ANTIFUNGAL ACTIVITIES OF THIOSEMICARBAZONES AND SEMICARBAZONES AGAINST MYCOTOXIGENIC FUNGI

Atividade antifúngica de tiosemicarbazonas e semicarbazonas frente a fungos micotoxigênicos

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ABSTRACT

Mycotoxigenic fungi can compromise the quality of food, exposing human and animal health at risk. The antifungal activity of eight thiosemicarbazones (1-8) and nine semicarbazones (9-17) was evaluated against *Aspergillus flavus, A. nomius, A. ochraceus, A. parasiticus* and *Fusarium verticillioides*. Thiosemicarbazones had MIC values of 125-500 µg/ml. The thiosemicarbazones 1 and 2 exerted fungistatic activity against *Aspergillus* spp., and thiosemicarbazone 2 exerted fungicidal activity against *F. verticillioides*. Compound 2 showed an iron chelating effect of 63%. The ergosterol content of *A. parasiticus* had a decrease of 28 and 71% for the 31.2 and 62.5 µg/ml concentrations of thiosemicarbazone 2 compared to the control. The obtained results of antifungal activity revealed that thiosemicarbazone class was more active when compared to semicarbazone class and, the thiosemicarbazone 2 was the most active compound, specially, against *Aspergillus* spp.

Index terms: *Aspergillus*, *Fusarium*, mycotoxicology, thiosemicarbazones.

RESUMO

Os fungos micotoxigênicos podem comprometer a qualidade dos alimentos colocando em risco a saúde do homem e dos animais. As atividades antifúngicas de oito tiosemicarbazonas (1-8) e nove semicarbazonas (9-17) foram avaliadas frente *Aspergillus flavus, A. nomius, A. ochraceus, A. parasiticus e Fusarium verticillioides*. As tiosemicarbazonas apresentaram valores de MIC entre 125 a 500 µg/ml. As tiosemicarbazonas 1 e 2 exerceram atividade fungistática frente *Aspergillus* spp., e enquanto, a substância 2 apresentou atividade fungicida frente *F. verticillioides*. O composto 2 também foi capaz de apresentar efeito quelante (63%) frente ao ferro e, reduzir 28 e 71% o conteúdo de ergosterol de *A. parasiticus* nas concentrações de 31,2 e 62,5 µg/ml, respectivamente. Os resultados obtidos para a atividade antifúngica revelaram que a classe das tiossemicarbazonas foi mais ativa quando comparada a classe das semicarbazonas e, a tiossemicarbazona 2 foi mais ativa frente *Aspergillus* spp.

Termos para indexação: *Aspergillus*, *Fusarium*, micotoxicologia, tiosemicarbazonas.

INTRODUCTION

Thiosemicarbazones (TS) and semicarbazones (SM) are a class with widespread biological activities including antiviral (Banerjee et al., 2011), antitumor (Easmon et al., 2001), antiparasitic (Oliveira et al., 2008) and antifungal (Tenório et al., 2005) activities. These compounds are able to complex with metal ions, and their biological activity is modulated by the structure of the ligand and the nature of the metal (Pelosi, 2010). Several papers described the antifungal activities of thiosemicarbazones complex, including the *Aspergillus* spp. (Al-Amiery et al., 2012) and thiosemicarbazones as free ligands detaching *A. parasiticus, Candida albicans, A. niger, Trichophyton mentagrophites*, *Rizocthonia solani* and *Stemphylium solani* (Reis et al., 2011; Brousse et al., 2004).

Some strategies are used to minimize the loss of food and other agricultural commodities (Palencia et al., 2010, Medeiros et al., 2012). Benzoic, ascorbic, propionic, formic and acetic acids are used to prevent food contamination. However, the intensive use of these compounds can enhance the expression of mycotoxins by toxigenic fungi, such as aflatoxin (Katerere et al., 2008). Several papers described the antifungal activities of thiosemicarbazones complex, including the *Aspergillus* spp. (Aljahdali; El-Sherif, 2013; Alomar et al., 2013), and thiosemicarbazones as free ligands detaching *A. parasiticus, Candida albicans and A. niger* (Al-Amiery et al., 2012; Reis et al., 2011; Kizilcikli et al., 2007). Further, this class of molecules presents no significant toxicity to the macrophage cells (Soares et al., 2011). *Aspergillus* and *Fusarium* genera encompass species with mycotoxigenic potential and are found in food and feed (Sundheim et al., 2013).

