.1 M, pH 7.2) at RT. Inhibition zones were measured and scored according to the manufacturer’s recommendations (CECON, Brazil).

To investigate whether the interaction between Ag-HPA salts and antifungal agents interfered with the melanization process of mycelial forms, the colonies were allowed to grow at RT in a dark environment. Colony melanization was photodocumented on days 15 and 30 of incubation.

**Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)**

For TEM, samples were postfixed using 1% osmium tetroxide (Sigma-Aldrich), diluted in potassium ferrocyanide (Sigma-Aldrich), dehydrated with a series of acetone and embedded in Epon resin Polybeded 812 (Polysciences). Ultrathin sections were stained with 2% uranyl acetate for 20 min and lead citrate for 5 min and examined using transmission electron microscopy (900-Zeiss Company, Germany) at an accelerating voltage of 80 kV. For SEM, samples treated as mentioned before were adhered to cover slips with poly-L-lysine, postfixed, dehydrated in ethanol, dried to critical point in CO₂, and covered with gold for observation with a scanning electron microscope (912-Zeiss Company) at an accelerating voltage of 50 kV.
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Statistics
Data were analyzed with the Prism program. Statistical significances are shown as \( p < 0.05 \) values by Tukey’s test at the 5% level of significance.

Results
Synthesis
The heteropolyacids \( \text{H}_3[\text{PW}_{12}\text{O}_{40}] \), \( \text{H}_3[\text{PMo}_{12}\text{O}_{40}] \), \( \text{H}_4[\text{SiW}_{12}\text{O}_{40}] \), and \( \text{H}_6[\text{SiW}_{10}\text{V}_{2}\text{O}_{40}] \) were obtained as large and brightly-colored crystals after two crystallization steps from water solutions, as shown in Fig. 1A for \( \text{H}_6[\text{SiW}_{10}\text{V}_{2}\text{O}_{40}] \). The salts obtained were subjected to FTIR analysis, and their structures were compared with the literature data \[20, 25\]. The HPA salts obtained were silver dodecamolybdophosphate (\( \text{Ag}_3[\text{PMo}_{12}\text{O}_{40}] \)), silver dodecatungstophosphate (\( \text{Ag}_3[\text{PW}_{12}\text{O}_{40}] \)), silver dodecatungstosilicate (\( \text{Ag}_4[\text{SiW}_{12}\text{O}_{40}] \)) and silver decatungstovanadosilicate (\( \text{Ag}_6[\text{SiW}_{10}\text{V}_{2}\text{O}_{40}] \)). The obtained spectral results are summarized in Table 1.

X-Ray Diffraction
According to the literature, no results for X-ray diffraction of HPA silver salts were found. However, the diffractogram shown in Fig. 1B is similar to the diffractogram of HPA \( \text{H}_3[\text{PW}_{12}\text{O}_{40}] \) described previously \[26\], suggesting that the crystal structure was preserved upon substitution of \( \text{H}^+ \) with \( \text{Ag}^+ \) ions. The diffractograms were compared with X-ray diffractometer software on the PCPDFWIN database.

Growth Inhibition Assay of HPAs on Sporothrix spp.
In our experimental conditions, diverse effects on cell growth were observed depending on the fungal strain. The Ag-HPA salts were tested at a concentration of 32 \( \mu \text{g/ml} \) against three strains of \( \text{Sporothrix} \) spp. Toward strain ATCC 32285, the Ag-HPA salt \( \text{Ag}_3[\text{PMo}_{12}\text{O}_{40}] \) showed the highest activity, with 93% inhibition of growth, followed by \( \text{Ag}_3[\text{PW}_{12}\text{O}_{40}] \) (80%), and \( \text{Ag}_4[\text{SiW}_{12}\text{O}_{40}] \) (68%) (Fig. 2A). Toward strain CMDB/IOC 01980599, \( \text{Ag}_3[\text{PW}_{12}\text{O}_{40}] \) caused 84% inhibition, followed by \( \text{Ag}_3[\text{PMo}_{12}\text{O}_{40}] \) and \( \text{Ag}_4[\text{SiW}_{12}\text{O}_{40}] \), presenting 77% and 71%, respectively (Fig. 2B). Toward clinical strain LSAsS01, the highest activity was demonstrated by \( \text{Ag}_3[\text{PMo}_{12}\text{O}_{40}] \), which caused 83% inhibition, followed by \( \text{Ag}_4[\text{SiW}_{12}\text{O}_{40}] \) (76%) and \( \text{Ag}_3[\text{PW}_{12}\text{O}_{40}] \) (69%) (Fig. 2C). Of the Ag-HPA salts, \( \text{Ag}_6[\text{SiW}_{10}\text{V}_{2}\text{O}_{40}] \) inhibited cells the least at approximately 57% (ATCC 32285), 48% (CMDB/IOC 01980599) and 36% (LSAsS01), respectively. The positive control in the presence of 500 \( \mu \text{g/ml} \) itraconazole presented total (100%) inhibition of growth against all strains tested. The negative controls, HPA acid in the form of \( \text{H}_3[\text{PMo}_{12}\text{O}_{40}] \) and HCl presented a weak effect and were ineffective, respectively, on the growth of the strains tested when compared to the other treatments. (Figs. 2A-2C).

