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Abstract

Tyrosinase is a metalloenzyme involved in *o*-hydroxylation of monophenols and oxidation of *o*-diphenols to *o*-quinones, with formation of brown or black pigments (melanines). Tyrosinase inhibitors are of great interest in medicine and cosmetics (skin whitening compounds), but also in food and beverage industry (antibrowning agents). Here we report on the activity as mushroom tyrosinase inhibitors of a series of hydroxyphenyl thiosemicarbazones (**1**-**5**): one of them revealed an inhibitory activity stronger than kojic acid, used as reference. Enzymatic inhibition activity was confirmed by colorimetric measurements on small wheels of Fuji apples treated with the hydroxyphenyl thiosemicarbazones. The mechanism of action of compounds **1-5** was investigated by molecular modelling and by studying in solution their speciation with Cu(II) ions, the ions in the active site of the enzyme. Finally, compounds **1-5** were tested on human fibroblasts: they are not cytotoxic and they do not activate cells in a pro-inflammatory way.

1. Introduction

Tyrosinase is a metalloenzyme that controls the production of melanin, a broad term for a group of natural pigments found in humans, animals and plants. Melanin is responsible for the color of human skin, but it is, for example, also involved in the enzymatic browning of fresh-cut fruits and vegetables. As a consequence, tyrosinase inhibitors could be of interest as skin whitening compounds, but also

as antibrowning agents for food and beverage industry (Zolghadri et al., 2019). In the last decades the change in lifestyle and the increase in health consciousness and in purchase power of developed countries lead to a fast growth of the ready-to-eat fruit and vegetable market. Notwithstanding the minimal processing that fresh fruits and vegetables undergo, these products present a reduced shelf life, due, among others, to enzymatic browning (Croguennec, T., 2016). In order to help to increase the shelf life and preserve natural and appealing appearance and organoleptic fresh-like characteristics, the fruit processing industry requires the development of new, efficient and safe tyrosinase inhibitors, to be included in the packaging materials or in edible coatings. Consumers are influenced in their choices by the color of fruits and vegetables, that are exposed to enzymatic browning because of brushing, peeling, and crushing operations (Loizzo, Tundis & Menichini, 2012).

A large number of tyrosinase inhibitors have already been reported in the literature, from both synthetic and natural sources (Zolghadri et al., 2019; Pillaiyar, Namasivayam, Manickam & Jung, 2018; Chan, Huang, Lee & Lin, 2014; Ullah, Son, Young Yun, Hyun Kim, Chun & Moon, 2016; Kostopoulou & Detsi, 2018), but most of them are not suitable for practical applications, because of safety reasons, their scarce activity or their off-flavors. Actually, only few antibrowning agents are commonly used by food industry, such as ascorbic and citric acids. Then, it has been recently noted that only a few of available studies are well sounded from chemical and mechanistic point of view (Tang, Cui, Li, Huang & Li, 2018; Xu, Liu, Zhu, Yu & Cao, 2017). Thiosemicarbazones (TSCs) have a lot of different biological activities, and they have been also proposed as inhibitors of mushroom tyrosinase (Yi et al. 2011; Xu, Liu, Zhu, Yu & Cao, 2017; Chen et al., 2012; Hałdys et al., 2018). Metalloenzymes could be inhibited by complexation of the metal ion/ions in their active site; in other words, the inhibition of the metalloenzyme depends on the possibility for the inhibitor to interact with the entire enzymatic cleft, and, in particular, to coordinate the metal ion/ions in the active site (Chen, Adamek, Dick, Credille, Morrison & Cohen, 2019). It is well known that the thiosemicarbazone group has a good affinity for the copper ions, and tyrosinase, that belongs to the Type-3 copper protein

