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Anti-tyrosinase, anti-elastase, and antioxidant activities of some symmetric bisthiocarbohydrazone compounds

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Symmetric bisthiocarbohydrazone compounds (1, 2, 3) were obtained by the condensation of thiocarbohydrazide with carbonyl compounds such as isatin (a heterocyclic ketone) and two hydroxyl aldehydes (2-hydroxybenzaldehyde, 2-hydroxy-1-naphthaldehyde) respectively, according to the previously reported methods. These synthesized compounds were evaluated in terms of their anti-tyrosinase, anti-elastase and antioxidant potentials in vitro. All of the tested compounds exhibited anti-tyrosinase and anti-elastase activities. It was observed that the inhibition increased with the increase of bisthiocarbohydrazone concentrations. Compound **1** showed the highest anti-tyrosinase activity. The anti-tyrosinase activity is decreasing in the following order; **2<3<1**. Compound **1** showed also the highest anti-elastase activity. The anti-elastase activity is decreasing as **3<2<1**. As a result, the most effective compound in terms of anti-tyrosinase and anti-elastase activities is bisthiocarbohydrazone derived from isatin. Compounds **2** and **3** with the hydroxyl substitution showed antioxidant activity close to Trolox. These compounds were found to have significant reducing effects and to be effective scavengers of DPPH.

Keywords: Thiocarbohydrazone, isatin, antityrosinase, antielastase, antioxidant

Thiocarbohydrazide (TCH) derivatives constitute an important class of N, S donor ligands possessing interesting chemical, biological and medicinal properties[1-3]. Both hydrazine groups of thiocarbohydrazide are very reactive and mainly form symmetric bisthiocarbohydrazone derivatives with aldehydes and ketones[4-6].Thiocarbohydrazones have attracted much attention due to their pharmacological activity[7-9]. These compounds possess high antibacterial activity and are potentially useful as antimicrobial agents against Gram-positive bacteria[10-12]. When the anticancer effects of isatin thiocarbohydrazone were examined, it was found that it showed promising cytotoxic activity. In particular, the high antifungal effects of its Co(II) complex were tested[3].

Enzymes are biological macromolecules that accelerate or catalyze chemical reactions in biological systems. The decrease or increase in the activities of some enzymes in the organism causes different types of damage. Enzyme inhibition studies focus on explicating the possible relationship between enzyme activity and various diseases[13-15].Tyrosinase (EC 1.14.18.1) is a copper containing monooxygenase, widely distributed in bacteria, fungi, plants and mammals[16]. This enzyme is also the key enzyme for the biosynthesis of melanin[17,18] and other pigments via the oxidation of L-tyrosine. Although melanin protects the human skin from UV radiation, an excessive accumulation of epidermal pigments causes serious esthetic problems and even skin diseases in human beings[19]. Moreover, more tyrosinase activity results in the browning and black spotting of food and further leads to undesirable changes in the flavor and nutritive value[20]. Interestingly, it was recently demonstrated that tyrosinase is also involved in the brain neuromelanin formation[21], which means that over tyrosinase activity is linked to Alzheimer's, Parkinson's and Hungtington's diseases and other neurodegenerative diseases[22-26]. Tyrosinase plays an important role in the initiation of different types of cancers such as melanoma or anorectal malignant melanoma[27,28]. For insects, tyrosinase activity was found to be closely associated with physiological processes including melanin formation, wound healing, resistance to parasites and skin keratinization[29]. Tyrosinase inhibitors were widely used in agriculture, food and cosmetics industries and in the control of the melting process of insects. Many

natural and synthetic tyrosinase inhibitors have been reported in the literature[30,31]. These inhibitors have high toxicity and low activity. For this reason, non-toxic, safe and effective inhibitors are necessary in the field of cosmetic, medicine and agriculture.

Elastase (EC 3.4.21.37) is a member of serine proteases that does the catalysis of elastin analysis, the main component of the connective tissues. Elastases are particularly abundant in the skin, lungs, arteries, and ligaments. Elastase is known to cause delayed wound healings, increased inflammation progress, cystic fibrosis, ischemia reperfusion injury, acute respiratory distress, Wegener's granulomatosis, and tissue permeability. Elastin is the protein widely distributed in the vertebrate tissues of human beings especially abundant in ligaments, lungs and skin, which is being hydrolyzed or cleaved by elastase enzyme belonging to the class of serine proteases[32]. It is also the key enzyme which attacks all major matrix protein of connective tissue[33]. Human neutrophil elastase has been implicated in the progression of various types of cancer and plays an important role in some processes like blood coagulation, apoptosis and inflammation[34].

