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Article

In vitro susceptibility of *Sporothrix* spp. to complexes coordinated with Co(II) and cobalt chloride hexahydrate

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Sporothrix spp. are the major dimorphic fungus associated with a type of subcutaneous mycosis, sporotrichosis. The limitation of antifungal availability and the past reports of *in vitro* resistance of *Sporothrix* spp. clinical isolates makes it important to search for new compounds with antifungal activities. In this study, we therefore evaluate the *in vitro* activities of complexes coordinated with Co(II) and cobalt chloride hexahydrate against clinical isolates of *Sporothrix* spp. Broth microdilution test was performed as per M38-A2 from CLSI (2008) in duplicate for 31 clinical isolates of *Sporothrix* spp. (27 *S. brasiliensis* e 04 *S. schenckii* stricto sensu). The antifungal activities of the complexes coordinated with Co(II) and cobalt chloride hexahydrate were detected at a concentration range of 32-128 µg/mL for all isolates. None of the compounds demonstrated any cytotoxicity (to macrophage cells) at the concentration of 200 µg/mL. The activity against *Sporothrix* spp. recorded in this study instigate the continuity of experimental studies with Co(II) to search for the mechanisms of antifungal action as well as to evaluate its interaction with the commercial antifungal drugs.

KEYWORDS: *Sporothrix brasiliensis. S. schenckii* stricto sensu. Itraconazole. Minimal inhibitory concentration.

INTRODUCTION

Sporothrix spp. are dimorphic fungi of great importance in the tropical and subtropical countries as the causal agent of sporotrichosis (Mesa-Arango *et al*., 2002). This disease is considered as one of the main subcutaneous mycosis in Brazil, and its etiology is related with the source of infection. Sapronotic cases are mainly related to *S. schenckii* stricto sensu; on the other hand, almost all zoonotic cases are associated with *S. brasiliensis* (Barros, Paes, Schubach, 2011; Rodrigues, Hoog, Camargo, 2016).

Virulence factors, such as enzyme and melanin production, differ between these two main pathogenic species of *Sporothrix*, which can be attributed to the differences in their pathogenicity and antifungal susceptibilities (Marimon *et al*., 2008; Almeida-Paes *et al*., 2015). In fact, *S. brasiliensis*, which has a higher expression of virulence factors relative to *S. schenckii* stricto sensu, has been reported to be more aggressive in experimental models (Arrillaga-Moncrieff *et al*., 2009; Almeida-Paes *et al*., 2015) as well as more frequently associated with atypical cases of sporotrichosis in

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humans (Silva *et al.,* 2012; Almeida-Paes *et al*., 2014). On the other hand, *S. schenckii* stricto sensu has been associated with more incidences of *in vitro* resistance to classical antifungal drugs (Marimon *et al*., 2008; Stopiglia *et al*., 2014).

In the last decades, fungal diseases have gained attention due to their increase in incidence, representing a global health problem. In addition, the treatment of these diseases remain challenging because the therapeutic arsenal are limited to only 3 classes of antifungals for the treatment of all types of fungal diseases, including those caused by *Sporothrix* sp. (Robbins, Wright, Cowen, 2016). In addition, an antifungal treatment regime usually requires drug administration over a long period of time (Butts, Krysan, 2012; Roemer, Krysan, 2014; Scorzoni *et al*., 2017).

The exponential growth in the numbers of cases of sporotrichosis in the recent years is associated with the resistance rates to drugs presently available (Heidrich *et al.,* 2011; Oliveira *et al*., 2011; Gilaberte *et al*., 2014; Robbins, Wright, Cowen, 2016; Sanchotene *et al*., 2017), which instigates the need for the search for new compounds with antifungal activities. Search for specific molecular targets with no cross-reaction with the host, which is responsible for the toxicity related to antifungal therapy, is extremely difficult. Since the identification of arsenic-based compound for the treatment of syphilis by Paul Erlich, inorganic chemistry has demonstrated its potential in this field. In addition, several metal ions have demonstrated fundamental biological properties for structuring and activating enzymatic processes, protein synthesis, and the transfer of genetic information (Rizzotto, 2012).

In this context, transition metals, such as cobalt, have gained prominence, as they possess extensive therapeutic applications, including antifungal activities (Kaur *et al*., 2016). Thus, this study aimed to evaluate the *in vitro* activity of compounds coordinated with Co(II) against the fungi of the *Sporothrix* complex and relate it to the *in vitro* activity of itraconazole (ITC).