family, has two copper ions in the active site. We have decided to study the activity of a series of TSCs, introducing the hydroxyphenyl group on their skeleton, since phenolic compounds are known to be good tyrosinase inhibitors (Pillaiyar, Namasivayam, Manickam & Jung, 2018). Moreover, when the OH is in *ortho* on the phenyl ring, it can contribute to the chelation of the metal ion, or, if it is in a different position, it can establish interactions with the aminoacidic residues of the enzymatic cleft. Finally, the terminal thiosemicarbazone NH_2 was modified to slightly improve water solubility, obtaining the pool of compounds **1-5** (**Figure 1**), that were tested as mushroom tyrosinase inhibitors. Docking and solution studies were performed in order to have information about structural features impairing enzymatic inhibition activity. The cytotoxic profile was also evaluated. We studied the potential non-specific toxicity toward human tyrosinase-negative (fibroblasts) of compounds **1-5** as well as their ability to induce a pro-inflammatory activation in the same cells.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade and were used as received. The purity of the synthesized compounds was verified ≥95 % by elemental analysis. Technical data about NMR, ATR-IR, electrospray mass spectral analyses (ESI-MS), and ICP analyses were already reported (Rogolino et al., 2015).

2.2. Synthesis

The TSCs **1-5** were prepared following reported literature procedures (Rogolino et al., 2015). Briefly, thiosemicarbazide in hot absolute ethanol was mixed with an equimolar amount of aldehyde, in the same solvent. The solution was refluxed for 6 h, cooled at room temperature, and then concentrated

in vacuum. The precipitate was filtered off, washed with cold ethanol and dried in vacuum. Characterizations (^{1}H-NMR, IR, MS, elemental analysis) of the TSCs **1**-**5** are reported in the Supplementary Material.

2.3 Tyrosinase activity

The mushroom tyrosinase used for the bioassay was purchased from Sigma-Aldrich. The diphenolase activity of tyrosinase was checked according to the method of Masamoto (Masamoto, Ando, Murata, Shimoishi, Tada & Takahata, 2003) with minor modifications, using L-DOPA (Sigma-Aldrich) as substrate. Enzyme activity was determined at room temperature following the increase in absorbance at 475 nm (ε = 3700 M^{-1} cm^{-1}) determined by the formation of dopachrome by a Kontron Uvikon 860 spectrophotometer. Stock solutions of L-DOPA (15 mM), inhibitor (0.5 mM) and enzyme (2.5 U/μl) were prepared in Na_2HPO_4-NaH_2PO_4 25 mM buffer (pH 6.8) and then diluted to the appropriated test concentrations. The experiments were performed at a constant enzyme concentration (12.5 U/mL), varying inhibitor and substrate concentrations. The substrate was added at the last moment to the reaction mixture and immediately absorbance measurement started for measuring the initial reaction rate from the slope of the linear increase of optical density. 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (kojic acid, Sigma-Aldrich) was used as positive control.

2.4 Colorimetric measurements

The colorimetric measurements were performed using Fuji apples, following the method of Wu *et al*. (Wu, Cheng, Li, Wang & Ye, 2008). Apples were washed and cut into small wheels by a mandolin slicer. After that, they were dipped in 200 mL of 30 μM test solution, prepared in Na_2HPO_4-NaH_2PO_4 25 mM buffer (pH 6.8) for 3 min and then drained. This procedure was repeated for the compounds **1**, **2**, **3** and **5**. Control samples were dipped in Na_2HPO_4-NaH_2PO_4 25 mM buffer alone. Samples were

then placed on absorbent paper and stored at room temperature. Changes of color were measured with a tristimulus reflectance colorimeter (TECHKON SpectroDens – Spectro-Densitometer), calibrated using a standard white reflector plate. The readings were made for each replicate by changing the position of the apple small wheel to get uniform color measurements. The CIE *L* a* b** color space was assumed, where *L** indicates lightness, *a** indicates chromaticity on a green (-) to red (+) axis, and *b** chromaticity on a blue (–) to yellow (+) axis. Measurements were made immediately after dipping and at time intervals thereafter. Total color difference (ΔE*) calculated as follows:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

was also used to evaluate the antibrowning potential of different treatments.

