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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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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.



## 1. 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, gastroenteric, 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

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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 and Ouellet, 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 and Gambino, 2004; Bisceglie et al., 2014; Pervez et al., 2008) and also their metal complexes show an improved biological activity (Pelosi, 2010; Parrilha 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 and 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 biometal 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. Synthesis and characterization of S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone (molecule V)

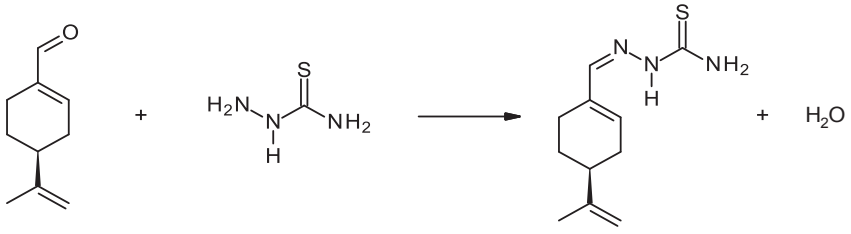
Scheme 1 describes the synthesis of S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone that, from here on, will be referred to as molecule V.

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 h, and monitored by TLC ($CH_3OH$: $CH_3CH_2OH$ = 2:1; Rf = 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 $C_{11}H_{17}N_3S$.

IR ($cm^{-1}$): 3411 (vs) $\nu$ $NH_2$, 3159 (vs) $\nu$ NH, 2919 (w) $\nu$ $CH_2$, 1592 (vs) $\nu$ C=C, 1529 (vs) $\nu$ CN, 950 (m) $\nu$ C=S, 886 (s) $\nu$ C=S.
UV-Vis ($CH_3OH$, $10^{-5}$ M): $\varepsilon_0$ (294 nm) = 17381; 294 nm: $n \rightarrow \pi^*$; 243 nm: $n \rightarrow \sigma^*$.
MS m/z (rel. int.%): 246 ($M + Na^+$; 85), 224 ($MH^+$; 100), 198 ($MH^+$-$C_2H_2$; 20).
$^1H$ NMR ($CDCl_3$; 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, $NH_2$), 6.88 (1H, m), 2.6 (2H,m), 2.2 (2H, m), 1.90 (3H, s), 1.40 (1H, m).




**Scheme 1.** Synthesis of S-4-isopropenylcyclohexen-1-carbaldehydethiosemicarbazone (molecule V).