Determination of MICs
The MICs for the salts of heteropolyacids with tungsten and molybdenum were tested against three clinical isolates of \( \text{Sporothrix} \) spp. and on the five standard strains as shown in Table 2. Among the Ag-HPA salts, the most

Table 1. FTIR spectrum main peaks assignment.

| Literature* | \( \text{Ag}_3[\text{PW}_{12}\text{O}_{40}] \) | \( \text{Ag}_3[\text{PMo}_{12}\text{O}_{40}] \) | \( \text{Ag}_4[\text{SiW}_{12}\text{O}_{40}] \) | \( \text{Ag}_6[\text{SiW}_{10}\text{V}_{2}\text{O}_{40}] \) | Assignment (cm\(^{-1}\)) |
|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1,055-1,100 | 1074            | 945             | 1109            | 1006            | \( n \text{ P-O} \) |
| 900-1,000   | 892             | 896             | 938             | 909             | \( n \text{ M-O} \) |
| 700-850     | 807             | 654             | 837             | 795             | \( n \text{ M-O-M} \) |

*Tsigidinos, 1978
expressive results were observed for the dodecamolybdophosphate of silver (Ag$_3$[PMo$_{12}$O$_{40}$]) with MIC values varying from 8 to 16 μg/ml. The dodecatungstophosphate of silver (Ag$_3$[PW$_{12}$O$_{40}$]) also presented expressive results with MIC values varying from 8 to 32 μg/ml. The MIC of dodecatungstosilicate of silver (Ag$_4$[SiW$_{12}$O$_{40}$]) also varied from 16 to 32 μg/ml, and the MIC of divanadodecatungstosilicate of silver (Ag$_6$[SiW$_{10}$V$_2$O$_{40}$]) varied from 16 to 128 μg/ml. All antifungal drugs used as controls presented activity superior to Ag-HP A salts. Miconazole nitrate and terbinafine exhibited MIC values as low as 0.125-0.5 μg/ml and 0.5-2 μg/ml, respectively, which were even better than the MIC values of amphotericin B (1 to 8 μg/ml) and itraconazole (1 to 32 μg/ml), the recognized agents for the treatment of sporotrichosis (Table 2).

### Interactions of Ag-HPA Salts and Antifungal Drugs

Interactions among Ag-HPA salts and antifungal drugs were determined against CMDB/IOC 01980599 and LSAS01 clinical strains using the agar diffusion method. For some interactions between Ag-HPA salts and antifungal drugs, the inhibition zone results were significant but remained sensitive, as shown in Table 3. Table 3 (hatching) shows synergistic-like and antagonistic-like effects, as shown by the variation of Ag-HPA salts and antifungal combinations. Interactions of Ag-HPA salts and the antifungal drugs ITC and AMB changed their activity toward mycelial forms on both strains CMDB/IOC01980599 and clinical LSASs01, as shown in Table 4. The results show that for fungi treated with silver salts and further submitted to antifungal challenge, the activity between these two compounds against both mycelia and yeast cells remained synergistic-like when compared to untreated cells.

