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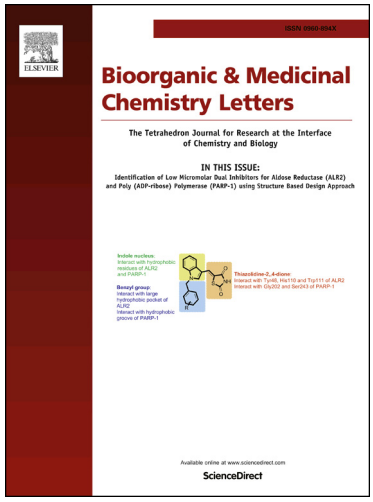

Thiosemicarbazones as Inhibitors of Tyrosinase Enzyme

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Thiosemicarbazones as Inhibitors of Tyrosinase Enzyme

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ABSTRACT

In the search for compounds which may inhibit the development of melanomas, a series of thiosemicarbazones has been investigated as possible inhibitors of the tyrosinase enzyme. The results showed that all the thiosemicarbazones tested exhibited significant inhibitory effects on the enzyme. Thiosemicarbazones **Thio-1**, **Thio-2**, **Thio-3** and **Thio-4** substituted with oxygenate moieties, were better inhibitors (IC_{50} 0.42, 0.35, 0.36 and 0.44 mM, respectively) than **Thio-5**, **Thio-6**, **Thio-7** and **Thio-8**. For the better inhibitors, molecular docking results suggested that the oxygen present in the *para* position of the aromatic ring is essential for the tyrosinase inhibition, due its high ability for complexation with Cu^{2+} ions. Inside the active protein pocket, **Thio-2** – the best studied inhibitor - is able to interact with the amino acid residues His-155, Gly-170 and Val-172 *via* hydrogen bonding and hydrophobic force. **Thio-2**, containing a substituent on the aromatic ring similar to the substrate L-DOPA, showed a competitive inhibition mechanism as viewed in a Lineweaver–Burk plot. The same results were observed in the UV-Vis curves.

Melanin pigments are found in mammalian skin and extracutaneous tissue including the brain, eye and inner ear. Cutaneous melanin is formed in neural crest-derived melanocytes. Melanin produced in skin melanocytes provides protection from the sun's ultraviolet radiation. People with black skin are well protected from the mutagenic effects of ultraviolet radiation while those with fair skin are at high risk for developing skin cancer. Melanoma is the most aggressive and potentially lethal form of skin tumor. It originates in pigment-producing melanocytes that are found in the basal layer of the epidermis and in the eye. The number of melanoma cases and deaths worldwide has increased faster than many other cancers though, lately, the trend has stabilized.[1,2]

Pigmentary disorders occur for various reasons such as loss of melanocytes and increased or decreased melanocyte activity. Hyperpigmentation in the epidermis is caused by excessive melanin synthesis (increased melanocyte activity) which is controlled by the rate-limiting tyrosinase enzyme (EC 1.14.18.1, phenol oxidase), which is found in melanocytes localized to the membrane of the unique melanocyte organelle, the melanosome.[3]

The active site of tyrosinase consists of two copper atoms and it is involved in the transformation of L-tyrosine to dopaquinone which occurs through two steps: hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), then oxidation of the latter to an *ortho*-quinone (dopaquinone). Dopaquinone is further transformed through several reactions to yield brown to black melanin.[4]

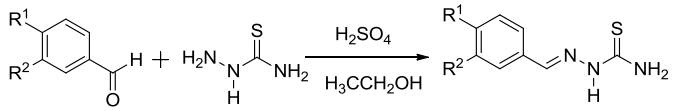
Several tyrosinase inhibitors have also been used in the cosmetic industry as skin-whitening agents. Most of the inhibitors of tyrosinase are, therefore, copper chelators or phenolic compounds structurally analogous to the substrates L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA).[5–7] It is well known that tyrosinase can be inhibited by aromatic aldehydes and aromatic acids, tropolone, alkoxybenzoic acids and kojic acid; recently, dialkylphosphorylhydrazones have been reported as potential tyrosinase inhibitors.[8–12]

Thiosemicarbazones have received considerable attention because of their potential therapeutic activity, such as antitumoral, antibacterial and antimalarial.[13–16] Several times, the transition metal complexes of thiosemicarbazones showed greater biological activity than uncomplexed ligands.[17,18]

The aim of this study was to investigate seven benzaldehyde-substituted thiosemicarbazones and pyridinecarboxaldehyde-thiosemicarbazone for tyrosinase inhibitory capacity. Evaluation of the inhibitory activity was made through kinetic study of the diphenolase inhibition and the inhibition constants that characterized the system. The inhibition mechanism involved was also investigated by two

methods, Lineweaver–Burk plot and UV-Vis spectrophotometry. In order to offer a molecular level description for the inhibitory ability of the studied compounds, and suggest the main amino acid residues responsible for the stabilization of the interaction between the enzyme and its best inhibitor, molecular docking exercises were carried out.