MATERIAL AND METHODS

Synthesis and characterization of cobalt(II) metallodrugs

The synthesis of 4-benzilydene-aminobenzoic acid (Compound 3): In a round-bottom flask, 1 g of 4-aminobenzoic acid (Compound 2) and 0.8 mL of benzaldehyde with 5 mL of absolute ethanol were added. The mixture was then heated under reflux for 7 h, followed by cooling to the room temperature. Within an hour, colorless crystals were filtered off and dried at the room temperature.

catena-poly-{trans(diaqua-*kO*)bis[*m*-*p*-(benzilydenamino)benzoato-*k²O,O'*]cobalt(II)} (Compound 4): 150 mg of 4-benzilydene-aminobenzoic acid were mixed with methanol. Two drops of NH_4OH were then added. After dissolution, 74 mg of $CoCl_2 \cdot 6H_2O$ (Compound 1) in methanol were added dropwise. A pink solid was filtered off after 30 min and dried at room temperature.

catena-poly-[trans(diaqua-*kO*)bis(*m*-*p*-aminobenzoato-*k²O,O'*)cobalt(II)] (Compound 5): The same procedure as mentioned above was applied, with the use of 1 g of Compound 2 and 860 mg of $CoCl_2 \cdot 6H_2O$. A pink solid product was filtered off after 30 min and dried at the room temperature. 4-Aminobenzoic acid was purchased from Aldrich and used as received.

Vibrational Spectroscopy (FT-IR)

Infrared spectroscopy was employed for the characterization of the studied compounds. Spectra were registered using the Shimadzu IR PRESTIGE-21 spectrophotometer from 4000 cm^{-1} to 400 cm^{-1}. Solid samples were grounded and mixed with spectroscopic grade KBr for analysis by diffuse reflectance method with the *DRIFTS* accessory.

Electronic spectroscopy (UV-Vis)

Electronic spectra were measured using the Shimadzu UV-2550 double beam spectrophotometer at 200-800 nm and a quartz cuvette with 1-cm optical path.

Solutions of the compounds were prepared with dimethyl sulfoxide (DMSO; Sigma®).

Scanning electron microscopy (SEM)

The SEM images were obtained using the JEOL JSM 6610 LV instrument operating at 10 kV coupled with an energy dispersive spectroscopy (EDS) system. The samples were deposited on a stub and coated using the Denton Vacuum carbon coater prior to exposure (60 s of exposure with 40 mA).

Preparation of the evaluated compounds

Compounds 1-5 were maintained at 4°C and solubilized in 99.5% DMSO to a concentration of 10 mg/mL. Then, the solution was diluted in RPMI until a concentration of 512 µg/mL. ITC (Janssen Pharmaceutical®) was also diluted in DMSO to prepare the solution of 32 µg/mL.

Sporothrix spp. isolates investigated

A total of 31 clinical isolates of *Sporothrix* spp. were analyzed in this study. All of them (including 27 *S. brasiliensis* and 04 *S. schenckii* stricto sensu) were obtained from the fungal collection of the Micology Lab from the Faculty of Medicine of the Federal University of Rio Grande (FAMED-FURG).

From the inoculum preparation, young colonies were obtained after 7 days of incubation at 30ºC in potato dextrose agar (PDA). The colonies were scratched from the plates and added to sterile tubes containing saline solution (3 mL). The resultant fungal suspension was transferred to another tube and centrifuged (10 min at 3500 x g). After 10 min, the supernatant was collected and used as the standard inoculum. The suspension density was adjusted by spectrophotometer (530 nm) to 0.09-0.13 of optical density and then diluted to 1:50 in RPMI 1640 medium buffered with 165 mM of orpholinepropanesulfonic acid (MOPS), providing a solution with a concentration twice greater than the test solution (0.4 x 10^4 a 5 x 10^4 UFC/mL). Pour-plate technique was used to confirm the inoculum concentration for all tested isolates.

Determination of the *in vitro* susceptibility

Broth microdilution test was performed according to the M38-A2 of the Clinical and Laboratory Standards Institute (CLSI, 2008) with ITC (0.0313 a 16 µg/mL) and 5 compounds at a concentration range of 4-256 µg/mL. Microplates were incubated at 30°C for 72 h, and the results were obtained by visual reading of the Minimal Inhibitory Concentration (MIC), which is defined as the lower concentration at which 100% of the fungal growth is inhibited. All tests were performed in duplicate. Then, Minimal Fungicide Concentration (MFC) was evaluated by using a subcultive of 10-µL aliquot from each well of the MIC test, with concentrations equal to or greater than those of the MIC, which were plated in PDA in duplicate and then incubated for 10 days at 30°C, followed by determination of the MFC, as the minimal concentration that makes it unfeasible for the fungus to grow.

Cytotoxicity assay

The cytotoxicity of the compounds was evaluated on adherent J774A.1 macrophage cell line (ATCC TIB-67). Briefly, in each well of a 96-well plate, 200 µL of the cell suspension (concentration 1 x 105 cells/mL) prepared in Dulbecco's Modified Eagle Medium (Vitrocell Embriolife®) supplemented with 10% fetal bovine serum was added, followed by incubation for 24 h at 37°C in a humid atmosphere of 5% CO2. Next, the cells were exposed to different concentrations of compounds (200 to 0.8 µg/mL) and the plate was re-incubated for 24 h. Posteriorly, 30 µL of 0.01% resazurin was added and incubated for 6h, followed by fluorescence reading to determine the half-maximun inhibitory concentration (IC50) (Ahmed, Gogal, Walsh, 1994).