The pattern of pigmentation of mycelial forms of both strains (CMDB/IOC 01980599 and clinical LSASs01) presented considerable alterations after Ag-HPA salts and the antifungal drugs ITC and AMB were combined, as

### Table 2. In vitro antifungal activity of Ag-HPA salts and reference antifungals.

| Strains Sporothrix | Agente test   | CMDB/IOC 01980599 | CMDB/IOC 01990699 | CMDB/IOC 02020699 | CMDB/IOC 02030799 | ATCC 32285 | LSAS01 | LSAS02 | LSAS03 |
|--------------------|---------------|--------------------|--------------------|--------------------|--------------------|------------|--------|--------|--------|
|                    | Polyoxotungstates |                   |                    |                    |                    |            |        |        |        |
|                    | Ag$_3$[PW$_{12}$O$_{40}$] | [μg/ml] | 8 | 8 | 32 | 32 | 16 | 32 | 32 | 32 |
|                    |     | [nmol/ml] | 22.5 | 2.5 | 9.9 | 9.9 | 4.9 | 9.9 | 9.9 | 9.9 |
|                    | Ag$_3$[SiW$_{10}$V$_2$O$_{40}$] | [μg/ml] | 16 | 16 | 32 | 32 | 16 | 32 | 64 | 64 |
|                    |     | [nmol/ml] | 4.8 | 4.8 | 9.6 | 4.8 | 9.6 | 19.1 | 38.2 | 19.1 |
|                    | Ag$_4$[SiW$_{12}$O$_{40}$] | [μg/ml] | 16 | 32 | 16 | 16 | 16 | 16 | 16 | 16 |
|                    |     | [nmol/ml] | 4.8 | 9.6 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |
|                    | Polyoxomolybdate |                   |                    |                    |                    |            |        |        |        |
|                    | Ag$_3$[PMo$_{12}$O$_{40}$] | [μg/ml] | 8 | 8 | 16 | 16 | 16 | 16 | 16 | 8 |
|                    |     | [nmol/ml] | 3.7 | 3.7 | 7.4 | 7.4 | 7.4 | 7.4 | 7.4 | 3.7 |
|                    | Antifungals |                   |                    |                    |                    |            |        |        |        |
|                    | ICZ  | [μg/ml] | 1 | 16 | 16 | 16 | 16 | 16 | 16 | 4 |
|                    |     | [nmol/ml] | 1.4 | 22.6 | 45.3 | 22.6 | 5.7 | 22.6 | 5.7 | 5.7 |
|                    | MCZ  | [μg/ml] | 0.125 | 0.125 | 0.5 | 0.5 | 0.25 | 0.5 | 0.5 | 0.5 |
|                    |     | [nmol/ml] | 0.3 | 0.3 | 1 | 1 | 0.5 | 1 | 1 | 1 |
|                    | TBF  | [μg/ml] | 0.5 | 0.5 | 2 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
|                    |     | [nmol/ml] | 1.5 | 1.5 | 6 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
|                    | AMB  | [μg/ml] | 2 | 1 | 4 | 4 | 1 | 8 | 8 | 4 |
|                    |     | [nmol/ml] | 2.16 | 1.1 | 4.3 | 4.3 | 4.3 | 8.6 | 8.6 | 4.3 |
Table 3. Inhibition zone values (in mm) showing synergism and antagonism between Ag-HPA salts at concentrations 128 and 256 μg/ml and commercial antifungal, by the disk diffusion assay on yeast form.