2.5 Potentiometry

The Cu(II) stock solution was prepared from $CuCl_2 \cdot 2H_2O$ (Merck). Its concentration was determined by titrating the solution with EDTA in presence of concentrate ammonia; Fast Sulfon Black is used as indicator. Equilibrium constants at 25 ± 0.1 °C for protonation and complexation reactions were determined as detailed in Rogolino et al., 2015. Temperature was controlled to ±0.1 °C by a ISCO GTR 2000 IIx bath. Appropriate amounts of ligand solution, prepared by weight, were titrated with standard KOH or HCl (when starting from the alkaline solution pH ≈11), with and without metal ions, under magnetic stirring. The protonation constants were obtained by titrating 20 or 50 ml of samples of each ligand (0.05 mmol). The speciation was defined by performing the titrations at different ligand/metal ratios (1 up to 4). At least two measurements (about 60 experimental points each) were performed for each system. The electrodic chain was calibrated, again, as detailed in Rogolino et al.. Speciation and the logarithm of the stability constants (log β_{pqr}) are obtained by using the program HYPERQUAD (Gans, Sabatini, & Vacca, 1996). In $\beta_{pqr} = [M_pL_qH_r]/[M]^p\,[L]^q\,[H]^r$, the equilibrium

constant for $pM + qL + rH = M_pL_qH_r$, M is the metal, L the fully deprotonated ligand, H the proton, and charges are not reported for simplicity.

2.6 Cellular assays

Human skin fibroblasts, obtained with informed consent from a healthy male subject, were cultured in standard culture medium, i.e. DMEM (Lonza), supplemented with 100 U/mL penicillin/streptomycin (Life Technologies) and 10% fetal calf serum (Sigma Aldrich), in 96-well plates at 5% CO_2 and 37°C. When confluent, cells were incubated in triplicate with sterile filtered standard culture medium with or without **1**-**5** 100 μM for 24 hours. Cell viability was then evaluated through the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1993). Briefly, cells were incubated with 1 mg/ml MTT (Sigma Aldrich) for two hours. Cells were then washed with phosphate buffer saline at 37 °C and treated with dimethyl sulfoxide (Sigma Aldrich) in the dark. Cell lysates absorbance at 570 nm was measured with a Spark 10M TECAN microplate reader.

Cell pro-inflammatory activation was evaluated measuring the concentration of interleukin-6 (IL-6) in cell supernatants after 24 hours incubation with standard culture medium with or without 100 μM solutions of **1**-**5**. IL-6 was measured using a commercially available ELISA kit (Thermo Scientific).

2.7 Molecular modelling

2.7.1 Ligands preparation

Molecular Operating Environment software package platform (MOE) was used for the current computational study (Molecular Operating Environment, MOE 2009). A single database file containing the ligands for molecular docking studies was prepared using MOE builder mask. The geometries of the selected ligands were optimized by an energy minimization pass until a

convergence gradient criterion of 0.0001 kcal mol^{-1} $Å^{-1}$ was reached, using the MMFF94x force field. Solvent effect was calculated using the Generalized Born Solvation Model.

2.7.2 Protein preparation

The X-ray coordinates of a tyrosinase from *Agaricus bisporus* in complex with inhibitor tropolone (PDB code: 2Y9X) (Ismaya et al., 2011) were retrieved from RCSB Protein Data Bank (http://www.rcsb.org/). Before docking protein termini were capped and solvent water molecules were removed; then the system was 3D protonated and partial atomic charges were assigned according OPLS_AA force field.

2.7.3 Docking procedures

The active site pocket was defined in order to contain the residues within 15 Å from the co-crystallized tropolone molecule. The different docking methods implemented in MOE platform were tested by re-docking using different combinations of placement, rescoring and refinement features, and the following conditions were chosen: Alpha Triangle as placement method, London dG as rescoring 1 function, Forcefield as refinement, and Affinity dG as second rescoring procedure. The same docking procedure was applied to the ligands, and the highest docking pose of each ligand was retained.

3. Results and discussion

The TSCs **1**-**5** were easily prepared in good yields by condensation of the hydroxyphenyl aldehyde and thiosemicarbazide (**5**) or 4-ethyl-3-thiosemicarbazide (**1**-**4**) (**Figure 1**) (Rogolino et al., 2015). The ethyl derivatives are slightly more soluble in water, where tyrosinase inhibition tests are carried out. **1**-**5** differ for number (two or three) and positions of the OH groups on the phenyl moiety bound to the TSC scaffold, offering different possibilities for the interactions with the enzyme.