Statistical analysis

Descriptive analyses were performed and the geometric mean of MIC and MFC were calculated. Kruskal-Wallis and Friedman was used to compare the MIC values between the species (*S. brasiliensis* e *S. schenckii* stricto sensu), compounds, and isolates related to their resistance to ITC. All tests were performed using the SPSS 20.0 software,

and statistical significance was considered at a level of 95%. For each compound tested and for ITC, the MIC50 and MIC90 (i.e., the lowest antifungal concentrations that can inhibit 50% and 90%, respectively, of the fungal growth of the isolates) were calculated.

RESULTS

Infrared spectroscopy is an extremely powerful and economical technique for characterization. Compounds containing the C=N group presents a characteristic absorption band in the infrared spectrum, which can be used to identify these compounds. Our identification is based on this characteristic absorption parameter. In addition, the coordination mode in the cobalt(II) complex could also be identified by infrared spectroscopy. In this study, the difference between symmetric and asymmetric carboxylate stretching was found to be compatible with the bidentate chelate coordination mode. Analyses including infrared spectroscopy, as discussed in this study, are widely presented in the literature for similar molecules. We found that our spectroscopic data was compatible with that described previously for similar chemical structures.

In the IR spectra, the main stretch for imine compound (Compound 3, Figure 1) was observed at 1622 cm^{-1}, which agrees with the Schiff base group (C=N) reported elsewhere (Kalaivani, Priya, Arunachalam, 2012). In addition, the absence of stretches from the amine group at (cm^{-1}) 3458($\upsilon_{asym}NH_2$) and 3362(υ_{sym} NH_2) confirms its expected chemical structure (Figure 2) (Joseyphus, Nair, 2010). For the cobalt(II) complexes, an extremely broad absorption band was observed near 3000 cm^{-1}, compatible with the O–H stretch of water molecules. The stretches of carboxylate for *catena*-poly-{[trans(diaqua-*kO*) cobalt(II)]-bis(*m*-*p*-aminobenzoato-*k²O,O'*)} (Figures 3 and 4) and *catena*-poly-{[trans(diaqua-*kO*)cobalt(II)]-bis(*m*-*p*-(benzylideneamino)benzoato-*k²O,O'*)} (Figures 5 and 6) were detected respectively at (cm^{-1}): 1520 ($\upsilon_{asym}COO^-$) and 1390 ($\upsilon_{sym}COO^-$), 1537 ($\upsilon_{asym}COO^-$) and 1390 ($\upsilon_{sym}COO^-$). The D(COO^-) values are in agreement with the bridging coordination mode for the carboxylate ligand (Zelenák, Vargová, Gyoryová, 2007). Our spectroscopic results are in agreement for the coordination polymer proposed structures, where two water molecules may be coordinated to the metal generating octahedral sites. In addition, the pink color exhibited by Co(II) complexes in our study is a well-known characteristic for octahedral geometry.

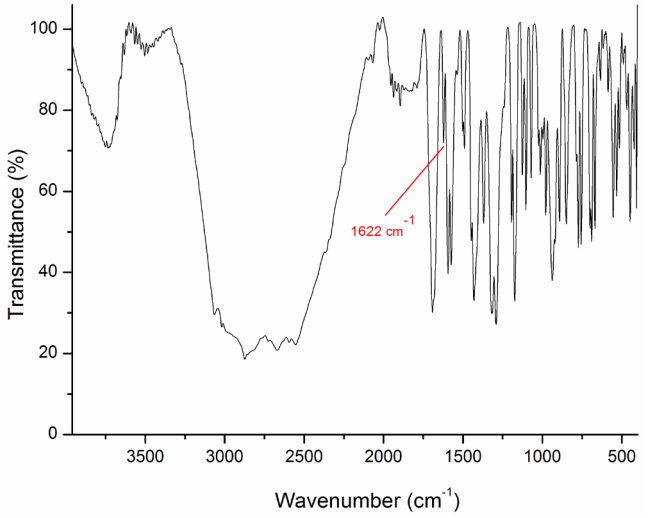

FIGURE 1 – FT-IR spectrum of 4-(benzylideneamino)benzoic Acid.

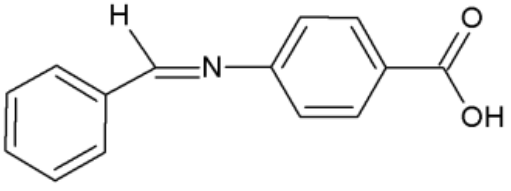

FIGURE 2 – Structural representation of 4-(benzylideneamino) benzoic Acid.