The thiosemicarbazones were prepared according to the general Scheme 1, using the thiosemicarbazide and respective substituted benzaldehydes and pyridinecarboxaldehyde.[19] The substituted benzaldehydes used to prepare the target compounds were obtained from commercial sources. All the compounds above were purified by recrystallization from methanol. Additionally, all the synthetic compounds were characterized by spectroscopic data, which were in full accordance with their proposed structures.[20,21] The compounds tested are presented in **Table 1**.

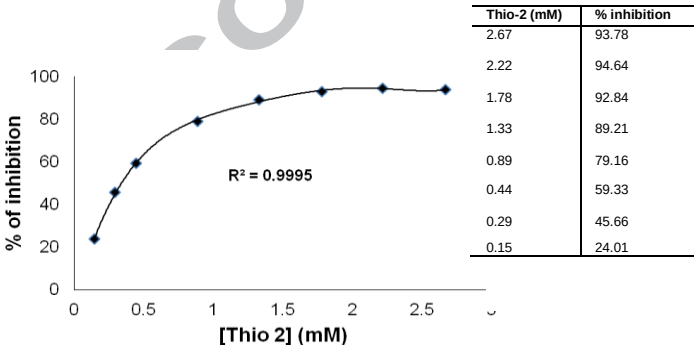


Scheme 1 Synthetic route for thiosemicarbazone synthesis.

In the present study, each thiosemicarbazone and then thiosemicarbazide was examined for its ability to inhibit tyrosinase activity with L-DOPA, in several concentrations, according to the assay protocol described by Caxeiro *et al*.[12,22] The percentage inhibition values were calculated from the equation:

$$\% \text{ inhib} = \{[(B_{30} - B_0) - (Am_{30} - Am_0)] / (B_{30} - B_0)\} \times 100$$

where B_0, B_{30}, Am_0 and Am_{30}, are absorbance of the control sample at time zero, absorbance of the control sample after 30 min, absorbance of the test sample at time zero and absorbance of the test sample after 30 min, respectively. In the equation, the interference of the possible absorbance of organic compounds was subtracted. An increase of dose-dependent inhibition percentage was observed through exponential model curves for all thiosemicarbazones. **Fig. 1** shows the graphic obtained for **Thio-2** - the compound that showed the highest inhibitory effect.



Thio-2 (mM)	% inhibition
2.67	93.78
2.22	94.64
1.78	92.84
1.33	89.21
0.89	79.16
0.44	59.33
0.29	45.66
0.15	24.01

Fig. 1. Inhibitory effect of **Thio-2** on the diphenolase activity of mushroom tyrosinase for the catalysis of L-DOPA at 25°C.

The IC_{50} values obtained from equations generated through percentage inhibition against concentration curves for each thiosemicarbazones are summarized in **Table 1**. For comparison between the studied compounds, **ascorbic acid** (IC_{50} 0.26 mM) was used as positive control. As shown in **Table 1** all compounds exhibited tyrosinase inhibitory effects, with IC_{50} values ranging from 0.35 to 1.42 mM. Among the assayed compounds, **Thio-1**, **Thio-2**, **Thio-3** and **Thio-4** showed the best inhibitory effect, with IC_{50} values in the range of 0.35 mM to 0.44 mM, at use L-DOPA as substrate for diphenolase activity of mushroom tyrosinase. The *p*-hydroxybenzaldehyde-thiosemicarbazone (**Thio-1**) was previously evaluated;[23,24] however, the results were very different, but one of them was consistent with those observed in this work for the same compound.[24]

Table 1
Concentrations of thiosemicarbazones and ascorbic acid leading to 50% activity loss (IC_{50}) of tyrosinase and substrate L-DOPA

Compounds	Substituents		IC_{50} (mM)
	R^1	R^2	
Thio-1	HO	H	0.42
Thio-2	HO	OCH_3	0.35
Thio-3	OC_2H_5	H	0.36
Thio-4	OCH_3	H	0.44
Thio-5	H	H	0.82
Thio-6	Br	H	1.42
Thio-7	Cl	H	0.84
Thio-8	Pyridine 2 carboxaldehyde thiosemicarbazone		1.19
Ascorbic acid	-	-	0.26

