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A battery of assays as an integrated approach to evaluate fungal and mycotoxin inhibition properties and cytotoxic/genotoxic side-effects for the prioritization in the screening of thiosemicarbazone derivatives

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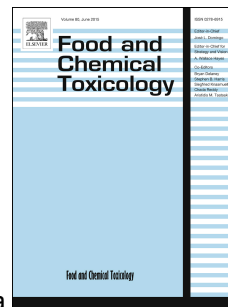
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# Accepted Manuscript



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**A battery of assays as an integrated approach to evaluate fungal and mycotoxin inhibition properties and cytotoxic/genotoxic side-effects for the prioritization in the screening of thiosemicarbazone derivatives**

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### Authors 'contributions

The authors contributed equally to this work. All authors have taken part in the academic discussions of the manuscript's content. All authors have approved the final version.

### Abstract:

Aflatoxins represent a serious problem for a food economy based on cereal cultivations used to fodder animal and for human nutrition. The aims of our work are two-fold: first, to perform an evaluation of the activity of newly synthesized thiosemicarbazone compounds as antifungal and anti-mycotoxin agents and, second, to conduct studies on the toxic and genotoxic hazard potentials with a battery of tests with different endpoints. In this paper we report an initial study on two molecules: S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone and its metal complex, bis(S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazonato)nickel (II). The outcome of the assays on fungi growth and aflatoxin production inhibition show that both molecules possess good antifungal activities, without inducing mutagenic effects on bacteria. From the assays to ascertain that the compounds have no adverse effects on human cells, we have found that they are cytotoxic and, in the case of the nickel compound, they also present genotoxic effects.

## Introduction

Aflatoxins are a class of mycotoxins produced principally by two species of *Aspergillus*, namely *A. flavus* and *A. parasiticus*. In particular, depending on environmental conditions, such as hot and humid climates, these fungi proliferate and can produce aflatoxins (IARC, 2012). The proliferation of these molds has a dramatic influence on the bioeconomy since they grow on carbon-rich substrates like polysaccharides. Consequently, they are commonly found on starch-rich substrates, such as cereals, and their presence causes serious economic losses. Moreover, residues of aflatoxin and their metabolites can enter the food chain, since they can also be present in the meat, offals and eggs of animals fed with aflatoxin contaminated fodder (Richard, 2007). The presence of aflatoxins in food is known to be hazardous for human and animal health because it is at the origin of mutagenic and teratogenic effects, and consequently of tumorigenicity, and also of estrogenic, gastro-enteric, renal and hepatic disorders. In addition, it has been demonstrated that some mycotoxins induce immunodeficiency and reduce the resistance to infective diseases (Marin et al., 2013).

Among good agricultural practices, the use of synthetic fungicides is still the most effective way to intervene. A few molecules are known to inhibit, to a major or minor extent, aflatoxin biosynthesis, but their mechanism of action is still poorly understood (Holmes et al., 2008). It seems that the biosynthesis of mycotoxins is strictly connected to the redox equilibrium within the cell, and that the production of reactive oxygen species (ROS) by the mold and by the host, during the mold/plant interactions, is able to modulate the aflatoxin synthesis pathway (Holmes et al., 2008). Moreover, some studies highlight the importance of the role played by metal ions in the aflatoxin biosynthesis (Cuero, 2005).

The aim of our study is the evaluation of the antifungal activity of compounds possessing a thiosemicarbazone moiety. These substances are known to present significant inhibition activity on proliferating cells (Beraldo et al., 2004; Pervez et al., 2008) and also their metal complexes show an improved biological activity (Parilha et al., 2011, Al-Amiery et al., 2012).

The new compounds we have been synthesizing must be not only efficient in their antifungal activity, but also harmless to the environment, to the ecosystems, and ultimately to human beings. The use of biocides has the undoubted advantage to preserve food, but may represent a risk to human health since consumers could be exposed to residues in food. Today, the use of chemical pesticides is strictly regulated and the removal of the most hazardous chemicals from the market is highly recommended and encouraged (Pal & Gardener, 2006). In recent years, the need to develop disease control measures has become a priority for scientists worldwide and, although restrictions have been imposed to protect food quality and the environment, chemicals are still our main resource to prevent food crop diseases.

The European Food Safety Authority (EFSA) reported that pesticide residues were detected in 46.7% of the food samples analyzed throughout the European Union in 2008. Residues of at least two pesticides were found in 27% of the samples analyzed, among which one-third contained residues of more than four pesticides (EFSA, 2010). Yet the risk assessment of pesticides for humans is based on the hazard characterization of individual active molecules, without taking into account possible combined effects of multiple residues in the diet. Today, more than 300 active substances are used to protect crops. A large number of studies have been published on the possible harmful effects of pesticides for human health especially among occupational exposed subjects (Bolognesi et al., 2011; Weichenthal et al., 2010), but only limited evidence exists regarding the risk for the general population through the consumption of contaminated food.