In this work, the antifungal activities of eight thiosemicarbazones (1-8) and nine semicarbazones (9-17) were evaluated against *Aspergillus* spp. and *Fusarium*

verticillioides. For compounds with antifungal activity, the Fe^{2+} chelating effect was evaluated, and their ability to alter fungal ergosterol levels was evaluated by HPTLC method.

MATERIALS AND METHODS

Preparation of synthetic substances

Thiosemicarbazones were synthesized using the appropriate aldehydes and thiosemicarbazide, with drops of concentrated sulfuric and ethanol as a solvent, as previously described in the literature (Oliveira et al., 2008). Semicarbazones were prepared using aldehydes and semicarbazide hydrochloride in the presence of sodium acetate, with ethanol as a solvent (Guerra et al., 2006). All products were characterized by infrared, mass, ^{1}H and ^{13}C NMR spectroscopies.

The compounds were solubilized in DMSO (Merck, Darmstad, Germany): Tween ® 20 (Merck, Darmstad, Germany): RPMI 1640 (Invitrogen, USA) diluted in the ratio 1:1:8. The culture medium RPMI 1640, containing L-glutamine without sodium bicarbonate, was buffered with 3-(*N*-morpholino) propanesulfonic acid (MOPS) (Merck, Darmstad, Germany) at a final concentration of 0.165 mol/l, and pH 7.0.

Antifungal activity assays

All fungal strains were obtained from the Mycological Collection *Trichocomaceae* of the Oswaldo Cruz Institute-Fiocruz/RJ. *Aspergillus flavus* MCT 00040, *A. nomius* MCT 00328, *A. ochraceus* MCT 00435, *A. parasiticus* MCT 00334, and *Fusarium verticillioides* MCT 00177 were rehydrated and activated in Sabouraud dextrose agar (SDA) culture medium and incubated for seven days at 25°C. To induce conidia formation, cultures were grown in potato dextrose agar (PDA) medium for seven days at 35°C. *A. ochraceus* was incubated for seven days at 25°C, and *F. verticillioides* was maintained at 35°C for 48 h and then incubated at 25°C until day seven.

Minimal inhibitory concentrations (MIC) and IC$_{50}$ determination

Antifungal susceptibility testing was performed as described in the M38-A document for filamentous fungi (CLSI, 2002) using 96-well microtiter assay plates containing RPMI 1640 medium at pH 7.0 buffered with MOPS. The compounds were diluted to obtain final concentrations ranging from 3.9 to 500 μg/ml, and the maximum concentration of DMSO was 2.5% (v/v). Conidia of *Aspergillus* spp. and *F. verticillioides* were inoculated into the appropriate wells at a final concentration of $0.4-5x10^{4}$ CFU/ml. Control wells were inoculated with fungi without (solvent and medium alone) the addition of antifungal compounds. Solvent and medium alone were also prepared to be used as control. The minimum inhibitory concentration (MIC) of each drug was determined visually after incubation at 35°C for 48 h. The MIC was accepted as the lowest concentration of the substance able to completely inhibit (100%) the visible growth of the fungus. Amphotericin B (AMB) (Sigma Chemical Co., Missouri, USA) was used as a reference at final concentrations ranging from 16 to 0.12 μg/ml. Each experiment was performed in triplicate. The half maximal inhibitory concentration (IC$_{50}$) values were determined for thiosemicarbazones 1 and 2 using the same microplate assays. Data processing and calculation of the IC$_{50}$ values were performed in Excel 2007 (Microsoft Co., Redmond, WA) after dose-response curve determination. The antifungal activity (AA) was calculated by applying expression (1) to absorbance values obtained at 490nm (Ueda-Nakamura et al., 2006).