As tyrosinase is a copper-containing enzyme, it is expected that potential tyrosinase inhibitors should show high binding affinity for Cu^{2+} ions.[25] In order to offer a molecular level description for the inhibitory ability of the studied compounds, molecular docking exercises for mushroom *Agaricus bisporus* tyrosinase (PDB 2Y9X)[2] were performed with GOLD 5.2 program (CCDC).[26]

Fig. 2A and **2B** show the best docking poses inside the tyrosinase active site, for **Thio-1**/**Thio-4** and **Thio-5**/**Thio-8**, respectively. All studied compounds are buried inside the protein binding site and both are close to the dicopper center (center responsible for the tyrosinase activity) with an average distance of 1.18 Å – 2.26 Å, however the hydroxyl group from phenol moiety for **Thio-1**/**Thio-4** and the sulfur/nitrogen groups from thiosemicarbazone moiety for **Thio-5**/**Thio-8**, are the main chemical groups responsible for the complexation with Cu^{2+} ions. As **Thio-1**/**Thio-4** showed better tyrosinase inhibition than **Thio-5**/**Thio-8** (**Table 1**), molecular docking results suggest that it occurs probably due the presence of hydroxyl groups, that are better chemical functions than sulfur/nitrogen for the complexation with Cu^{2+} ions presented inside the tyrosinase structure.

Fig. 2C depicted the overlap between the best tyrosinase inhibitor (**Thio-2**) with the standard sample (**ascorbic acid**). Note that one of the hydroxyl groups of the standard sample is overlap with the hydroxyl group of the **Thio-2**. It suggests that the same chemical group (-OH) of both samples can interact equally with the Cu^{2+} ions, resulting in a quite similar experimental IC_{50} values. **Fig. 2C** also shows the main amino acid residues responsible for the stabilization of the complex tyrosinase/**Thio-2**. The molecular docking result suggest that the amino groups from the thiosemicarbazone moiety can interact *via* hydrogen bonding with the peptidic C=O oxygen and N-H hydrogen of Gly-170 and Val-171 residues, within distances of 1.84 Å and 3.07 Å, respectively. Hydrophobic force, *via* π-

stacking, between the aromatic ring of the ligand with the amino acid residue His-155, was also detected by molecular docking, within a distance of 3.21 Å.