| CMBD/IOC | 01980299 | Ag₆[SiW₁₀V₂O₄₀] | Ag₃[PMo₁₂O₄₀] | Ag₆[SiW₁₀V₂O₄₀] | Ag₃[PMo₁₂O₄₀] |
|-----------|-----------|------------------|------------------|------------------|------------------|
| Antifungal | Control | 128 μg/ml | 128 μg/ml | 128 μg/ml | 128 μg/ml |
| ITC       | 20 (S)c   | 22 (S)d | 22 (S)d | 22 (S)d | 25 (S)c | 25 (S)c | 28 (S)b | 28 (S)b | 32 (S)a |
| AMB       | 16 (S)d   | 20 (S)b | 23 (S)a | 16 (S)d | 22 (S)c | 16 (S)d | 21 (S)b | 21 (S)b | 25 (S)a |
| KTC       | 42 (S)d   | 48 (S)b | 49 (S)b | 52 (S)a | 53 (S)a | 45 (S)d | 50 (S)b | 48 (S)b | 51 (S)b |
| FLC       | 20 (S)a   | ND (R)d | 6 (R)c | 8 (R)c | 8 (R)c | 8 (R)c | 6 (R)d | 13 (R)b | 15 (I)b |
| M CZ      | 45 (S)c   | 51 (S)e | 54 (S)d | 58 (S)c | 63 (S)a | 48 (S)f | 50 (S)e | 56 (S)c | 61 (S)b |
| LSAS01    | 40 (S)c   | 35 (S)b | 32 (S)c | 36 (S)c | 36 (S)c | 36 (S)c | 35 (S)d | 35 (S)d |

shown in Figs. 3 and 4, suggesting interference in the melanization process. Only Ag₆[SiW₁₀V₂O₄₀] did not present a difference in pigmentation (Figs. 3C4 and 4C4) for either strain when compared to controls (Figs. 3A1 and 4A1).

SYTOX Green Uptake Assay

Membrane permeabilization of the yeast after 96 h incubation with Ag-HPA salts was observed on a light microscope by using vital dye staining. As shown in Fig. 5, no fluorescence was observed in the control test in the absence of Ag-HPA salt. For all treatments with Ag-HPA salts, fungal cells presented marked fluorescence due to the presence of SYTOX Green in their interior. Meanwhile, the panels with control and ITC-treated cells were clearly unchanged or less affected, respectively, when compared with the silver salt-treated cells.

TEM Analysis

For TEM images of control samples, budding cells presented intact cell walls, normal cytoplasmic density and dense microfibrillar material (Fig. 6A). In contrast, in the presence of Ag₃[PMo₁₂O₄₀], yeast cells presented large abnormal vacuoles and less dense microfibrillar material (Figs. 6B and 6C). Treatment with Ag₃[PW₁₂O₄₀] induced cell wall rupture and membrane detachment from the cell wall (Fig. 6D), cell wall thickness and detachment of the cell wall in many layers, which gave a “layer cake” aspect of the cell wall, and vacuolization and reduction of microfibrillar material (Figs. 6E-6F, black arrow). In the presence of Ag₆[SiW₁₀V₂O₄₀], cells presented cytoplasmic disorganization, cell wall thickening, loss of microfibrillar material (Fig. 6G, arrow) and membrane detachment from the cell wall (Fig. 6H, black arrow). In the case of Ag₃[PMo₁₂O₄₀] treatment, yeast cells tended to present a more extreme membrane detachment (black arrow), leading to significant disorganization of membrane components, cytoplasm disorder, vacuolation, and the presence of vesicles in the space formed between the membrane and the vacuole (white star), despite the preserved external microfibrillar material (Fig. 6I, black arrow).

Table 4. Inhibition zone values (in mm) showing synergistic-like activity between Ag-HPA salts at concentration 128 μg/ml and commercial antifungal, by the disk diffusion assay on mycelia form.

| CMBD/IOC | 01980599 | Ag₆[SiW₁₀V₂O₄₀] | Ag₃[PMo₁₂O₄₀] | Ag₆[SiW₁₀V₂O₄₀] | Ag₃[PMo₁₂O₄₀] |
|-----------|-----------|------------------|------------------|------------------|------------------|
| Antifungal | Control | 128 μg/ml | 128 μg/ml | 128 μg/ml | 128 μg/ml |
| ITC       | 18 (I)c   | 30 (S)c | 34 (S)c | 36 (S)a | 36 (S)a | 21 (S)d | 21 (S)d | 21 (S)d | 21 (S)d | 23 (S)d |
| AMB       | 22 (S)c   | 22 (S)b | 25 (S)a | 15 (S)d | 20 (S)c | 11 (S)e | 11 (S)e | 11 (S)e | 11 (S)e | 14 (S)d |
| KTC       | 62 (S)a   | 38 (S)d | 40 (S)d | 58 (S)b | 63 (S)b | 32 (S)d | 34 (S)f | 36 (S)f | 40 (S)d |
| FLC       | 40 (S)a   | 15 (I)c | 26 (S)b | 10 (R)d | 11 (R)d | ND (R)e | ND (R)e | ND (R)e | ND (R)e | ND (R)e |
| M CZ      | 41 (S)cde | 39 (S)cde | 43 (S)cde | 53 (S)b | 62 (S)a | 40 (S)d | 40 (S)c | 44 (S)d | 49 (S)b |
| LSAS01    | 40 (S)d | 35 (S)b | 32 (S)c | 36 (S)c | 36 (S)c | 36 (S)c | 35 (S)d | 35 (S)d |