$$AA\left(\%\right)=100-\left(\frac{\left(A_{sample}-A_{mean\ of\ growth\ inhibition}\right)}{\left(A_{mean\ of\ 100\%\ growth}-A_{mean\ of\ 100\%\ growth\ inhibition}\right)}\right)x100 \quad (1)$$

Minimal fungicidal concentration (MFC) determination

To determine the minimal fungicidal concentration (MFC) the contents of each well were homogenized and an aliquot (1 μl) from each well was transferred onto SDA. The plates were incubated at 30ºC for nine days. The MFC was defined as the lowest concentration without visible growth of fungal colonies. The fungicidal effect was considered when the values of the MFC was less than or equal to four times the MIC value, as described by Pfaller et al. (2004).

Determination of iron chelating activity

To assess the Fe^{2+} chelating activity, solutions containing 1 an 2 (3.75 μM) in distilled water: DMSO (3:1, 5.0 ml) were used. FeSO$_4$ (3.75μM) in distilled water was added to each solution. Spectrophotometric determinations, scanning interval of 190-500nm, were obtained using a UV-Vis spectrophotometer (Shimadzu UV-Vis Mini 1240) and a quartz cuvette (1.7 ml). The ability of the samples to chelate Fe^{2+} ions was calculated using equation 2 and expressed as a percentage (Lim et al., 2009).

$$Chelating\ effect\left(\%\right)=-\left[1-\left(A_{sample}\,/\,A_{control}\right)\right]x100\%\quad(2)$$

Determination of the stoichiometry of the thiosemicarbazone – Fe²⁺ complex

To determine the stoichiometry of the thiosemicarbazone-Fe²⁺ complex, the L-M Mollard Method was used, with Fe²⁺ ranging from 25 to 225μM and thiosemicarbazones ranging from 225 to 25 μM, according to equation 3 (Guerra et al., 2006; Farias, 2009).

$$l\,/\,m=C_l\,x\,A_m\,/\,C_m\,x\,A_l\quad(3)$$

l/m = ratio between metal and ligand concentrations; C_l = ligand concentration; C_m = metal concentration; A_l = ligand absorbance; A_m = metal absorbance

Ergosterol extraction and evaluation by high performance thin-layer chromatography

A. parasiticus conidia ($1x10^7$/ml) were incubated at 26ºC in RPMI medium in the presence or absence (control) of sub-inhibitory concentrations of antifungal compounds (i.e., 125 and 250 μg/ml of 1, and 31.2 and 62.5 μg/ml of 2). After 48h, conidia were harvested by centrifugation and washed 3 times with 0.85% NaCl. Total lipids were extracted using chloroform: methanol (2:1; 1:1; and 1:2) mixtures (Soares et al., 1995). Combined extracts were dried under a stream of nitrogen and submitted to Folch partition (Folch et al., 1957). The lower phase (neutral lipids) was dried, resolubilized in chloroform and subjected to high performance thin-layer chromatography (HPTLC). The chromatography was carried out on silica gel 60 plates (Sigma Chemical Co., Missouri, USA) using hexane:diethyl ether:acetic acid (60:30:1.5) as a solvent, and the spots were visualized by dipping the plate in a chemical reagent (50 mg of iron chloride, 5 ml of acetic acid, 5 ml of sulfuric acid and 90 ml of distilled water) for 2s followed by heating (Larsen et al., 2004). Ergosterol (4 μg) and lanosterol (1 μg) purchased from Sigma Chemical Co. (Missouri, USA) were also subjected to HPTLC and were used as standards for sterols. Densitometric quantification of ergosterol was performed using Image J free software (Cabral et al., 2013). This experiment was performed three times, obtained from independent triplicate culture/extraction. Compounds concentrations used in this experiment not affected the viability of *A. parasiticus* conidia.