So far, some enzyme activities of some hydrazone compounds of isatin, which is the component of compound **1**, have been studied[35,36]. In another study, a series of 2-acetylpyridine thiocarbohydrazones as an inactivator of HSV-1 ribonucleotide reductase are found to possess better activity than that of corresponding thiosemicarbazones[37]. In our previous study, some enzyme activities of asymmetric bisthiocarbohydrazone derived from isatin and their Pd(II) complexes were investigated and high inhibition values were obtained[38]. But, no study was found examining the anti-tyrosinase and anti-elastase and activities of symmetrical bisthiocarbohydrazone compounds. In this study, anti-tyrosinase and anti-elastase activities of these compounds were determined for the first time.

Free radicals and reactive oxygen species are considered to be associated with some diseases such as cancer, inflammation, neurodegenerative disorders, aging, diabetes, atherosclerosis, autoimmune disease and osteoporosis[39-44]. Antioxidants are important inhibitors against oxidative damage. They prevent or delay the damage caused by free radicals[34]. Numerous synthetic or natural antioxidant substances have been tested with success in various disease models. Synthetic antioxidants damage the liver and other organs due to their toxic effects. Therefore, the preparation of new, effective antioxidants is very important. The development of novel synthetic compounds, capable of scavenging free radicals, has been a great success, especially in many pathological conditions such as cancer, diabetes, neurodegenerative disorders, inflammation, vascular disease[34].

In our previous study on benzophenone-based bisthiocarbohydrazone synthesis and its antioxidant properties, it was found that these compounds showed good antioxidant activity[4]. Again, the antioxidant activities of salicylaldehyde-based mono and bisthiocarbohydrazones in different solvents were investigated, and it was observed that the activity increased with the addition of water to the organic solvent[45]. In this study, antioxidant activities of these compounds, as well as tyrosinase and elastase inhibitory effects, are determined and the results are discussed.

Results and Discussion

Symmetrical bisthiocarbohydrazones (1, 2 and 3) were prepared by the reaction of thiocarbohydrazide and carbonyl compounds. The structures of the compounds were determined by TLC, melting point, elemental analysis, IR and 1H-NMR spectroscopy. Compounds 2 and 3 soluble in common organic solvents, but compound 1 is slightly soluble in these solvents. Tyrosinase inhibitory activity of the compounds are given in Table I. All bisthiocarbohydrazones showed anti-tyrosinase activity. Tyrosinase inhibitory activity of the compounds was found to increase in a dose dependent manner. A higher tyosinase inhibitory activity is associated with a lower IC_{50}. A high tyrosinase inhibitory activity was seen at compound **1**. IC_{50} value was $3.13x10^{-5}$ mg/mL. The tyrosinase inhibitory activity of the bisthiocarbohydrazones and standard compound have decreased in the order; **2** < Kojic acid <**3** <**1** (Table I).

Elastase inhibitory activity of the bis-thiocarbohydrazones; **1, 2,** and **3** are given in Table I.

Table I — Tyrosinase and elastase inhibitory activities of the compounds.

Compounds	IC_{50}(mg/mL)*	IC_{50}(mg/mL)*
	Tyrosinase	Elastase
1	$3.13x10^{-5} \pm 5.77x10^{-7}$	$1.13x10^{-7} \pm 5.77x10^{-9}$
2	3.41 ± 0.17	$7.53x10^{-5} \pm 5.77x10^{-7}$
3	$8.34x10^{-5} \pm 2.23x10^{-5}$	$9.00x10^{-5} \pm 5.29x10^{-6}$
Kojic Acid	0.68 ± 0.07	-
Ursolic Acid	-	0.0048 ± 0.0003

*Mean ± SD

All compounds exhibit anti-elastase activity. Compound **1** showed the highest anti-elastase activity. IC_{50} value was found 1.13 x 10^{-7} mg/mL. A low elastase inhibition activity was seen in compound **3**. IC_{50} value was found 9.00 x 10^{-5} mg/mL. The anti-elastase activity of the bisthiocarbohydrazones and standard compound have decreased as Ursolic acid <**3** <**2** <**1** (Table I).