S = Sensitive; R = Resistant; I = Intermediary; ND = not determined. Values of mean of inhibition zones in the same line, followed by identical lower case letters do not differ among themselves Tukey Test (p ≤ 0.05).

ITC ≥ 20 = Sensitive; ITC ≥ 12-19 = Intermediary; ITC ≤ 11 = Resistant; AMB > 10 = Sensitive; AMB ≤ 10 = Intermediary or resistant; KTC > 20 = Sensitive; KET ≥ 10 = Intermediary; KET < 10 = Resistant; FLC ≥ 14 = Sensitive; FLC ≥ 14 = Intermediary; FLC < 14 = Resistant; M CZ > 20 = Sensitive; M CZ > 20 = Intermediary; M CZ < 10 = Resistant.

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SEM images of untreated yeast cells (control) showed a normal morphology with budding and regular surfaces (Fig. 7A). After treatment with Ag₃[PMo₁₂O₄₀] or Ag₃[PW₁₂O₄₀], Ag₆[SiW₁₀V₂O₄₀], and Ag₄[SiW₁₂O₄₀], respectively (Fig. 7B), cells presented a crimping surface. In the presence of Ag₄[SiW₁₂O₄₀], cells presented many hyphae structures with little and few budding cells (Fig. 7C), in contrast to the smooth surface and a higher number of budding structures in control cells.

**Scanning Electron Microscopy – SEM**

SEM images of untreated yeast cells (control) showed a normal morphology with budding and regular surfaces (Fig. 7A). After treatment with Ag₃[PMo₁₂O₄₀] (Fig. 7B) or Ag₆[SiW₁₀V₂O₄₀] (Fig. 7C), cells presented a crimping surface. In the presence of Ag₄[SiW₁₂O₄₀], cells presented many hyphae structures with little and few budding cells (Fig. 7C), in contrast to the smooth surface and a higher number of budding structures in control cells.

**Fig. 3. Sensibility, synergistic effects, and reduction in melanization of the mycelial form of the Sporothrix spp. strain CMDB/IOC 01980599 in the presence of Ag-HPA salts and antifungal drug disks by the diffusion agar method.** (A1) Untreated control; (A2-A5) Growth in the presence of Ag-HPA salts Ag₃[PMo₁₂O₄₀], Ag₃[PW₁₂O₄₀], Ag₆[SiW₁₀V₂O₄₀], and Ag₄[SiW₁₂O₄₀], respectively; (B1) Growth in the presence of amphotericin B (AMB); (B2-B5) Growth in the presence of combinations of AMB and Ag-HPA salts; (C1) Growth in the presence of itraconazole (ITC); (C2-C5) Growth in the presence of a combination of ITC and Ag-HPA salts. Figures correspond to 15 days (inferior small plates) and 30 days (superior big plates) of incubation.

**Fig. 4. Sensibility, synergistic effects, and reduction in melanization of the mycelial form of the Sporothrix spp. clinical strain LSASs01 in the presence of Ag-HPA salts and antifungal drug disks by the diffusion agar method.** (A1) Untreated control; (A2-A5) Growth in the presence of Ag-HPA salts Ag₃[PMo₁₂O₄₀], Ag₃[PW₁₂O₄₀], Ag₆[SiW₁₀V₂O₄₀], and Ag₄[SiW₁₂O₄₀], respectively; (B1) Growth in the presence of amphotericin B (AMB); (B2-B5) Growth in the presence of combinations of AMB and Ag-HPA salts; (C1) Growth in the presence of itraconazole (ITC); (C2-C5) Growth in the presence of a combination of ITC and Ag-HPA salts. Figures correspond to 15 days (inferior small plates) and 30 days (superior big plates) of incubation.
Discussion