RESULTS AND DISCUSSION

The thiosemicarbazones (1-8) were synthesized from appropriate aldehydes and thiosemicarbazide under acid catalysis as previously described (Oliveira et al., 2008), and the semicarbazones (9-17) from aldehydes, semicarbazide hydrochloride and sodium acetate in ethanol as a solvent (Guerra et al., 2006) (Figure 1). The thiosemicarbazones were obtained with 60-95% yield and the semicarbazones with 45-95% yield.
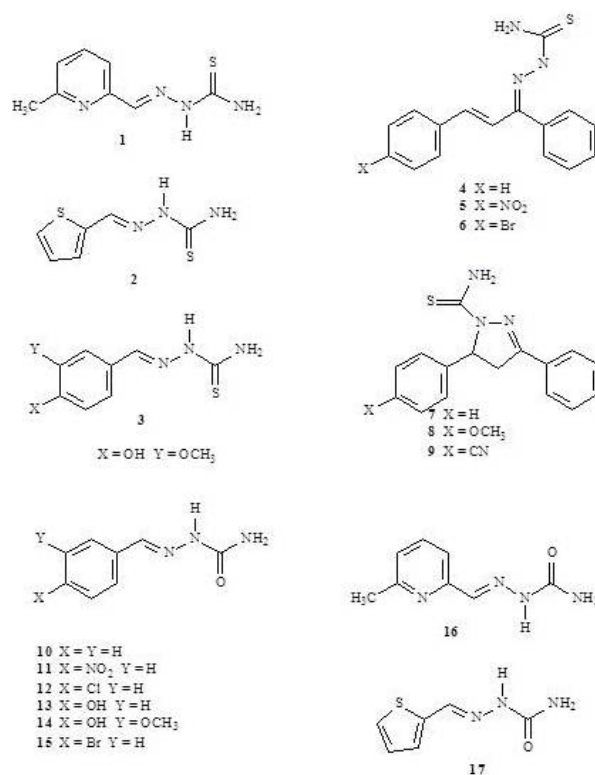

Figure 1 – Chemical structures of the synthesized thiosemicarbazones and semicarbazones.

Thiosemicarbazones 1-9 and semicarbazones 10-17 were assayed against Aspergillus spp. and F. verticillioides using the broth microdilution method (CLSI, 2002). The results indicated that thiosemicarbazone class is more effective at inhibiting fungal growth than those of the semicarbazone class. Thiosemicarbazone 2 inhibited the growth of all fungal samples with an MIC of 125 μg/ml against A. nomius, A. ochraceus and A. parasiticus and with MICs of 250 μg/ml and 500 μg/ml against A. flavus and F. verticillioides, respectively. Thiosemicarbazone 1 inhibited

100% of fungal growth at a concentration of 500 µg/ml for *A. flavus*, *A. parasiticus* and *F. verticillioides*. The fungus most sensitive to the action of the thiosemicarbazones was *A. parasiticus*; thiosemicarbazone 2 showed the best inhibitory activity (MIC=125 µg/ml) against this strain, while thiosemicarbazones 1, 3, 4, 5, and 6 had MICs of 500 µg/ml. The second most sensitive fungus to the thiosemicarbazones was *A. flavus*. Semicarbazones 9 and 11 showed weak growth inhibition only against A. *flavus* and *F. verticillioides*. AMB was used as a reference standard and had an MIC of 2 µg/ml for most *Aspergillus* species and for *F. verticillioides*.

The obtained results for aromatic ketone thiosemicarbazone derivatives showed MIC values 128 mg/ml against *A. niger* (Brousse et al., 2004). The thiosemicarbazones *S*-methyl substituted and their zinc complex showed antifungal activity against *Candida albicans* with MIC values in the range of 19.5 and 312 mg/l, respectively (Kizilcikli et al., 2007), and our previous results for N_1,N_4-disubstituted thiosemicarbazones presented against *C. albicans* MIC values of 250 µg/ml (Reis et al., 2011). Thus, the results obtained for thiosemicarbazone 2 with MIC values of 125 µg/ml against *A. nomius*, *A. ochraceus* and *A. parasiticus* are in the same range of antifungal activity described in the literature.

After the MIC values were determined for thiosemicarbazones 1 and 2, the same microplates were evaluated in turbidity assays using an Elisa reader (490 nm), and the IC_{50} values were calculated to be 86.5 µg/ml and 66.7 µg/ml for 1 and 2, respectively.

The antifungal activities of the thiosemicarbazones and semicarbazones were evaluated against all mycotoxigenic fungi. However, only the compound 2 had fungicidal effect (MFC 500 µg/ml) against *F. verticillioides*, as seen in figure 2.