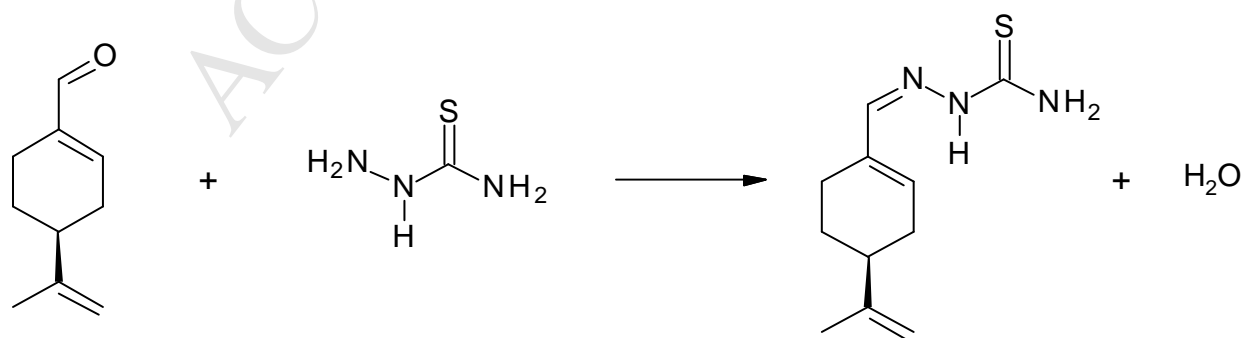
Pesticides residues which contaminate food vegetables could also present mutagenic/genotoxic effects on different cell types (Feretti et al., 2007; Altintop et al., 2012, 2016; Dos Santos et al., 2016), may act as endocrine disrupting chemicals and could affect reproductive activity in human (Chiu et al., 2015). Chronic exposure to low levels of pesticide residues may affect human health and in particular children can be exposed to pesticides residues by dietary ingestion because they eat more food per body mass than an adult and their diet is often rich in food contained high levels of pesticides residues, such as fruit juices or baby foods. For this reason, the second step of our

research was to evaluate the harmlessness of these molecules and we assessed the toxic and genotoxic activities.

Until now, alternative methods to the use of chemicals have not given satisfactory results. It is in this perspective that our research is aimed at the identification of new compounds, based on natural molecules and functionalized so as to make them ligands for bio-metal ions, in the hope to obtain species highly active already at extremely low concentrations but harmless to the health of animals and to the environment. To this aim, we have created a new study approach and optimized a protocol that allows us to synthesize and rapidly evaluate the activity of newly synthesized molecules using a battery of assays, as described in Zani et al. (2015) and to prioritize molecules that deserve further studies. In this paper, we report the initial outcomes of our study on two molecules that have shown interesting results: S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone and its metal complex, bis(S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazonato)nickel (II).

## 2. Materials and Methods.

### 2.1.1 Synthesis and characterization of S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone (molecule V).



Scheme 1

Scheme 1 describes the synthesis of S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone that, from here on, will be referred to as molecule V.

Molecule V was synthesized following this procedure: 0.18 g of thiosemicarbazide (1.9 mmol) were dissolved in 20 mL of EtOH at reflux temperature. An equimolar amount of perillaldehyde (0.35 mL) was subsequently added dropwise. The resulting solution was left under magnetic stirring and refluxing for 14 hours, and monitored by TLC (CH<sub>3</sub>OH: CH<sub>3</sub>CH<sub>2</sub>OH= 2:1; R<sub>f</sub> = 0.78). The pale yellow solution was then poured into a crystallizer, left evaporating at room temperature and a yellow product was isolated as crystals and characterized as reported below:

Yield: 85%.

M.P.: 147 °C.

Elemental Analysis % (theoretical): C 59.49 (59.10), H 7.46 (7.61), N 18.29 (18.80), S 14.49 (14.35), in agreement with a molecular weight of 223.35 corresponding to the anhydrous form of the ligand with formula C<sub>11</sub>H<sub>17</sub>N<sub>3</sub>S.

IR (cm<sup>-1</sup>): 3411 (vs) ν NH<sub>2</sub>, 3159 (vs) ν NH, 2919 (w) ν CH<sub>2</sub>, 1592 (vs) ν C=C, 1529 (vs) ν CN, 950 (m) ν C=S, 886 (s) ν C=S.

UV-Vis (CH<sub>3</sub>OH, 10<sup>-5</sup>M): ε<sub>0</sub> (294 nm) = 17381; 294 nm: n→π\*; 243 nm: n→σ\*.