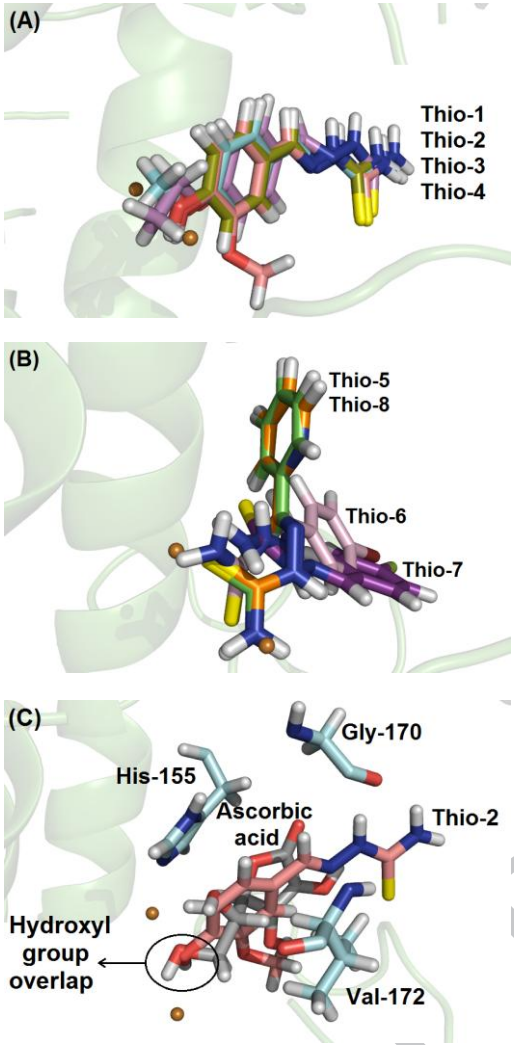


Fig. 2 (A) Overlap among the thiosemicarbazones **Thio-1**, **Thio-2**, **Thio-3** and **Thio-4**, inside the tyrosinase active site. (B) Overlap among the thiosemicarbazones **Thio-5**, **Thio-6**, **Thio-7** and **Thio-8**, inside the tyrosinase active site. (C) Overlap between the best tyrosinase inhibitor (**Thio-2**) with the standard compound (**ascorbic acid**). Tyrosinase structure is in cartoon representation (PDB: 2Y9X); Carbon atoms for **Thio-1**, **Thio-2**, **Thio-3**, **Thio-4**, **Thio-5**, **Thio-6**, **Thio-7**, **Thio-8** and **ascorbic acid** are in moss green, beige, violet, light blue, dark green, lilac, purple, orange and gray, respectively. The selected amino acid residues are in cyan. Copper ions (Cu^{2+}) are represented as spheres in brown. Element colors: hydrogen: white; oxygen: red; nitrogen: dark blue; sulfur: yellow, chloro: limon and bromo: firebrick.

The inhibitory mechanism of thiosemicarbazones on mushroom tyrosinase for oxidation of L-DOPA was determined from Lineweaver–Burk double reciprocal plots. Graphs were constructed and some are shown in **Fig. 3**.

The equations used to determine the inhibition constant K_i for competitive, uncompetitive and uncompetitive mixed inhibition are described below.[28]

Competitive inhibition: $K_{map} = [(K_m/K_i)[I] + K_m]$

Uncompetitive inhibition: $1/V_m = [(1/V_mK_i)\,[I] + 1/V_m]$

Uncompetitive mixed inhibition: $-1/K_{map} = -(1 + [I]/K_i)/K_m$

where K_m, K_{map} and K_i are the Michaelis–Menten constant, the apparent Michaelis-Menten constant and the inhibition constant, respectively. V_m is the maximum velocity in the presence of each inhibitor. **Table 2** shows the K_{map}, K_i and V_m values, as well as the mechanism of tyrosinase inhibition. The presence of sulfur and nitrogen atoms in thiosemicarbazone structures may explain the inhibitory effect; however, the competitive mechanism of inhibition due to increased affinity for copper ions present in the active site was due to the presence of substituents that favored this interaction.

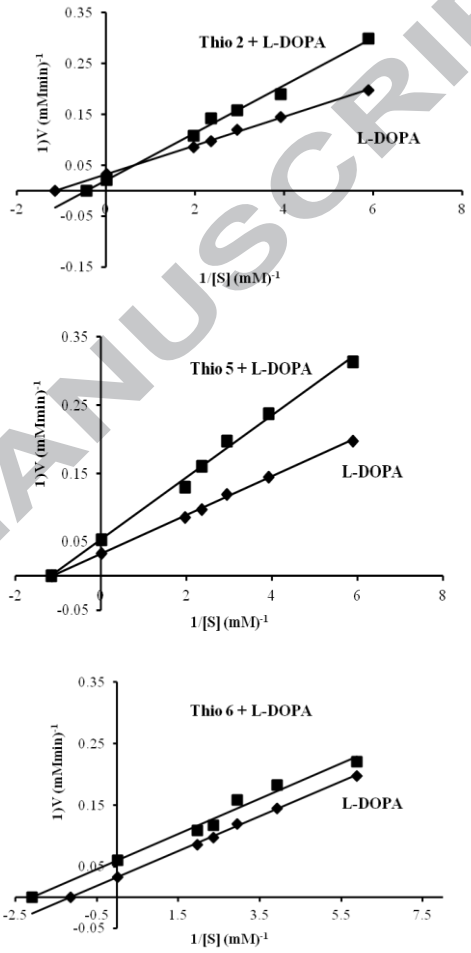


Fig. 3 Lineweaver–Burk plots of tyrosinase and L-DOPA in the presence and absence of thiosemicarbazones at 1.6 mM.

Table 2

Type of inhibition mechanism, as well as K_{map}, K_i and V_m values. K_m and V_m in the tyrosinase-catalyzed oxidation of L-DOPA were 0.87 mM and 30.58 mM/min.