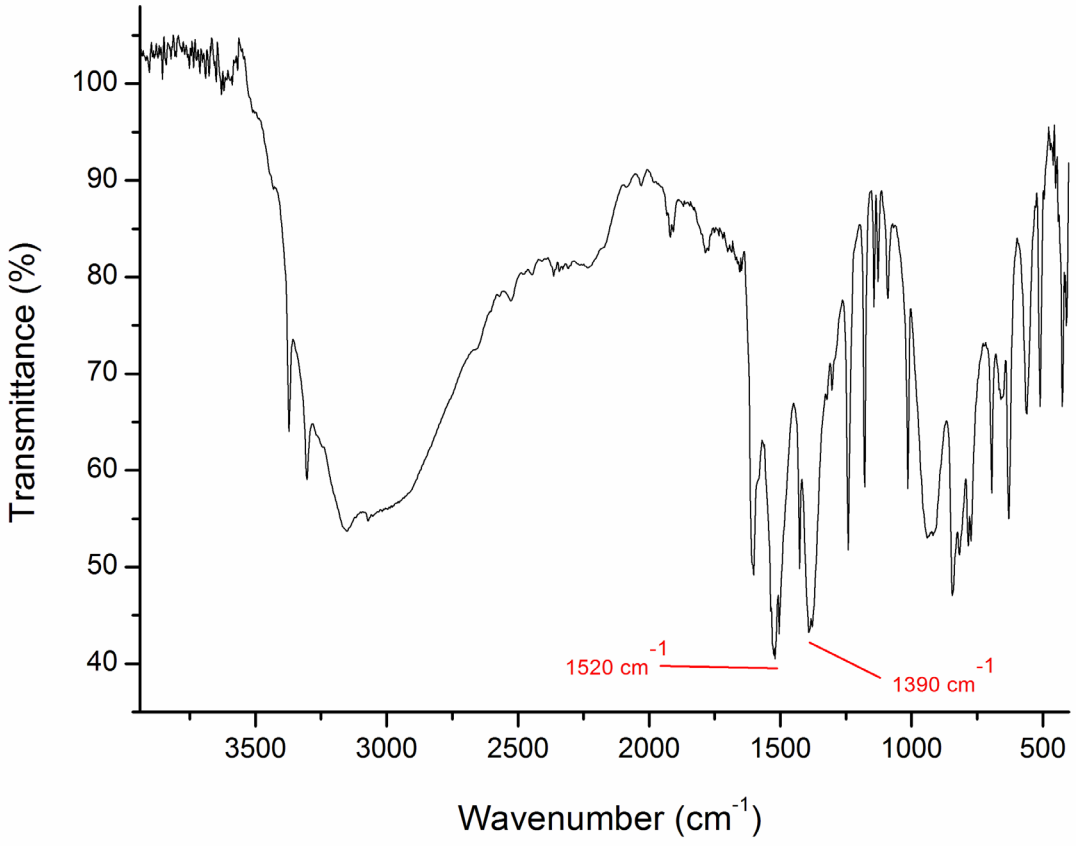

FIGURE 3 – FT-IR spectrum of *catena*-poly-{[trans(diaqua-*kO*)cobalt(II)]-bis(*m*-*p*-aminobenzoato-*k²O,O'*)}.

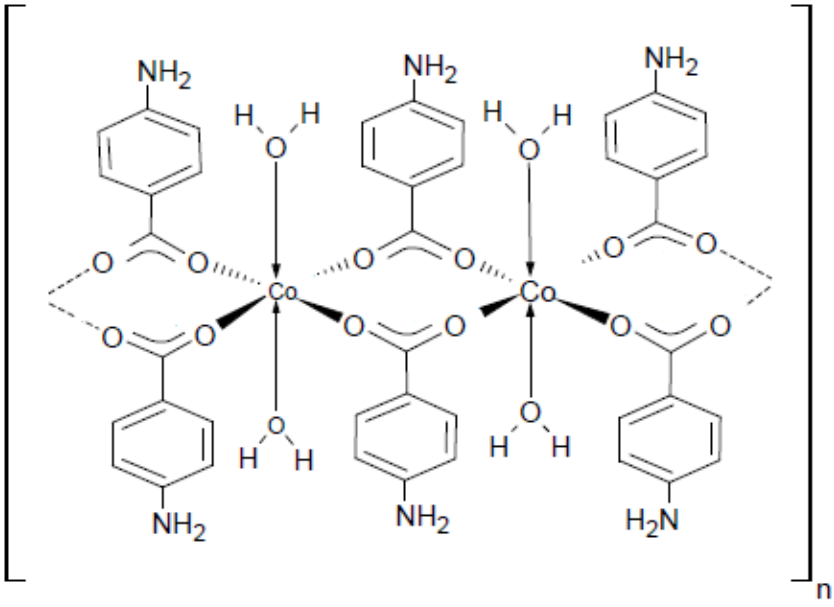

FIGURE 4 – Structural representation of *catena*-poly-{[trans(diaqua-*kO*)cobalt(II)]-bis(*m*-*p*-aminobenzoato-*k²O,O'*)}.