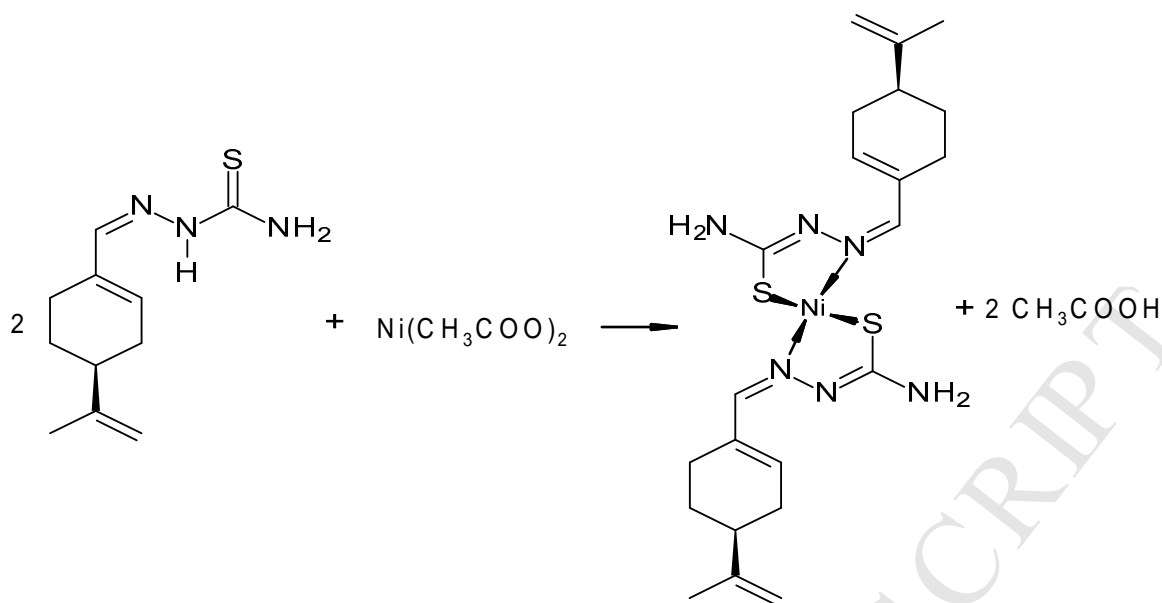
MS m/z (rel. int.%): 246 (M+Na<sup>+</sup>; 85), 224 (MH<sup>+</sup>; 100), 198 (MH<sup>+</sup>-C<sub>2</sub>H<sub>2</sub>; 20).

<sup>1</sup>H NMR (CDCl<sub>3</sub>; 300 MHz, ppm): 9.74 (1H, s, NHCS), 7.27 (1H, t, J=6.0 Hz, CH=N), 7.08 and 6.36 (2H, 2 br s, 1H each, NH<sub>2</sub>), 6.88 (1H, m), 2.6 (2H,m), 2.2 (2H, m), 1.90 (3H, s), 1.40 (1H, m).

### 2.1.2 Bis(S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazonato)nickel(II)

(Molecule T)





Scheme 2

Scheme 2 depicts the synthesis of the metal complex S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone nickel (II) that will be quoted as molecule T.

Molecule T was synthesized using the following procedure: 0.57 g of molecule V (2.55 mmol) were dissolved in 50 mL of EtOH at reflux temperature. Then, 0.32 g of  $\text{Ni}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$  (1.275 mmol) previously dissolved in 10 mL of water, were added to the ligand solution. The resulting mixture was left at reflux temperature under magnetic stirring for 4 hours. The solution became dark brown and was poured into a crystallizer and left evaporating at room temperature. A homogeneous brown powder was isolated and characterized as reported below.

M.P.: 195 °C.

Elemental Analysis % (theoretical): C 52.68 (52.30), H 6.44 (6.81), N 16.51 (16.60), S 12.85 (12.72), in agreement with a molecular weight of 501.39 corresponding to the hypothesized stoichiometry.

IR ( $\text{cm}^{-1}$ ): 3415 (vs)  $\nu$   $\text{NH}_2$ , 2920 (w)  $\nu$   $\text{CH}_2$ , 1592 (vs)  $\nu$   $\text{C}=\text{C}$ , 1515 (vs)  $\nu$   $\text{CN}$ , 946 (m)  $\nu$   $\text{C}=\text{S}$ , 871 (s)  $\nu$   $\text{C}=\text{S}$ .

MS  $m/z$  (rel. int.%): 554 ( $\text{M}+\text{Na}^+$ ; 100).

<sup>1</sup>H NMR (CDCl<sub>3</sub>; 300 MHz, ppm), 7.05 (1H, t, CH=N), 7.05 and 6.34 (2H, 2 br s, 1H each, NH<sub>2</sub>), 6.85 (1H, m), 2.62 (2H,m), 2.25 ppm (2H, m), 1.88 (3H, s), 1.40 (1H, m).

## 2.2. Evaluation of the effects of molecules V and T on *in vitro* mycelium growth

Strains of *A. flavus* used in this work were previously described in Degola et al. (2015). Conidia suspensions were obtained from 10-day YES-agar [2% (w/v) yeast extract (Difco, Detroit, MI), 5% (w/v) sucrose (Sigma, St Louis, MO), 2% (w/v) agar (Difco)] cultures incubated at 28 °C; conidia concentration (quantified by OD<sub>600</sub>) and viability (>90%) were determined according to Degola et al. (2011). Conidial germination rate and post-germination hyphal outgrowth were assessed by analyzing changes in optical density of spore suspensions over time: in a 96 well microtiter plate (Sarstedt, Newton, NC, USA) 10<sup>4</sup> spores were inoculated in a final volume of 200 μL of YES liquid medium amended with 100 μM of V and T molecules and incubated at 28 °C. The optical density at 620 nm (OD<sub>620</sub>) was recorded for each well between 38 and 46 h with a microplate reader (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finland) without shaking. Samples were inoculated in quadruplicate. The “Past 3.x” software was used to analyse statistical differences between samples. Analysis of variance was performed by Levene test. When p-values (p <0.05) were obtained, Kruskal-Wallis test was performed.