HPAs have been discussed as efficient, reusable, green and cost effective catalyst compounds that can be quickly synthesized [14, 27]. The present methodology used recrystallization, which has been used as the preferred method for the purification of HPAs [20]. The X-ray diffraction analysis (Fig. 1B) of Ag₃[P₂W₁₂O₄₀] is representative of the Ag-HPA salt profile used in the present study. A similar diffractogram of the POM H₃[P₂W₁₂O₄₀] was described [26] that resembles the results observed in this work but with different purposes and applications. Since 1972, the biological activities of POMs have been studied against tumor cells, viruses and bacteria [27]. A single report in the literature on the use of Keggin-type POMs against fungi was described by Curticăpean et al. [28], but the authors mentioned that it is only effective against bacteria. It has been known since ancient times that silver ions and silver salts can be used as antimicrobial agents because of their growth-inhibitory activity against microorganisms [29]. In this study, we examined the antifungal activity of four Keggin-type Ag-HPA salts, including one polyoxomolybdate (Ag₃[PMo₁₂O₄₀]), and three polyoxotungstates (Ag₃[P₂W₁₂O₄₀], Ag₄[SiW₁₂O₄₀], and Ag₆[SiW₁₀V₂O₄₀]) on yeast and mycelial forms of *Sporothrix* spp., and their interactions with antifungal drugs associated with cell damage. The results presented in Figs. 3 and 4 were overwhelming by the noticeable observation of both the pigmentation and zone of inhibition differences of the treatments and antifungal drugs used.

The growth inhibition test and range of MICs of Ag-HPA salts against yeast *Sporothrix* spp. depended not only on the HPA but also on the yeast strain used. Both features were expected; the first was due to the varying physical-chemical properties of the HPA salts with different formulations. The second is well known and shown in the literature as fungi dimorphism, thermotolerance, cell wall components, and presence of melanin involved in the pathogenicity of *Sporothrix schenckii* [1, 30, 31]. The data showed that Ag-HPA salt phosphorus-containing heteroatoms and metals of the transition molybdenum (Ag₃[PMo₁₂O₄₀]) and tungsten (Ag₃[P₂W₁₂O₄₀]) presented a higher inhibition, with 78-93% and 69-85%, in the yeast form, respectively, compared with salt silicon-containing heteroatoms and metals tungsten (Ag₄[SiW₁₂O₄₀]) and vanadium-substituent (Ag₆[SiW₁₀V₂O₄₀]), which inhibited yeast growth by 68-76% and 36-57%, respectively. In the MIC assay, P-containing Ag-HPA salts appeared to present overall lower MICs, although the values were higher than those presented by traditional antifungal agents (Table 2). Ag-HPA salt treatment revealed that the most resistant strain (ATCC 32285), which apparently did not present the ability to produce melanin pigment based on microscopy like the melanized clinical strains LSASs01, LSASs02 and LSASs03 (Table 2). In previous assays, strain ATCC 32285 showed pigmentation after growth in minimum medium with the addition of 1.0 M L-DOPA (3,4 dihydroxy-L-phenylalanine), a
substrate for melanin synthesis (data not shown). This observation shows that culture conditions and the presence of exogenous substrate for pigment synthesis may favor melanization if the fungus possesses the enzymatic capacity. Previous work has shown that the addition of L-DOPA and glucose in the culture medium positively interferes with the melanization of *S. schenckii* and *Sporothrix brasiliensis* [1]. It was also demonstrated that melanin caused resistance to amphotericin B and to caspofungin against *Cryptococcus neoformans* by reducing the cell permeability, limiting the intracellular concentrations of antifungal drugs, or escaping from free radical components [32] and from freezing-thawing stress of clinical strain [33, 34].