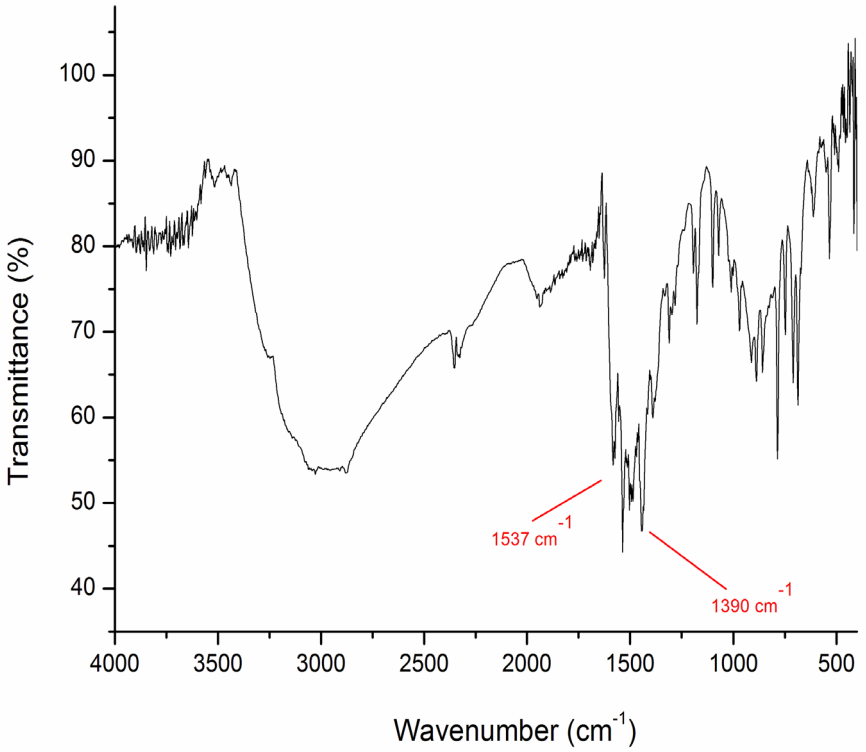

FIGURE 5 – FT-IR spectrum of *catena*-poly-{[trans(diaqua-*kO*)cobalt(II)]-bis(*m*-*p*-(benzylideneamino)benzoato-*k²O,O'*)}.

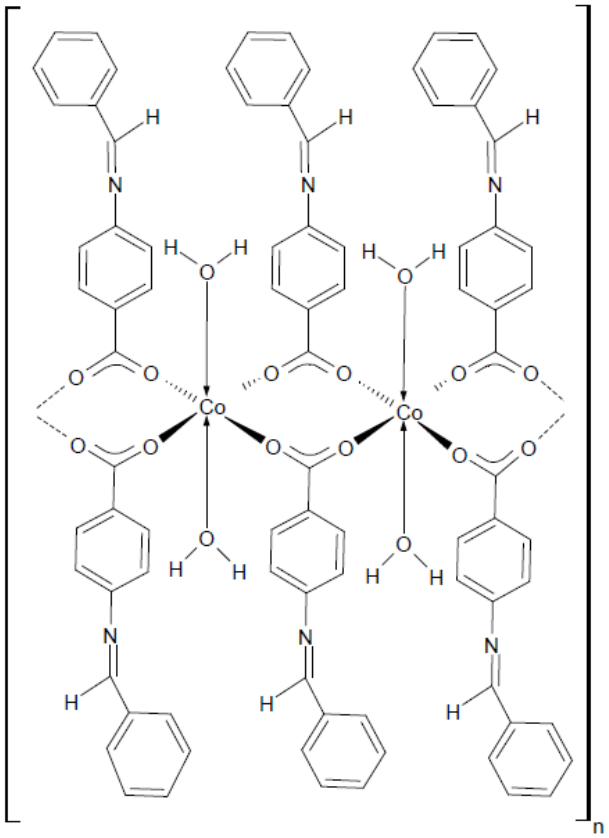

FIGURE 6 – Structural representation of *catena*-poly-{[trans(diaqua-*kO*)cobalt(II)]-bis(*m*-*p*-(benzylideneamino)benzoato-*k²O,O'*)}.

Both the Co(II) complexes electronic spectra (shown in Figure 7) revealed the characteristic p”p* transition in the ultraviolet region due to the aromatic systems. For Compound 4, the absorption at 327 nm can be attributed to the n”p* transition in the C=N group (Shaabani, Shaghaghi, 2010). At >500 nm, extremely weak absorptions could be assigned to the d” transitions characteristic of the octahedral systems for both Compounds 4 and 5. These results are also in agreement with the IR data for the proposed chemical structures.