## 2.3. Evaluation of the effect of molecules V and T on aflatoxin production

A high throughput procedure performed in a multiwell plate was used to assess aflatoxin accumulation in a coconut-milk derived medium (CCM) (Degola et al., 2011; 2012). The effect on aflatoxin biosynthesis was assessed by the above mentioned microplate fluorescence-based procedure as described in Degola et al. (2015). Briefly, suspensions of conidia were diluted to the appropriate concentrations and brought to the final concentration of 5 × 10<sup>2</sup> conidia/well; cultures were set in a final volume of 200 μL/well of CCM medium added with V and T molecules. The plates were incubated in the dark under stationary conditions for up to 6 days at 25 °C. Aflatoxin

accumulation was monitored by fluorescence emission determination: readings were performed directly from the bottom of wells of the culture plate with a microplate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland) using the following parameters:  $\lambda_{\text{ex}} = 360 \text{ nm}$ ;  $\lambda_{\text{em}} = 465 \text{ nm}$ ; manual gain = 83; lag time = 0  $\mu\text{s}$ ; number of flashes = 3; and integration time = 200  $\mu\text{s}$ .

The “Past 3.x” software was used to analyse statistical differences between samples. Analysis of variance was performed by Levene test. When p-values ( $p < 0.05$ ) were obtained, Kruskal-Wallis test was performed.

#### 2.4. Cytotoxicity of molecules V and T on human cells

The antiproliferative effects of the V and T molecules were evaluated by MTS assay (Cell Titer96® AQueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) towards different human cell lines: Hs27 foreskin fibroblasts, CRL1790 colon epithelial and U937 histiocytic lymphoma cells.

Hs27 (ATCC, CRL1634) and CRL 1790 (ATCC, CCD 841 CoN) were obtained from the American Type Culture Collection (ATCC). U937 cells were obtained from the American Tissue Culture Collection (Rockville, MD). Normal (Hs27 and CRL1790) and tumor (U937) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) and RPMI-1640 medium respectively, media were supplemented with 10% (v/v) fetal bovine serum, 1% L-glutamine (2 mM) and 1% penicillin (100 units  $\text{mL}^{-1}$ )/streptomycin (100  $\mu\text{g mL}^{-1}$ ). Hs27 and CRL1790 cells were used between passage numbers 5 and 20. Cells were maintained in a humidified atmosphere at 5%  $\text{CO}_2$  and 37 °C and culture medium was refreshed every two or three days during sub-culturing.

The cytotoxicity was evaluated according to the following method:  $5 \times 10^3$  cells/well were seeded in 96-well plates in 100  $\mu\text{L}$  of medium without phenol red, supplemented with 1% glutamine, 1% penicillin/streptomycin and 5% fetal bovine serum and then incubated at 37 °C in a humidified (95%)  $\text{CO}_2$  (5%) incubator. After 24 h, cells were treated, in quadruplicate, with increasing concentrations of the molecules in the range 0.5 to 100  $\mu\text{M}$  for further 24 h. The assay was

performed by adding 20  $\mu\text{L}$  of the CellTiter96® AQueousOne Solution Cell Proliferation Assay directly to the culture wells, incubating for 4 h and then recording the absorbance at 485 nm with a 96-well plate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland).

MTS assay was performed to identify  $\text{GI}_{50}$  value, that is the concentration of drug that causes a 50% reduction of cell growth (Malick et al., 1997).

## 2.5 Genotoxicity of molecules V and T on human cells

To assess primary DNA damage the alkaline version of Comet assay was performed with U937 cells as described in Buschini et al. (2009). Briefly, the cells were seeded at a concentration of  $1 \times 10^5$  cell/mL in 24-well plates in 1 mL of medium, supplemented with 1% glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum and then incubated at 37 °C in a humidified (95%)  $\text{CO}_2$  (5%) incubator. After 24 h cells were treated, in duplicate, with increasing concentrations of the molecules in the range 25 to 100  $\mu\text{M}$  for 1 and 24 h. After treatment, determination of cell numbers and viabilities were performed with the trypan blue exclusion method. Only the treatments that had a viability higher than 70% have been processed in the Comet assay. Positive and negative controls were represented by ethylmethanesulfonate (EMS), 2 mM, and DMSO, 100  $\mu\text{M}$ , respectively. DNA was stained with 75  $\mu\text{L}$  ethidium bromide (10  $\mu\text{g}/\text{mL}$ ) before the examination at 400 $\times$  magnification under a Leica DMLS fluorescence microscope (excitation filter BP 515–560 nm, barrier filter LP 580 nm), using an automatic image analysis system (Comet Assay IV – Perceptive Instruments Ltd, UK).

The “IBM SPSS Statistics 24” software was used to analyse statistical differences between samples. The mean values from the repeated experiments were used in a one-way analysis of variance (ANOVA). If significant F-values ( $p < 0.05$ ) were obtained, Student’s t test (Bonferroni’s version) was performed.

## 2.6. Mutagenicity of molecules V and T

The V and T molecules were dissolved in a compatible solvent (DMSO) and assayed with the Salmonella/microsome test (Ames test) at increasing doses (0.1, 1, 10, 50, 100  $\mu\text{M}/\text{plate}$ ), with *S. typhimurium* TA98 and TA100 strains, with and without metabolic activation (S9 mix) to highlight the presence of indirect and direct mutagenic activity. The experimental procedure was the standard plate incorporation method (Maron and Ames, 1983). Salmonella TA98 strain detects frame-shift mutagens and TA100 strain responds to base-pair substitution. According to what has been reported in the review of Mortelmans and Zeiger (2000) we tested the molecules only with strains TA98 and TA100 because it was considered as a first tier approach to be sufficient to identify approximately 90% of the mutagens. Moreover these strains are the most widely used for environmental studies (APHA, 2012).