187

188 *3.1 Effect on tyrosinase activity*

189 Measurements were carried out as a function of the substrate concentration at different inhibitor
190 concentrations, as described in paragraph *2.3*. Kojic acid, one of the most studied tyrosinase inhibitor
191 (Bentley, 2006), was assumed as reference.

192 Compounds **3** and **4**, with two hydroxyl groups in different positions of the phenyl ring (**Figure 1**),
193 do not exhibit any inhibiting activity against mushroom tyrosinase. The inhibitory effect of **1**, **2** and
194 **5** is concentration-dependent, the enzyme activity decreasing with the increasing of the concentration
195 of the tested compounds, without being completely suppressed. In **Figure 2** the Michaelis-Menten
196 plots for the reaction without inhibitor and in presence of different concentrations of compounds **1**
197 and **5** are shown (**2** behaves in a very similar way as **1** and it is omitted for clarity). The type of
198 inhibition on the diphenolase activity was defined by the Linweaver-Burk plot (Copeland, 2000). The
199 active compounds show a noncompetitive (mixed type) inhibition, that is they bind to the enzyme not
200 necessarily by competing with the substrate. Since the affinity (K_i) of the inhibitor for the free enzyme
201 E is different from the affinity of the inhibitor (αK_i) for the complex enzyme-substrate ES (this means
202 that α is finite but $\neq 1$), it can be defined mixed inhibition (**Table 1**). The Lineweaver-Burk plot for
203 **5**, the most active compound, is reported as an example (**Figure 2**); the same plots for **1** and **2** can be
204 found in SI (**Figure S1** and **Figure S2**).

205 Considering the reciprocal form of the most general velocity equation for noncompetitive inhibition
206 (Copeland, 2000):

207 $$\frac{1}{v} = \left((1 + \frac{[I]}{Ki}\right)\left(\frac{Km}{Vmax}\right) * \frac{1}{[S]} + \left(1 + \frac{[I]}{\alpha Ki}\right) * \frac{1}{Vmax}$$

208 ([I] and [S] are the concentrations of the inhibitor and the substrate, respectively), the inhibition
209 constant K_i is obtained from the dependence of the slope of the Lineweaver-Burk plot from the
210 inhibitor concentration, and the value αK_i from the dependence of the intercept of the same plot from

211 the inhibitor concentration. Both plots are linear (**Figures 2c** and **2d**) and the above mentioned
212 quantities are obtained from the *ratio* intercept/slope of the appropriate straight line. K_i is related to
213 the inhibitor activity, being the greater the smaller is the value of K_i.

214 Among the studied TSCs, only **5** possesses an inhibitory activity of practical interest, stronger than
215 those of the kojic acid assumed as reference, as revealed by the values of K_i and IC_{50} (**Table 1**). IC_{50}
216 is the concentration of the inhibitor able to halve the maximum reaction rate in the adopted
217 experimental conditions; the value reported for kojic acid agrees with the literature data, if normalized
218 for the tyrosinase concentration (Qamar et al. 2019; Sari, Barut, Özel, Kuruuzum-Uz & Şohretoğlu,
219 2019; Şöhretoğlua et al., 2018; Larik et al. 2017).

220 The position of the substituents on the phenyl group plays a negligible role on the inhibitor activity
221 (**Table 1**), whereas a little enhancement is obtained for the compounds **1** and **2** with three hydroxyls
222 instead of two. Note that **4** and **5** differ only for the ethyl group bound to the terminal nitrogen in **4**:
223 evidently, the removal of this group is essential for changing an inactive compound (**4**) in a strong
224 tyrosinase inhibitor (**5**).