Besides spectroscopic analyses, the morphological and qualitative elemental analyses were also performed (Figure 8). SEM images revealed plate/lamellar microcrystalline structures for both the Co(II) complexes in powder form. Coupled EDS analysis displayed only the characteristic lines for the expected elements in the samples.

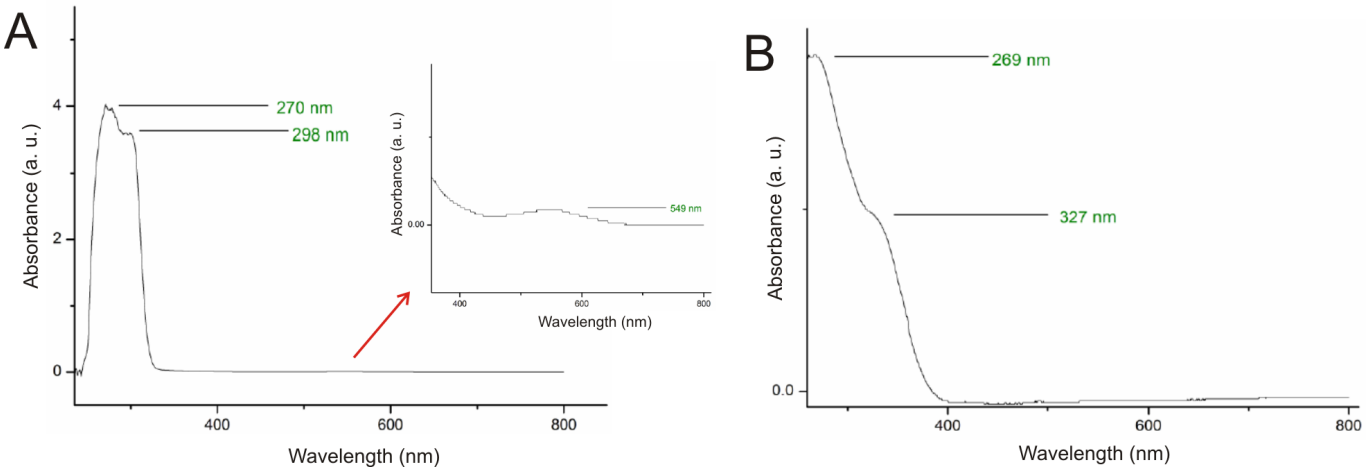

FIGURE 7 – Electronic spectra recorded for compound 5 (A) and for compound 4 (B).