Positive controls were 2-nitrofluorene (10  $\mu\text{g}/\text{plate}$ ) and sodium azide (10  $\mu\text{g}/\text{plate}$ ) for TA98 without S9 and TA100 without S9, respectively, and 2-aminofluorene (20  $\mu\text{g}/\text{plate}$ ) for both strains with S9. DMSO was tested as the negative controls. The data obtained are presented as revertants per plates computed by means of two replicates with their relative standard deviation. Moreover, the results were expressed as mutagenicity ratio (RM) dividing the revertants/plate by spontaneous mutation rate. Minimum significant ratio was used as a statistical parameter to characterise the fold-change between different doses tested of molecules. The results of the test were considered positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice that of the solvent control and at least two of these consecutive doses showed a dose-response relationship (APHA, 2012; Mortelmans and Zeiger, 2000).

### 3. Results

#### 3.1. Antifungal and antimycotoxigenic activities of molecules V and T

The data reported in Table 1 show that molecule V possesses a good growth inhibitory activity. In fact, we recorded a decrease of 48.7% in mycelial growth compared to the control at the concentration of 50  $\mu\text{M}$  and contextually, a decrease of mycotoxin accumulation of about 78%

(compared to control). Increasing the concentration of molecule V up to 100  $\mu\text{M}$ , both antifungal and antimycotoxigenic activities increased.

Differently from V, its nickel complex T showed a very limited activity on mycelia growth: 4.6% and 12.8% inhibition, at 50  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively, compared to control. Surprisingly, T displayed a comparable or even higher efficacy on preventing toxin accumulation (85% and 90% inhibition at 50  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively) when compared to V. Concentrations of thiosemicarbazone molecules over 100  $\mu\text{M}$  were not taken into account for fungi treatments, since an inhibitory effect of the solvent (DMSO) was observed on both mycelium growth and aflatoxin production.

### 3.2. Cytotoxicity of molecules V and T on human cells

To test cytotoxic effects of the new molecules we selected normal cell lines that represent the different routes of exposure by which this kind of chemicals can come in contact with, and/or enter human bodies by epidermal contact (Hs27) and ingestion (CRL1790). In addition, we have also used a tumoral cell line (U937), because it represents a good cell model used worldwide to identify cytotoxicity of drugs.

Both molecules, V and its nickel complex T, induced a stronger antiproliferative activity on normal cells (Hs27 and Crl1790) compared to tumor cells (U937) (Figure 1). Hs27 cells, human foreskin fibroblasts, showed the major sensitivity to the tested molecules. The  $\text{IC}_{50}$  induced by V resulted 34  $\mu\text{M}$  for Hs27 and 71  $\mu\text{M}$  for Crl1790; it is noteworthy that the proliferative activity of Hs27 cells was strongly reduced starting from the dose 5 $\mu\text{M}$ . The  $\text{IC}_{50}$  induced by T resulted to be 35  $\mu\text{M}$  for Hs27 and 49 for Crl1790; a mild antiproliferative activity was detected also on U937 cells, at the highest tested concentration (100 $\mu\text{M}$ ) the growth inhibition was around 55%.

### 3.3. Genotoxicity of molecules V and T on human cells

To identify if the newly synthesized molecules were able to induce subtoxic DNA alterations, their DNA damaging activity was assessed at different time of exposure (1h and 24h) through the Comet assay. We performed the alkaline version of Comet Assay on U937 cells, a good cell model used worldwide to identify genotoxic activity of drugs. This cell line showed to be less sensitive than normal cells to the toxic effects of the new molecules and for this reason was chosen to detect the subtoxic effects on DNA in human cells. No genotoxicity was detected on U937 cells treated with molecule V at both exposure times (Figure 2). Molecule T, after 1 h treatment, induced a significant dose dependent increase in the tail intensity percentage (TI%) (ANOVA,  $p < 0.001$ ), without perturbing cell viability. After 24h, no genotoxic activity was detected but the 100  $\mu$ M dose induced a remarkable cytotoxic effect (Figure 2). A severe DNA damage and/or an incomplete or altered repair could produce cytotoxic effects leading to cell death.

### 3.4. Mutagenicity of molecules V and T

In Table 2, the results of mutagenicity test carried out on V and T molecule are reported using TA98 and TA100 Salmonella strains expressed as number of revertants for plate (mean values  $\pm$  SD) and mutagenicity ratio (RM). The results were negative at all doses tested and did not show any mutagenic activity. In detail, both molecules induced neither frameshift nor base-pair substitution point mutations in *S. typhimurium* TA98 and TA100 strains, respectively, with and without metabolic activation ( $\pm$ S9).

## 4. Discussion

Thiosemicarbazones are molecules known to present a significant inhibition activity on proliferating cells (Beraldo et al., 2004; Pervez et al., 2008) and their metal complexes systematically show improved biological activity (Parilha et al., 2011, Al-Amiery et al., 2012, Kljun et al., 2014). Their antifungal properties have also been reported in the literature (Beraldo and

Gambino, 2004), but their effects on mycotoxin production is still a poorly investigated aspect (Degola et al, 2015). On this basis, we have envisaged the synthesis of new molecules obtained from natural substances with mild antifungal properties, by functionalization with the thiosemicarbazide moiety in order to make them able to bind metal ions.