When antimicrobial drugs that present only one mechanism of action are used, the high dosages required for efficacy often result in unwanted side effects and drug-resistance problems. Furthermore, to benefit from the effects of therapy, the synergy actions of antimicrobial agents may be evaluated to explore possible modes of action of new antibiotics or to generate antagonistic effects [35]. Using the agar diffusion method for synergism-like assays between antifungal drugs and Ag-HPA salts on yeast forms of *Sporothrix* spp., we also observed that their

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**Fig. 6. Activity of Ag-HPA salts on the internal structure of Sporothrix spp. yeast cells observed by TEM.** (A) Control of cells in the absence of treatment showed a compact cell wall (CW) and dense microfibrillar material (arrow); (B, C) Cells treated with Ag₃[PMo₁₂O₄₀]; (D, E, F) Cells treated with Ag₃[PWO₁₂O₄₀]; (G, H) Cells treated with Ag₆[SiW₁₀V₂O₄₀]. Treatment showed several ultrastructural alterations, such as reduction in microfibrillar material (B, C, E, arrows, I small arrow), disruption of cell wall (D, E, G, arrows), large vacuolization (B, C, G, H, I), membrane detachment from the cell wall (D, small arrow; E, H, I, arrows), and cell wall thickening (B, D-G). A larger space between membrane with the presence small vesicles (white star) and was seen in cells treated with Ag₆[SiW₁₀V₂O₄₀], as well as a strong cytoplasmic destructuration (I).

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**Fig. 7. Activity of Ag-HPA salts on the surface structure of Sporothrix spp. yeast cells observed by SEM.** (A) Control cells in the absence of treatment; (B) Cells treated with Ag₃[PWO₁₂O₄₀] showed hyphae-structure and slightly deformed conidia; (C) Cells treated with Ag₃[PMo₁₂O₄₀] showed wrinkling of the conidia; (D) Cells treated with Ag₆[SiW₁₀V₂O₄₀] showed thinner hyphae-structures with abnormal budding compared to untreated yeast. Bars are in μm or nm.
interactions depended on the strain tested. CMDB/IOC 010980599 and the clinical strain LSASs01 were sensitive to all antifungal drugs tested, except the last one, which presented intermediate sensitivity to ITC. The antifungal activities of combinations of ITC with the Ag-HPA salts reported here revealed that P-based heteroatoms with \( \text{Ag}_3[\text{PW}]]_{12}\text{O}_{40} \) and \( \text{Ag}_6[\text{PMo}]]_{10}\text{V}_{2}\text{O}_{40} \) were significantly more active against the macroscopic melanized clinical strain LSASs01. ITC and AMB were less active against the mycelial form of the CMDB/IOC 010980599 and LSASs01 strains when tested alone; however, when combined with Ag-HPA salts, their growth was retarded, and restricted pigmentation production suggested interference in melanization impairment after 15 days of incubation at room temperature. It is noteworthy to mention that ITC is a drug commonly used against \( C. \text{ neoformans} \) [33]. Conversely, Si-based heteroatoms with Ag-HPA salts \( \text{Ag}_3[\text{SiW}]]_{12}\text{O}_{40} \) and \( \text{Ag}_6[\text{SiW}]]_{10}\text{V}_{2}\text{O}_{40} \) were significantly more active against the CMDB/IOC 010980599 strain. Fungi also respond to stress stimuli by activating the SOS defense system by DNA repair, providing a survival mechanism [37]. In this manner, after contact of \( S. \text{ schenckii} \) with Ag-HPA salts in the presence of some fungicide, resistant cells were rendered (Tables 3 and 4, and Fig. 4), suggesting a reaction of defense of the fungus or even pigment melanin interference. The combination of antifungal agents may be a successful strategy against fungal strains after developing resistance against traditional fungicides. An antagonistic-like effect was observed when all Ag-HPA salts were used in combination with FLC against both strains, suggesting that the contact of Ag-HPA with cell surface promoted a distinguished alteration within the \( S. \text{ schenckii} \) cell. In the yeast \( \text{Candida albicans} \) the antagonistic interaction between FLC and silver nanoparticles (AgNP) was observed by others, and the authors attributed to release of silver ions that infiltrate into the yeast cells leading to the formation of NPs through reduction by organic compounds present in the cell wall and cytoplasm [47]. The LSASs01 strain also presented resistance in cotreatment with some Ag-HPA salts and AMB, KTC or MCZ (Table 3). The permeability of membranes of cells treated with Ag-salts has been described for some POMs. Kim et al. [38] inferred that Ag-NPs acted throughout the destruction of the membrane integrity of \( \text{Candida albicans} \), and the antifungal activity of the Ag-NPs might be due to one of several intracellular components released during membrane disruption. Silver can also interact with the DNA of microorganisms, preventing cells from replicating [39]. This work observed that Ag-HPA salts in lower concentrations inhibit yeast \( S. \text{ schenckii} \) spp., with MIC values ranging from 8 to 128 μg/ml. Data from fluorescence microscopy using SYTOX green dye suggest membrane permeabilization as one mode of action for all tested Ag-HPA salts on \( S. \text{ schenckii} \) yeast cells (Fig. 5), which was verified by the number of dead cells treated with POMs compared to control cells treated with ITC, with a visible difference in the number of fluorescent cells. SYTOX green was used to demonstrate the antifungal activity of plant-derived antimicrobial peptides against \( \text{Saccharomyces cerevisiae} \) and pathogenic yeasts \( \text{C. albicans} \) and \( \text{Candida tropicalis} \) [24, 40].