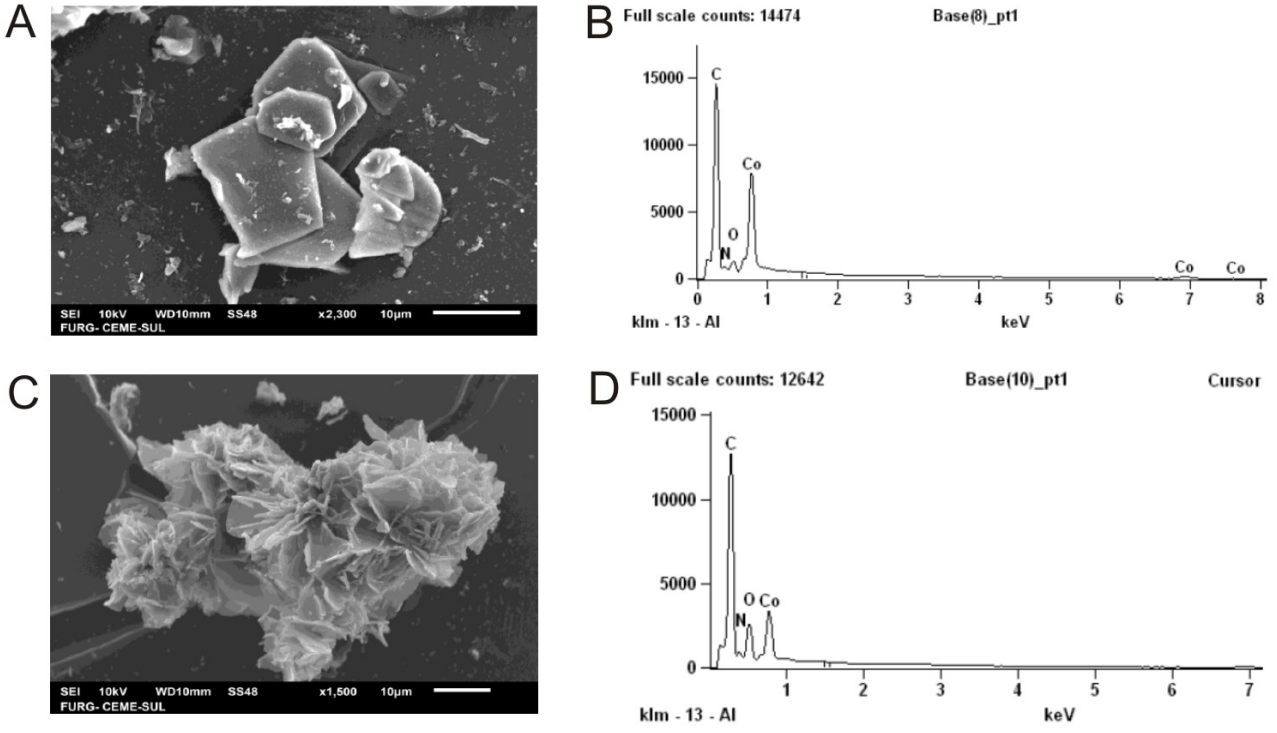

FIGURE 8 – SEM-EDS results obtained for compound 5 (A)/(B) and for compound 4 (C/D).

The complexes coordinated with Co(II) and cobalt chloride hexahydrate demonstrated antifungal activity at the concentration range of 64-128 µg/mL and 32-128 µg/mL, respectively, against all *Sporothrix* isolates, without any significant difference among fungal species (Table I). Nevertheless, by evaluating the average MIC found for each compound, it was possible to identify that the species *S. brasiliensis* was biologically more susceptible to all compounds evaluated, including the ITC.

Ligands (Compounds 2 and 3) did not demonstrate any activity at 256 µg/mL for all evaluated isolates. In addition, it was possible to verify that the co-ordination between $CoCl_2 \cdot 6H_2O$ (1) and the two ligands (2 and 3) influenced the activity of the divalent ion, since the MIC increased by approximately 2x when the compound was coordinated with 4-aminobenzoic acid and 4-(benzylideneamino) benzoic acid (Table I).

Five of the 31 isolates (16.1%) tested showed an MIC value of ≥2 µg/mL to ITC, which included 2 *S. brasiliensis* and 3 *S. schenckii* stricto sensu. Regarding this sensitivity profile of the isolates to the ITC, none of the compound was significantly different ($p > 0.05$) between the tested sensitive and resistant isolates.

Coordinated Compounds (4 and 5) and Compound 1 showed CFM value ≥256 µg/mL against all *S. schenckii* stricto sensu, and CFM ranged from 64 µg/mL to >256 µg/mL against *S. brasiliensis* isolates. Similarly, CFM value of ITC >16 µg/mL were recorded for all *S. schenckii* stricto sensu tested and to *S. brasiliensis* CFM ranging from 0.5 µg/mL to >16 µg/mL (GM = 1 µg/mL).

Table I - *In vitro* antifungal activity (µg/mL) data for Co^{II} (compound 1), their ligands (compounds 2-3) and coordinated compounds (compounds 4-5) against *Sporothrix* spp. strains (n=31)

			***Sporothrix brasiliensis*(n=27)**			***S. schenckii* stricto sensu(n=4)**		
Compound		**IC_{50}**	**MIC GM (range)**	**MIC_{50}**	**MIC_{90}**	**MIC GM (range)**	**MIC_{50}**	**MIC_{90}**
1	**$CoCl_2 \cdot 6H_2O$**	>200	64 (128- 32)^{Ac}	64	64	64 (64)^{Ab}	64	64
2	**$C_7H_7O_2N$**	>200	>256	NA	NA	>256	NA	NA
3	**$C_{14}H_{11}NO_2$**	>200	>256	NA	NA	>256	NA	NA
4	**$C_{14}H_{16}O_6N_2Co$**	>200	99 (128-64)^{Aa}	128	128	107.6 (128-64)^{Aab}	128	128
5	$C_{28}H_{20}O_4$ **N_2Co**	>200	112.6 (128-64)^{Ab}	128	128	128 (128) ^{Aa}	128	128
ITC		NA	0.5 (>16-0.0625) ^{A}	0.5	8	2 (>16 - 0.25)^{A}	16	>16

IC_{50}: inhibition concentration of 50% of the cells in the cytotoxicity assay. GM: geometric mean. MIC: minimum inhibitory concentration. ITC: itraconazole. NA – not available. Different capital letters correspond to $P < .05$ for the difference between the MIC values of the *Sporothrix* species (Kruskal-wallis test); and different small letters correspond to $P < .05$ for the difference between the Co compounds MICvalues (Friedman test)

DISCUSSION

Metallic complexes have been investigated for their pharmacological potential, since they can even act on biomolecules by the exchange of ligands, modifying the activity of the bound compounds and reducing their toxicity or acting in the bioreduction processes (Kaur *et al*., 2016). Changing or modifying the coordinated ligands can be applied as an important tool to provide clues about the structure-activity relationship questions. For example, in a previously published work, the structure of ligands demonstrated an extremely important role in the biological activity against *Streptococcus pneumoniae*. In that case, structural modifications were suggested to be crucial by both the experimental and simulated data to improve the enzymatic inhibition mechanisms (Gonçalves *et al.*, 2019).