Compound at 1.6 mM	Inhibitory mechanism	K_{map} (mM)	K_i (mM)	V_m (mM/min)
Thio-1	Uncompetitive mixed	0.7	7.3	9.5
Thio-2	Competitive	22.4	0.06	48.1
Thio-3	Competitive	21.7	0.06	38.4
Thio-4	Competitive	26.3	0.05	34.8
Thio-5	Uncompetitive mixed	0.8	4.5	18.9
Thio-6	Uncompetitive	0.5	0.6	16.6
Thio-7	Competitive	1.9	1.3	39.7
Thio-8	Competitive	8.3	0.2	27.1

It was interesting to note that **Thio-2**, **Thio-3** and **Thio-4** showed higher competitiveness with the active site (when compared to other compounds with the same mechanism of inhibition). These compounds with –OH, CH_3O– and CH_3CH_2O– moieties in the aromatic ring do not hydrogen bond with histidine residues present in the active site of the enzyme, favoring the interaction of **Thio-2**, **Thio-3** and **Thio-4** in the active site. **Thio-6**, with bromo as substituent, showed uncompetitive inhibition; this is probably because the bulky halide that causes distortion in the active site, making the enzyme catalytically inactive.

Further, the low K_i value, indicate a high inhibition at a given concentration of substrate and inhibitor; therefore, the results for this constant for **Thio-2**, **Thio-3** and **Thio-4** corroborate to the above discussion.

The results obtained from double reciprocal Lineweaver–Burk plots were compared with UV-Vis curves obtained from the thiosemicarbazone without and with the presence of enzyme + L-DOPA.

Generally, the methods used to determine the mechanism of inhibition of enzymes are Lineweaver–Burk and Dixon. In both methods, inhibitors and different substrate concentrations are required, as well as the addition of the increase in the enzyme consumption. Therefore, UV-Vis curves can indicate the type of inhibition mechanism in short time and with economy of reagents when compared to the traditional techniques. In this case, the substrate was L-DOPA and the thiosemicarbazones **Thio-1**, **Thio-5** and **Thio-6** showed similar mechanisms of inhibition, according to Lineweaver–Burk plots (uncompetitive and uncompetitive mixed). The UV-Vis curves were superimposed onto **Thio-5** and **Thio-6**; however, these two thiosemicarbazones did not present similarity with the substrate L-DOPA. Furthermore, the bromine moiety in **Thio-6** could cause distortion of the enzyme active site. **Thio-5** showed a tendency to a competitive type of inhibition mechanism, when compared to pure uncompetitive, as well as **Thio-1**, which has a hydroxyl moiety on the aromatic ring.

Thiosemicarbazones substituted with oxygenate moieties (**Thio-2**, **Thio-3** and **Thio-4**), which can form complexes with copper ions, had the same UV-Vis profile and the same mechanism of inhibition (competitive). Furthermore, the curves for the chloro-substituted thiosemicarbazone (**Thio-7**) and the pyridine 2 carboxaldehyde thiosemicarbazone (**Thio-8**) (compounds with different molecular structure compared with substrate) were similar, due to interaction of the side chain with the active site of the enzyme **(Fig. 4)**.

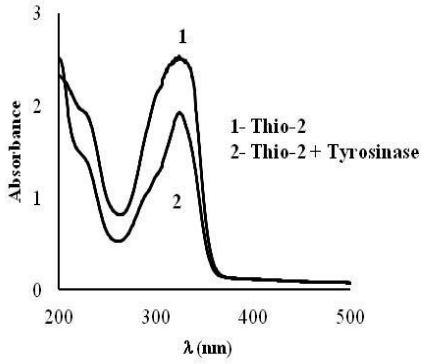


Fig. 4 UV curves of thiosemicarbazone with competitive inhibition type for Lineweaver–Burk.

Kinetic studies of the steady state of the pathway showed the low catalytic efficiency of tyrosinase on monophenols than *o*-diphenols.[28] The thiosemicarbazones were used in the evaluation of enzyme activity to determine the enzymatic kinetics. The evolution of the enzyme activity was monitored by readings taken with an UV-Vis spectrophotometer at 475 nm for 60 min at 10 min intervals. The good inhibitors maintained inhibition for the majority of the time **(Fig. 5)**.