In this work, two molecules, namely V, which is the parent thiosemicarbazone ligand, and T, the corresponding nickel complex, were synthesized and tested for their antifungal and antimycotoxigenic activities, and subsequently for their cytotoxic and genotoxic/mutagenic effects. Our study has shown that the nickel complex (molecule T) is a good aflatoxin production inhibitor, but quite unexpectedly it is less effective than the parent organic molecule (Table 2) in inhibiting mycelium growth. As far as these two properties are concerned, both molecules could in principle be used as antifungal products, but candidate hits must be not only efficient, but also harmless to the environment, the ecosystems, and ultimately to human beings. To this aim, we tested the antiproliferative activity of molecules V and T on three human cell lines (Hs27, CRL1790, U937) representative of tissues with which the compounds could come in contact. The results for both molecules showed a high cytotoxicity on normal cell lines (Hs27, CRL1790). Molecule V showed no genotoxic activity in the Comet assay on human cell lines and no mutagenic activity in Ames test on bacteria. The nickel complex (T), already at the dose of 50  $\mu$ M (the first dose tested for antifungal activity), induced a 50% cell growth inhibition in normal cell lines. Molecule T did not induce gene mutation on bacteria, but it was able to produce DNA damaging effects on U937 cells. This behavior is quite similar to the toxicological activity of other nickel thiosemicarbazones already found by the authors (Buschini et al., 2009).

Table 3 qualitatively summarizes all results obtained in tests with different end-points. Based on these results, we can conclude that even though molecules V and T present a remarkable effectiveness as regards their antifungal activity, they cannot be considered “safe” and therefore, as such, must be discarded. Nevertheless, these findings open up the way to the design of similar molecules equally effective, but without side-effects on biological processes and/or organisms. In



addition, these molecules could also be modified to study the effects of the molecular shape on the mechanisms of toxin biosynthesis, and this could allow to find more specific inhibitors or new strategies to fight mycotoxin contamination of food and feed commodities.

Thiosemicarbazones derivatives represent very attractive molecules for a great variety of biological applications not only in the agronomic field, but also in medicine for their pharmacological activities (Altıntop et al., 2012; 2013; Dos Santos et al., 2016). To our knowledge, many studies showed good anti-mycotic activity of thiosemicarbazones derivatives for human pathogens, but only a few studies examined their mutagenicity and cytotoxicity (Altıntop et al., 2016) and their potential use for agronomic applications (Degola et al., 2015).

The direct control of mycotoxin-producing fungi by using synthetic fungicides is still the most effective way to intervene, but the extensive use of fungicides generates long term residues in food and in the environment. To date the consumption of vegetables is the major source of non-occupational pesticide exposure for humans. Many studies have shown that low levels of pesticide exposure is associated with an increased risk of cancer and other chronic diseases (Fortes et al., 2011). On the other hands, aflatoxin contamination of crops is a significant health problem and efficacy and safety aspects have to be reconciled. Molecules more effective but less toxic for health and environment are continuously studied to control mycelium growth and aflatoxin production (Yang et al., 2016). The use of natural molecules and their chemical analogues is desirable as crop protective agents to promote food quality and reduce the health risks to consumers.

To sum up, this research utilized a new approach for the evaluation of the antifungal activity and aflatoxin inhibition properties of new molecules and for the study of their toxicity (Zani et al., 2015) by means of a battery of tests on human and bacterial cells with different genetic end-points. It allowed us also to assess the potential risks related to the use of these new molecules. It must also be stressed that this approach was in line with the requirements of European Directive 2010/63/EU on the use of alternative methods to animals models in toxicological studies.

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Table 1. Antifungal and anti-aflatoxigenic activities of V and T molecules expressed respectively as mean percentage inhibition of growth and of aflatoxin production (in comparison with non-treated controls; mean  $\pm$  SD). Statistical differences between V and T were reported.

	Inhibition of mycelium growth (%)		Inhibition of aflatoxin production (%)	
	50 $\mu$ M	100 $\mu$ M	50 $\mu$ M	100 $\mu$ M
V	48.3 $\pm$ 7.2*	75.5 $\pm$ 4.7*	71.3 $\pm$ 3.5*	86.7 $\pm$ 1.6
T	4.5 $\pm$ 1.9	11.8 $\pm$ 2.8	84.5 $\pm$ 1.8	89.6 $\pm$ 6.8

\*p<0.05

Table 2. Mutagenicity of V and T molecules evaluated with TA98 and TA100 ( $\pm$ S9) strains in Ames test expressed as number of revertants/plate (mean  $\pm$  SD) and mutagenicity ratio (MR).