Thus, the antimicrobial activity of transition metals divalent ions, such as Co(II), have been reported in the literature against gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi*, *Shigella dysentriae*), gram-positive bacteria (*Bacillus cereus, Corynebacterium diphtheriae, Staphylococcus aureus, Streptococcus pyogenes*) and even against fungi, such as *Candida*, *Aspergillus, Microsporum, Fusarium, Trichophyton,* and *Cryptococcus* (Chohan *et al*., 2006; Hoffman *et al*., 2015; Helsel *et al*., 2017). Our study brings data to increase this applicability, demonstrating, for the first time, the *in vitro* activity of Co(II) against two of the main species of *Sporothrix*.

Complexes containing cobalt have been widely applied in the search for antimicrobial agents, because they present low cost and low toxicity to the host (Heffern *et al*., 2013). On comparing the metal alone to the metal-ligand complexes, the ionic metal presented with the lowest MIC value. In addition, the activity of cobalt chloride against the two *Sporothrix* species suggested that the metal center is essential for the susceptibilities of the complexes. Accordingly, Kaur *et al.* (2016) identified that compounds containing the surfactant Hexadecylpyridinium chloride + copper did not have their potential increased against *Curvularia lunata, Helminthosporium oryzae, Aspergillus fumigates, Aspergillus niger,* and *Cladosporium herbarum*, demonstrating, in turn, that the antifungal activity of metallic complexes was lower than that in the ion alone.

In order to evaluate the antifungal activity of copper and cobalt-containing complexes against 3 *Candida* species, Hoffman *et al.* (2015) identified that cobalt-containing compounds generally possess lower inhibitory activity to copper and, when associated with phenyl, increased the MIC, indicating that metal-phenyl complexes are less active than ligand alone (Helsel *et al*., 2017). Differently from that found in this study, where ligands (Compounds 2 and 3) were not effective either alone or in potentiating antifungal action of Co(II), exhibited similar or even greater MIC values than Compound 1 alone.

However, the cobalt complex (Compounds 4 and 5) improved the activity by complexation in relation of ligands (Compounds 2 and 3), corroborating previous works that demonstrate the cooperation of metals, such as cobalt, for antimicrobial activity in relation to the ligands alone (Nagababu *et al*., 2008; Sekhon, 2010; Raja *et al.,* 2013; Turecka *et al*., 2018; Gałczyńska *et al*., 2019). One explanation for this selective sensitivity is the possibility of Co(II) favoring the chelating activity, lipophilicity of these complexes and, consequently, the intracellular transport of the compounds (Saha *et al.,* 2002; Melnic *et al.,* 2010; Kharadi *et al*., 2011; Castillo *et al.,* 2016).

Ligands 2 and 3 have been used for the synthesis of compounds in order to reduce the toxicity of salt and/or to potentiate its action. In fact, bis (4-aminobenzoate) cobalt (II) acts as a substrate for folic acid synthesis by microorganisms, which is an essential factor for microbial growth, a site that has been shared with sulfonamides (Rizzotto, 2012). However, Compound 2, substituted-PABA, presents in its chemical structure the addition of a phenyl group, which could potentiate the activity of the complexes. Because sulfonamides coordinated with the transition metals have had their activity related to compounds containing greater amount of heterocyclic rings, and, consequently, more carbons (Rizzotto, 2012).

In our study, Compound 5, although it was not sufficiently better than the divalent íon, presented greater activity, with lower MIC values, than Compound 4. This difference is possibly owing to its chemical structure, where the octahedral geometry together with the chelate ring could provide this complex with improved biological characteristics such as high lipophilicity of the central metal atoms relative to smaller compounds and free ions, which would favor the solubility of the molecules and penetration into the cell membranes (Rizzotto, 2012).

Studies using transition metals, such as cobalt, as a metal to construct active complexes against *Cryptococcus neoformans*, have identified the activity of these compounds related to the inhibition of the production of melanin or interfering in the action of enzymatic, mainly fungal urease (Helsel *et al*., 2017), such mechanisms of virulence are also present in *Sporothrix* sp. (Almeida-Paes *et al*., 2009). These findings suggested that it could be one of the fungal targets for cobalt compounds, shared between two species, albeit further studies are necessary

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to address the possible target site and/or mechanism of action and its possible applicability when interacting with or without antifungal.

Finally, considering the activities of the compounds, especially of Compound 1, recorded against all the isolates tested and their low cytotoxicity against macrophagic cells, continuity of the studies with cobalt is suggested, as a potential scaffold in the design of novel antifungal agents.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest pertaining to this work.

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