Interestingly, a strong decrease of **Thio-1** inhibition with time was observed in the kinetic graphs, and inhibitors with similar UV-Vis behavior also showed similar kinetic curves. Despite of the IC_{50} values for **Thio-2**, **Thio-3** and **Thio-4** being higher than for the others inhibitors, these compounds showed good enzymatic kinetics, since the inhibition power remained beyond 30 min, similarly to the positive control **ascorbic acid**.[28]

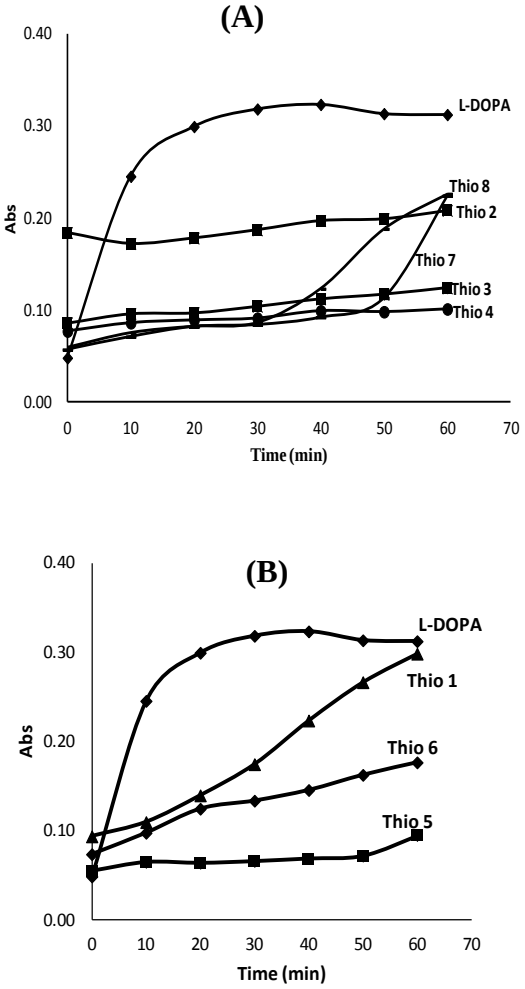


Fig. 5 Course for oxidation of L-DOPA (0.17 mM) by mushroom tyrosinase in the absence and presence of inhibitors (1.6 mM). (A) competitive inhibitors; (B) uncompetitive and uncompetitive mixed inhibitors.

In this study, a series of thiosemicarbazones was synthesized and their inhibitory activity against tyrosinase enzyme was evaluated, by experimental and theoretical methods. The thiosemicarbazones with oxygenate substituents in the aromatic ring exhibited strong inhibition on the activity of mushroom tyrosinase and IC_{50} values were similar to the standard **ascorbic acid**. Molecular docking results suggest that it occurs probably due the higher affinity

of hydroxyl groups than sulfur/nitrogen groups for Cu^{2+} ions. For the best tyrosinase inhibitor (**Thio-2**), it was able to interact with His-155, Gly-170 and Val-172 residues. The values of K_{map} and K_i were in accordance with the inhibitory power and the type of inhibition mechanism investigated for each compound. **Thio-3** and **Thio-4** showed the best inhibitory effects at maintained the inhibition for a significant time. Curves obtained by UV-Vis spectroscopy indicated inhibition of the competitive type of mechanism for compounds which structurally resembled the substrate used, but in other cases the double reciprocal technique was more appropriate. Further investigations are warranted to explore their potential application in the management of hyperpigmentation and melanoma cases.

Acknowledgments

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22. The organic compounds were solubilized in dimethylsulfoxide (VETEC) (10 mM) and different aliquots of the stock solution were added to the reaction medium containing the tyrosinase mushroom enzyme (50–100 units), EDTA (0.022 mmol/L) and L-DOPA (0.17 mmol/L), all acquired for Sigma-Aldrich, in PBS (50 mmol/L, pH 6.8) at room temperature. The time for diphenolase to oxidize L-DOPA was 30 min and the readings were performed in a UV-Vis spectrophotometer (Shimadzu, model Mini 1240, Japan) at 475 nm.
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26. Thiosemicarbazones structures (**Thio-1**, **Thio-2**, **Thio-3**, **Thio-4**, **Thio-5**, **Thio-6**, **Thio-7** and **Thio-8**) were built and energy-minimized with the Density Functional Theory (DFT), method Becke-3-Lee Yang Parr (B3LYP) with the standard 6-31G* basis set, available in Spartan'14 program (*Wavefunction*, *Inc*.). The molecular docking exercises were performed with GOLD 5.2 program. Hydrogen atoms were added to the protein (PDB 2Y9X) according to the data inferred by the GOLD 5.2 program on the ionization and tautomeric states. A 10 Å radius spherical cavity around the dicopper center - which is responsible for the tyrosinase activity - was defined as the binding site for the molecular docking calculations. The scoring function used was '*GoldScore*', due the best result obtained by the redocking studies - ligand tropolone. The figures of the docking poses for the largest docking score value were generated with the PyMOL Delano Scientific LLC program.
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