Samples	DOSE ( $\mu$ M/ plate)	TA 98-S9		TA98+S9		TA100-S9		TA100+S9	
		mean $\pm$ SD	MR	mean $\pm$ SD	MR	mean $\pm$ SD	MR	mean $\pm$ SD	MR
V	0.1	19.5 $\pm$ 2.1	0.9	37.5 $\pm$ 6.4	1.2	117.5 $\pm$ 10.6	1.2	93.0 $\pm$ 0.0	0.9
	1	24.0 $\pm$ 4.2	1.1	42.0 $\pm$ 14.1	1.3	89.0 $\pm$ 2.8	0.9	115.0 $\pm$ 4.2	1.1
	10	19.5 $\pm$ 3.5	0.9	40.0 $\pm$ 5.7	1.3	105.0 $\pm$ 5.7	1.0	113.0 $\pm$ 18.4	1.1
	50	15.0 $\pm$ 0.0	0.7	35.0 $\pm$ 7.1	1.1	99.5 $\pm$ 3.5	1.0	104.5 $\pm$ 17.7	1.0
	100	20.5 $\pm$ 6.4	1.0	35.5 $\pm$ 0.7	1.1	103.5 $\pm$ 3.5	1.0	112.5 $\pm$ 3.5	1.1
T	0.1	20.0 $\pm$ 1.4	0.9	24.5 $\pm$ 0.7	0.8	100.5 $\pm$ 14.8	1.0	113.0 $\pm$ 24.0	1.1
	1	18.0 $\pm$ 1.4	0.8	33.5 $\pm$ 2.1	1.1	109.0 $\pm$ 18.1	1.1	118.5 $\pm$ 3.5	1.2
	10	16.5 $\pm$ 6.4	0.8	33.5 $\pm$ 0.7	1.1	92.0 $\pm$ 2.8	0.9	99.0 $\pm$ 9.9	1.0
	50	30.5 $\pm$ 10.6	1.4	31.0 $\pm$ 9.9	1.0	110.5 $\pm$ 0.7	1.1	110.5 $\pm$ 10.6	1.1
	100	23.0 $\pm$ 4.2	1.1	25.0 $\pm$ 5.7	0.8	97.5 $\pm$ 3.5	1.0	123.5 $\pm$ 9.2	1.2
Negative Control		21.4 $\pm$ 7.5		32.0 $\pm$ 8.5		102.0 $\pm$ 13.1		102.0 $\pm$ 8.8	

Positive controls for TA98 ( $\pm$ S9) and TA100 ( $\pm$ S9): >1000

Table 3. Summary of the results obtained in tests with different end-points.

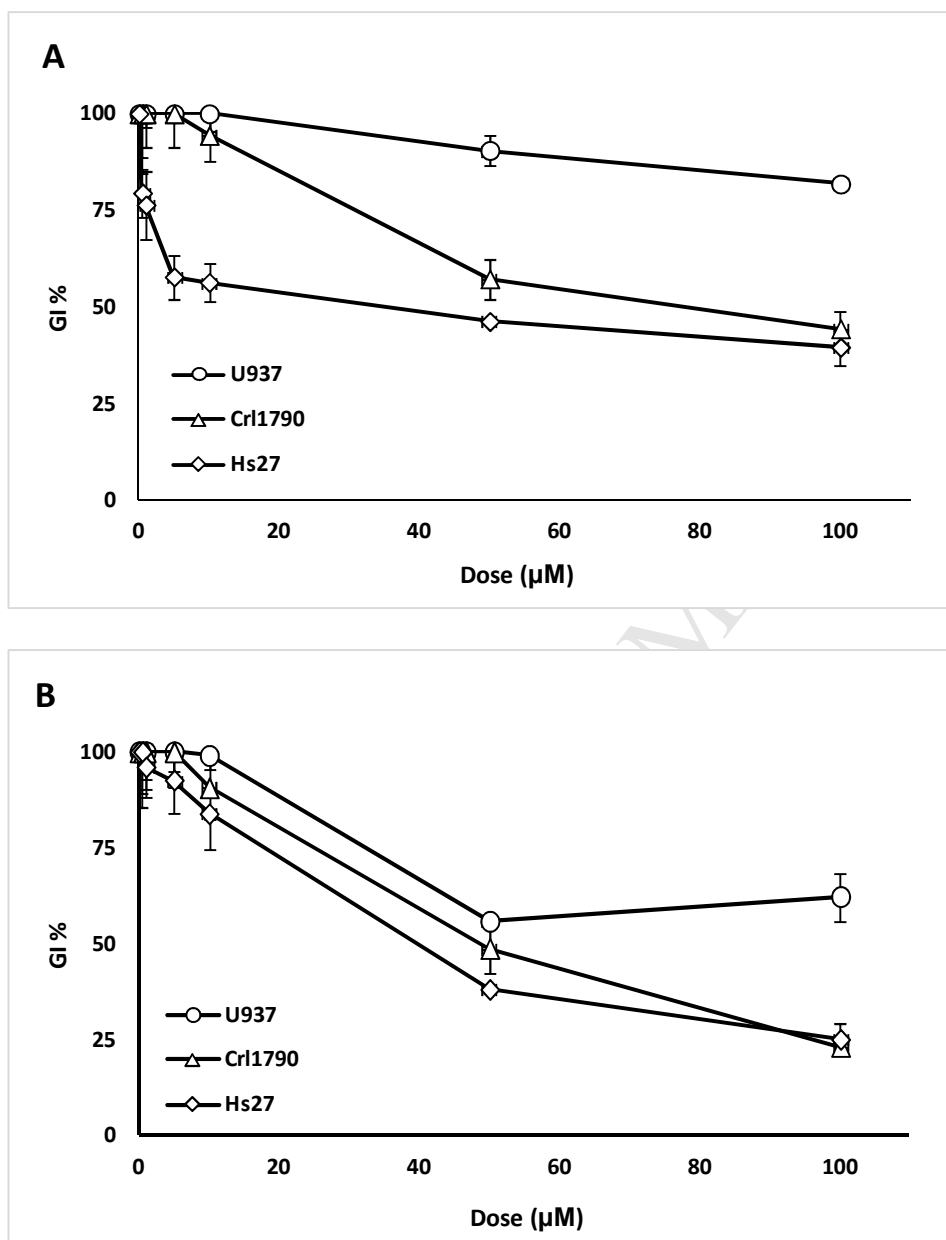
Molecules	Inhibition of mycelium growth	Inhibition of aflatoxin production	Cytotoxicity#		Genotoxicity	Mutagenicity
			Normal cells	Tumour cells		
V	+	+	+	-	-	-
T	-	+	+	-	+	-