225

226 *3.2 Antibrowning treatment of Fuji apple small wheels*

227 The effects of the dipping in 30 μM solutions of **1**, **2**, **3**, and **5** are qualitatively shown in **Figure 3**,
228 where they are compared after 28 hours from dipping. Compound **3** was used to check if it has an
229 antibrowning effect on the apple wheels, even if it does not inhibit tyrosinase activity in the enzyme
230 assay. The increase of browning follows, as expected, the reverse order of the inhibitory activity
231 against tyrosinase. The inactive compound **3**, as expected, behaves in the same way as the control
232 sample. On the contrary **5** seems to show the greatest antibrowning effect. It is much more difficult
233 to quantify the effect in terms of color differences by means of colorimetric measurements. Because
234 an increase of the parameter a* is related with an increase in redness, it has been suggested (Abbott

& Buta, 2002; Saper & Miller, 1998; Rojas-Grau, Sobrino-Lopez, Tapia & Martin-Belloso, 2006) that this parameter could be the more discriminating one. However, our results show that all the colorimetric parameters are able to provide a similar information, and in particular a* (chromaticity on a green (-) to red (+) axis) and b* (chromaticity on a blue (–) to yellow (+) axis); see in the supporting information **Figure S3**. The samples are divided in two groups: those completely without antibrowning activity (control sample and sample treated with **3**) and those treated with a thiosemicarbazone with antityrosinase activity (**1**, **2** and **5**). Within the last group, the differences are not statistically significant, even if they are appreciable at naked eye.

3.3 Potentiometry

Tyrosinases, belonging to the type 3 copper-containing protein, contain two copper ions in the conserved active site, coordinated by six histidine residues (Deri et al., 2016; Ramsden & Riley, 2014). It has been suggested (Sendovski, Kanteev, Ben-Yosef, Adir & Fishman, 2011; Noh, Kwak, Seo, Seo, Kim & Lee, 2009; Lima et al., 2014; Bochot et al., 2014) that a possible mechanism of inhibition of tyrosinase activity is the chelation of the metal ions in the active site. This possibility was deeply investigated for kojic acid, a well-known tyrosinase inhibitor (Noh, Kwak, Seo, Seo, Kim & Lee, 2009; Lima et al., 2014; Bochot et al., 2014). Starting from these considerations, we have studied the solution equilibria of the TSCs **2**, **4** and **5** with H^+ and Cu(II) ions by potentiometry in water at ionic strength 0.1 M KCl. In water these ligands are slightly soluble at acid pH, but they are perfectly soluble in the millimolar range at alkaline pH; for this reason, the protonation constants were obtained starting from pH ≈11 and titrating by HCl. The TSCs **4** (pK_1=8.51, pK_2=10.53) and **5** (pK_1=8.37, pK_2=10.42) result to be diprotic acids with similar values of pK_a (**Table 2**). These values, particularly for pK_2, are lower than those reported for salicylaldehyde thiosemicarbazone, the difference being due also to the different solvent used in the titrations (Enyedy et al. 2012). Compound **5**, with three -OH groups on the phenyl ring, is a triprotic acid (**Table 2**: pK_1=7.33, pK_2=7.95,

260 pK_3=11.79). Even if the different proton-dissociation equilibria are difficult to separate, for **4** and **5**,
261 the first dissociation constant can be reasonably attributed to one of the OH and the second one to the
262 dissociation of the N-H group of the thiosemicarbazide moiety (Enyedy et al. 2012). For **2** both the
263 constants pK_1 and pK_2, very near as value, are probably attributable to two OH groups, and the third
264 one to the N-H proton; the presence of two ionizable substituents reduces the acidity of the N-H group
265 of about one order of magnitude.

266 The speciation and the values of the formation constants of the copper complexes were obtained by
267 titrating **2**, **4** and **5** in presence of Cu(II) ions at different metal:ligand ratios (from 1 up to 6). Some
268 problems arise from the low solubility of the copper(II) complexes. In fact, the system **4**:Cu(II) was
269 soluble in all the pH range studied, while a precipitate was formed from pH=3 to pH=7 for all the
270 ligand:metal ratios investigated for **2**. Unfortunately, the most active thiosemicarbazone **5** gives rise
271 to the formation of insoluble complexes in the whole examined pH range. It must be outlined that all
272 **2**, **4** and **5** possess in *orto* on the phenyl ring at least one OH group involved in the chelation of Cu(II)
273 ions. **Table 2** displays the models for the speciation obtained by the Hyperquad software, showing
274 the best statistical parameters and the best fit between experimental and computed potentiometric
275 titration curves. Other models are also accepted by the software, in particular including only the 1:1
276 metal:ligand stoichiometry (Enyedy et al. 2012), but with worse statistical parameters and greater
277 standard deviations of the formation constants.