The results suggested that heterocyclic thiosemicarbazone derivatives (compounds 1 and 2) have increased antifungal activity. When compared the results of the thiosemicarbazone 2 and the semicarbazone 17 results, both thiophene derivatives, is shown the importance of the sulphur instead oxygen atom affording to greatest antifungal activity. Further, when compared our result with other thiosemicarbazone as free ligand, in general way, can be observed MIC values in the same range (Serda et al., 2012). Generally, food and feed commodities are naturally contaminated with mycotoxin-producing fungi, and the antifungal activity of thiosemicarbazones, as ligand free, studied in this work against *Aspergillus flavus*, *A. nomius*, *A. ochraceus*, *A. parasiticus* and *Fusarium verticillioides*, revealed a new control alternative.
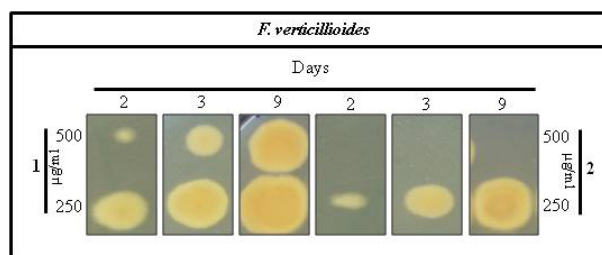

Figure 2 – Minimum fungicidal concentrations (MFCs) of thiosemicarbazones 1 and 2 against *Fusarium verticillioides*. Conidia were added to 96-well microtiter plates containing RPMI 1640 medium, pH 7.0, and 500 or 250µg/ml of thiosemicarbazones 1 and 2. After a 48 h incubation at 35°C and MIC determination, each well was homogenized, and an aliquot (1 µl) was added to SDA medium. After incubation at 35°C for 2, 3 and 9 days, the MFC was determined as the lowest concentration without visible growth of fungal colonies.

The metal chelating activity of thiosemicarbazones is well known (Beraldo, 2004). Iron plays an important role in many essential biological processes (Tenório et al., 2005). Thiosemicarbazones inactivate the non-heme iron subunits of several iron-dependent enzymes, such as the ribonucleotide reductase, a key enzyme for fungal survival (Soares et al., 2011). Thus, thiosemicarbazones 1 and 2 were evaluated for Fe^{2+} chelating activity using UV-visible spectroscopy (Soares et al., 1995). Figure 3 shows the superimposed spectra of thiosemicarbazones 1 and 2 in the presence and absence of Fe^{2+}, and a chelating effect is indicated by increased absorbance. The observed chelating effects of 63% for thiosemicarbazone 2 and 6% for thiosemicarbazone 1 correlated with the observed antifungal activities and suggested a possible mechanism of action for the thiosemicarbazones. The Fe^{2+} coordination number was also determined using the method of Mollard, which indicated a value of 3 for the two thiosemicarbazones with planar pyramidal geometry. These results indicated that the thiosemicarbazones act as dinuclear ligands and share two molecules (French; Blanzy, 1966), and iron chelating effect by antifungal drugs may be useful for prevention and treatment of fungal infections (Zarember et al., 2009).

The ergosterol is an important membrane sterol essential for fungal growth, such as for membrane fluidity and cellular cycle regulation. The mechanism of action of antifungal agents may involve changes in sterol biosynthesis that reduce the amount of