+ = positive results in test; - = negative results in test

# a positive results was identified when GI<sub>50</sub> was <100 µM.

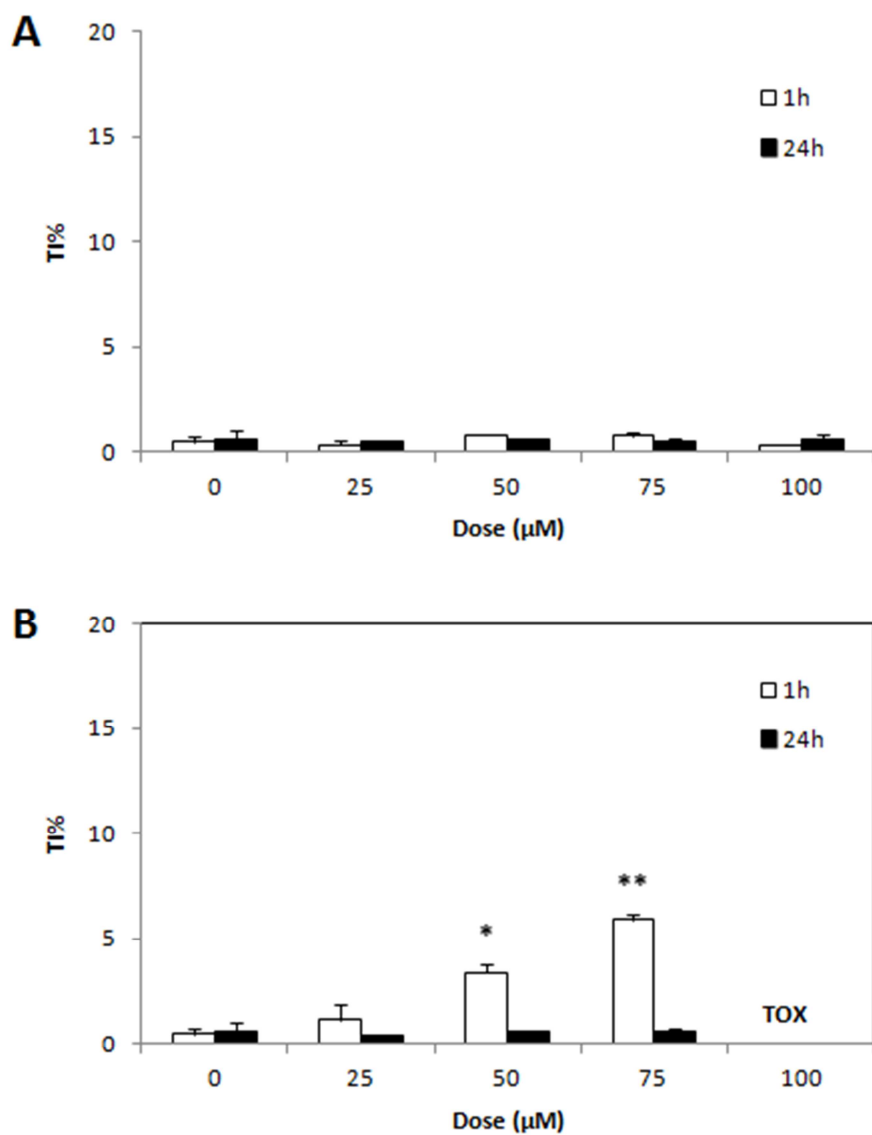


Figure 1. Antiproliferative activity detected by MTS assay on human cell lines (Hs27, Cr11790, U937) treated for 24h with V (A) or T (B).



GI%: percent of growth inhibition.

**Figure 2.** Genotoxicity activity of V (A) and T (B) molecules using alkaline Comet assay on U937 cells treated for 1h and 24h. As positive control was used EMS (2mM):  $TI\%_{1h} = 4.26 \pm 1.32$ ;  $TI\%_{24h} = 45.14 \pm 2.00$ .



TOX: viability  $\leq 70\%$

TI%: percentage of tail intensity.

\*  $p < 0.05$ ; \*\*  $p < 0.01$

Article reference: FCT\_FCT-D-16-01835

Article title: A battery of assays as an integrated approach to evaluate fungal and mycotoxin inhibition properties and cytotoxic/genotoxic side-effects for the prioritization in the screening of thiosemicarbazone derivatives

To be published in: Food and Chemical Toxicology

Highlights:

- 1) Synthesis of new bioactive molecules to reduce the risk of food contamination by aflatoxin;
- 2) Synthesis of new bioactive molecules efficient in antifungal activity and harmless for environment and human health;
- 3) An innovative approach to assess toxicity and genotoxicity of new compounds before their diffusion in environment.

**Competing interests**

The authors declare that they have no competing interests.

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ACCEPTED MANUSCRIPT