278 The complexes of **4** are already present at acidic pH with formation of the neutral CuL species, having
279 a very high stability; at near pH = 8 CuL_2 begins to form. For a better comparison, we can consider
280 also **2** as a diprotic acid, setting L'= LH. With this substitution it is easy to see that the sets of
281 complexes formed in solution have the same stoichiometry for **2** and **4**, as expected, due to the
282 presence of the same chelating moiety. For **2** the insoluble species seem to be CuL' (i.e. CuLH),
283 corresponding to CuL for **4**. The distribution diagrams as a function of pH for L:Cu = 4:1 at a Cu(II)
284 concentration equal to 1.25 mM clearly show that formation of CuL (CuLH, in the case of **2**) starts at

low pH, confirming that TSCs are very efficient Cu(II) chelators (**Figure S4**) (Sendovski, Kanteev, Ben-Yosef, Adir & Fishman, 2011). The chelation of the Cu(II) ion greatly increases the dissociation of the hydrazine group in the complexes of **2** and **4**, particularly for **2**. The solubility of the Cu(II) complexes follows a reverse order in comparison with the inhibitory activity, the complexes with **4** (without inhibitory activity) being soluble, and those with **5** (the most potent inhibitor) completely insoluble. Copper complexes of kojic acid are also completely insoluble in the full range of pH, not allowing to be studied in solution (Bhatia, Kociok-Kohn & Molloy, 2015).

In the active site of the enzyme, the inhibitor could coordinate both the copper(II) ions at the same time. To gain experimental evidence for the possible formation of bimetallic species in solution, we have performed the titrations even in great excess of Cu(II) in respect to the ligand, but the software always rejected the polinuclear species.

3.4 Molecular modelling

To predict the putative binding mode of the compounds **1- 5** with the tyrosinase catalytic site at molecular level, a series of computational docking studies was performed. First, optimized conformations of the compounds were obtained using the MOE (Molecular Operating Environment, MOE 2009) minimization feature. Next, the resulting ligand conformations were docked into the catalytic pocket of the available crystal structure of *Agaricus bisporus* tyrosinase, obtained from RCSB Protein Data Bank (http://www.rcsb.org).

As displayed in **Figure 4**, docking results for **1**-**5** revealed a common disposition within the catalytic residues, thus producing comparable binding modes. We expected that both, their structural similarity and the limited size of the binding pocket, mutually contribute in inducing the same alignment. In fact, the catalytic residues appear as a narrow hydrophobic funnel, with the metal Cu^{2+} cofactors located at the bottom of the cavity surrounding the active site. Structurally, the walls of the pocket

are formed, from one side, by six histidines (His61, His85, His94, His259, His263, and His296), which are involved in the coordination of two copper ions (**Figure 4**). The pocket is then completed by the following residues: Gly86, Phe90, His244, Glu256, Asn260, Phe264, Arg268, Ser282, Val283, Ala286, and Phe292.

Compounds **1–5** behaved similarly to that reported in Dong et al. (Dong, Liu, Liu, Yu & Cao, 2018), who investigated different series of TSCs, substituted on the C-N double bond. Specifically, docking results showed that the sulfur atom of the thiourea group of all compounds is directed toward the metal ions, positioning between the two metal cofactors. The resulting coordination bonds among sulfur atom and both copper ions thus ensure the formation of the ligand-protein complex. Such interaction orientates the distal benzene ring in a narrow hydrophobic cleft formed by residues Asn260, His263, Phe264, Arg268, Ser282, and Val283. The optimal disposition for a metal coordination, combined with the engagement of the aromatic platform into the hydrophobic cavity, would primarily contribute in forming the ligand-protein complex, and therefore in influencing the potency of the inhibitor. However, other structural features could be invoked in determining / affecting the inhibitory activities.