ergosterol produced by the fungus (Alcazar-Fuoli; Mellado, 2013). Synthetic substances can also form complexes with ergosterol and disrupt the fungal plasma membrane, resulting in increased membrane permeability, leakage of cytoplasmic contents and, ultimately, death of the fungal cell (Kathiravan et al., 2012).
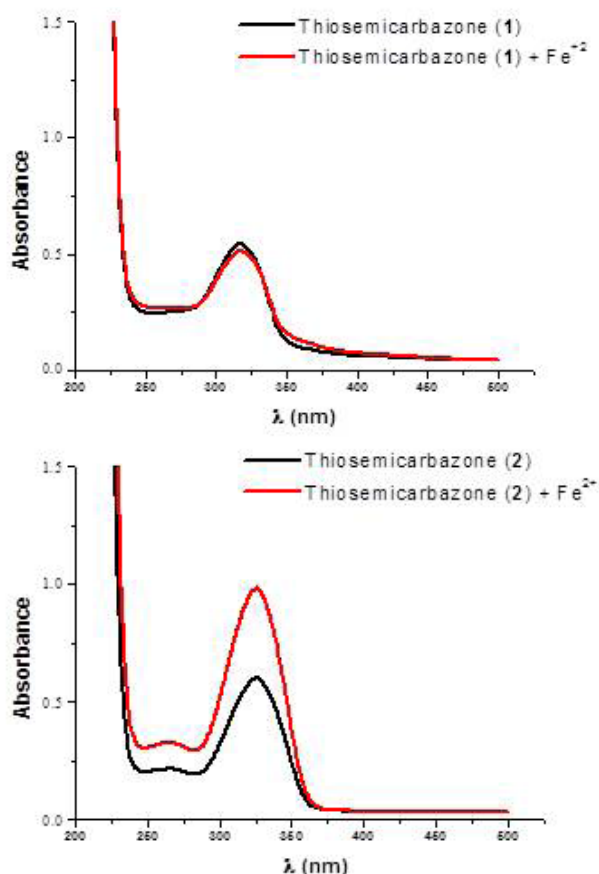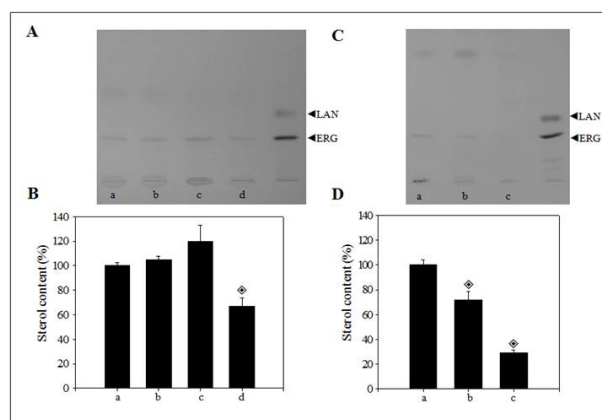

Figure 4 – Effect of thiosemicarbazones 1 and 2 on ergosterol production by *A. parasiticus*. (A) Conidia were incubated at 26ºC for 48 h in RPMI 1640 medium in the absence (a) or presence of 125μg/ml (c) and 250μg/mL (d) thiosemicarbazone 1 (A) and of 31.2μg/ml (b) and 62.5μg/ml (c) thiosemicarbazone 2 (C). The ergosterol (ERG) and lanosterol (LAN) standards were also applied to HPTLC plates, as indicated by arrows. The use of 1% DMSO as an eluent for the thiosemicarbazones did not alter ergosterol levels (b, panel A). Densitometric quantifications (B and D) correspond to each plate above. Graphical representation of HPTLC data was analyzed by means of the Image J software. Sterols content referred to the control was taken as 100%. Symbols denote significant differences (◈, P <0.05 Student's t test) in comparison with control cells (no treatment).

Figure 3 – Superimposed UV-visible spectra of thiosemicarbazone 1 (30μM) in the absence and presence of Fe^{2+} (3.75μM). Superimposed UV-visible spectra of thiosemicarbazone 2 (30 μM) in the absence and presence of Fe^{2+} (3.75 μM).

The treatment with 250 μg/ml of thiosemicarbazone 1 decreased *A. parasiticus* ergosterol content to approximately 33% of that of untreated conidia, while thiosemicarbazone 2 reduced fungal ergosterol content to 28% and 71% at 31.2 and 62.5μg/ml, respectively, indicating a dose-dependent response (Figure 4).

CONCLUSIONS

The obtained results of antifungal activity revealed that thiosemicarbazone class was more active specially, against *Aspergillus* spp. and showed chelating effect and decreased the ergosterol in lipidic content. Further, the compound 2 showed fungicidal effect against *F. verticillioides*.

ACKNOWLEDGMENTS

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