Additionally, TEM analysis showed an interaction between Ag-HPA salts and the membrane structure and cytoplasm of the fungus. The ultrastructural alterations promoted by Ag-HPA salts showed modification of cell wall structure, cell vacuolization, cytoplasm disorder and membrane detachment. Overall, the normal cell wall of \( S. \text{ schenckii} \) yeast presents an electron-transparent capsular or slime layer associated with electron-dense microfibrils [41]. In the present study, these microfibrils were preserved after treatment with \( \text{Ag}_6[\text{SiW}]]_{10}\text{V}_{2}\text{O}_{40} \) (Fig. 6), while the other treatments showed a clear reduction in microfibrillar material (Figs. 6B-6G). SEM images showed changes in the surface appearance of the cell surface from smooth to rough thus indicating outer cell wall damage with wrinkling after cells were exposed to \( \text{Ag}_6[\text{PW}]]_{12}\text{O}_{40} \), reflecting drastic internal ultrastructure changes, such as cell wall thickening and fraying (Figs. 6D and 6E). The larger surface area of silver compounds, providing better contact with microorganisms [42] and affecting yeast cells by attacking their membranes, thus disrupting the membrane potential [18], could explain the results presented in this work. Through TEM, other authors observed the formation of ‘pits’ on the membrane surfaces of \( \text{C. albicans} \), leading to the formation of pores and culminating with cell death after silver treatment [18], and considerable accumulation of silver nanoparticles (AgNPs) outside the yeast, with a further releasing of silver ions into the cytoplasm of \( \text{C. albicans} \), also observed by TEM [47]. Finally, pH may interfere with the presence of microorganisms at the site of infection, as well as enhance the activity of drugs during treatment [43]. It is likely that the moderate acidity of Ag-HPA salts and consequently the pH may favor their activity on \( S. \text{ schenckii} \), as evidenced in the data presented with vital dye and electronic microscopy.

Due to the increasing number of drug-resistant fungi, as well as the rise in immunocompromised patients, it has become of major importance to discover alternative antifungal substances to use against emerging mycoses such as sporotrichosis, notably in low-income and social index regions of developing countries such as Brazil [48, 49]), and against relevant mycotic diseases caused by other yeasts, such as \( \text{C. albicans} \) [50]. The Ag-HPA salts showed a consistent activity against \( S. \text{ schenckii} \) spp. yeast cells causing growth inhibition and changes in cell structure, leading to their permeabilization, vacuolization and cytoplasmic disorders, in addition to pigmentation alterations with growth inhibition of their mycelial forms. The results herein strongly suggest that Keggin-type Ag-HPA salts are a promising class of compounds to be explored in the search for new antifungal drugs. The significance of this work relies on the first report concerning the effect of Ag-HPA salts against yeast cells of the pathogenic fungus \( S. \text{ schenckii} \). 

### Acknowledgments

This work was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil, Grant Process 147407/2010-9) and by Fundação de Amparo e Pesquisa do Estado do Rio de Janeiro-FAPERJ (Grant...
Process E-26/110.611/2012; Financial support E-26/110.229/2008). This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) Finance Code 001.

Conflict of Interest
The authors have no financial conflicts of interest to declare.

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