The active compounds **5** (**Figure 4**) and **2** (**Figure S5B**) bear a hydroxyl group at the *ortho* position. Visual inspection of **2** in complex with tyrosinase (**Figure S5B**) revealed that the *ortho* hydroxyl group is able to establish a hydrogen bond (donor) with the oxygen of the amide group of Met280. This feature could presumably contribute to the activity, but it is not sufficient to ensure it. In fact, **4** (**Figure S5A**), which also carries an *ortho* hydroxyl group, is not active. An additional hydrogen bond interaction between the third hydroxyl group (absent in compound **4**) in position 6 and the carbonyl side chain of Asn260 participates in stabilizing **2** within the active pocket, thus contributing in its moderate inhibitory activity. An analysis of the docking results of the most active compound **5** seems to suggest that many features would mutually concur to explain its highest tyrosinase inhibitory activity. They include: a) the above-mentioned hydrogen bond between the *ortho* hydroxyl group and

the amide carbonyl of Met280, b) an arene-arene stacking interaction between the benzene ring and the imidazole ring of the His263, and moreover c) the smaller size of the unsubstituted thioamide group, that allows it a deeper arrangement of the sulfur atom between the metal cofactors. The best docking poses of the compounds with an ethyl substitution on the terminal nitrogen of the thiourea group showed, in fact, that their sulfur atoms are slightly away from the straight line directed between the metal cofactors. This displacement is due to the N-substitution, which forces the entire molecule, also hindering the formation of the arene-arene stacking between the catechol ring and the imidazole ring of His263. This is particularly evident for compound **4** (**Figure S5A**), the N-ethyl substituted derivative of **5**, which is inactive.

Compounds **1** and **3** (**Figures S5C** and **S5D**, respectively), lacking of the *ortho* hydroxyl group, did not show any significant additional interactions. The predicted binding mode for compound **1** only indicates an arene-arene stacking interaction with the imidazole ring of His263, which orientates the three hydroxyl groups of the benzene ring towards a hydrophobic region of the enzymatic pocket.

3.5 Cellular assays

None of the compounds showed cytotoxicity (**Figure S6**) nor ability to stimulate cells to secrete IL-6 (**Figure S7**). IL-6 secretion expressed as ratio to viable cells in each condition was similar between control cells and cells treated with **1 - 5**. The lack of a significant impact of any of these compounds, although tested at the highest concentration, on the viability of human fibroblasts, that do not express tyrosinase, excludes their non-specific toxicity towards human cells. Similarly, data on IL-6 secretion of human fibroblasts upon incubation with **1 - 5** indicate that they do not activate cells in a pro-inflammatory fashion either.

4. Conclusions

Major part of tyrosinase inhibitors are polyphenolic compounds or thiourea derivatives (Pillaiyar, Manickam & Namasivayam, 2017). TSCs **1**-**5** combine some relevant chemical characters of both the classes: the polyhydroxyl unit and the C=S group. Effectively, **1**, **2**, and **5** are inhibitors of mushroom tyrosinase, and in particular **5** has an inhibitory activity stronger than kojic acid used as reference. Compounds **1**, **2**, and **5** are mixed-type inhibitors just as kojic acid (Deri et al., 2016). These compounds are evidently able to coordinate Cu(II) ions, as predicted by molecular modelling, and as confirmed by potentiometric studies, but care has to be devoted not to oversimplify the inhibition mechanism overstressing this aspect: subtle differences in the structure of the molecule, such there are between **5** and **4**, or **1** and **3**, change drastically the inhibition power, even if they do not alter sensibly the complexation ability. Another relevant point to note is that the inhibitor activity is lost when the NH_2 group of compound **5** is alkylated as in **4**, and the same happens in thiourea derivatives (Thanigaimalai et al., 2010).

Obviously, if it possible to think about practical applications of these tyrosinase inhibitors, they have to be safe. Compounds **1**-**5** resulted non-toxic on human fibroblasts and they did not induce a proinflammatory response in cells. These data and others in the literature (Xu, Liu, Zhu, Yu & Cao 2017; Song et al. 2017) are encouraging in considering TSCs tyrosinase inhibitors suitable for practical applications.

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