There are two copper atoms in the active center of the tyrosinase enzyme[46]. The inhibition of metalloenzymes such as tyrosinase depends in part on the ability to coordinate the metal ions in the active site[47]. Park and Sung[48] suggested that copper ions in the active center of the tyrosinase enzyme are necessary for the activity of the tyrosinase enzyme and that there will be losses in activity with the change of the structure of the copper atom in this active center. Compounds with sulfur atoms in their structure form chelates with transition metal ions such as copper. Thiourea, thiosemicarbazone and thiocarbonyl compounds had been reported to be efficient tyrosinase inhibitors[49,50]. Liu et al. found that thiosemicarbazide and its analogue compounds showed high tyrosinase inhibition which suggested that the sulfur and nitrogen atoms in the molecule exhibited strong affinity for copper ion[51]. Thiosemicarbazone and thiocarbohydrazone compounds contain nitrogen and sulfur atoms in their structure. The presence of these atoms in the structure of compounds is related to their biological activities[52]. In our study, compounds **1** and **3** showed high level tyrosinase activity that rivaled the kojic acid. Compound **1**, containing sulfur and nitrogen atoms as well as isatin, a heterocyclic bioactive compound, showed excellent inhibitor activity (Table I). It is predicted that the sulfur and nitrogen atoms in compound **1** bind to the copper ion in the active site and inhibit the enzyme.

The elastase enzyme has a serine amino acid in its active center. The hydroxyl groups of the serine amino acid and the S and N atoms can form hydrogen bonds. In our study, all compounds showed a more effective inhibition effect than the standard substance, ursolic acid. They inactivate the enzyme by binding to the hydroxyl groups of the serine amino acid in the active center of the enzyme through the sulfur, nitrogen and hydroxyl groups in their structure. Some researchers have shown that compounds containing sulfur and nitrogen atoms in their structure have high inhibition of elastase enzyme[53-56]. This indicates that sulfur and nitrogen bind to active sites of the elastase enzyme. In our study, compound **1**, which carries the bioactive isatin molecule in addition to sulfur and nitrogen atoms, has the highest anti-tyrosinase and anti-elastase activities. Compound **1** could be a new lead for further modifications to discover more potent inhibitors.

In this study, the radical scavenging activities of the bisthiocarbohydrazone compounds in an organic solvent were investigated, and the results were interpreted depending on the presence of hydroxyl groups in the compounds. Antioxidant activities were determined by DPPH radical scavenging method and the results are given in Table II. When Table II is examined, it is seen that compound **3** shows the highest antioxidant activity and compound **1** shows the lowest activity. The reason why compound **1** shows the lowest activity may be the absence of hydroxyl groups in its molecule structure. Compounds **2** and **3** exhibit good radical scavenging activity compared to Trolox. The fact that both compounds contain two phenolic hydroxyl groups in their structure can be interpreted as increasing antioxidant activity.

Experimental Section

Thiocarbohydrazide was prepared according to a reported method by the reaction of carbon disulfide with hydrazine hydrate[57]. Isatin, 2-hydroxy benzaldehyde, 2-hydroxy-1-naphthaldehyde and other chemicals were purchased from Sigma Aldrich or Merck chemical companies.

Physical measurements

^{1}H-NMR spectra were recorded on a Varian UNITY INOVA 500MHz NMR spectrophotometer using deuterated DMSO as solvent. IR spectra were recorded on an Agilent Cary 630 FTIR-ATR spectrometer in the 4000-600 cm^{-1} range. The elemental analyses were analyzed with a Thermo Finnigan Flash EA 1112 Series elemental analyzer.

Table II — The radical scavenging activities of the compounds, as inhibition ratio % (initial concentration: 10^{-4} M; sample volume: 1 mL).

Compounds	DPPH Radical Scavenging Activity (%)*
1	4.18 ± 0.65
2	69.43 ± 2.86
3	86.74 ± 0.10
Trolox	85.81 ± 0.65

*Mean ± SD

Synthesis

Symmetric bisthiocarbohydrazones (1, 2, 3) were obtained by the condensation of thiocarbohydrazide with carbonyl compounds such as isatin[3], 2-hydroxybenzaldehyde[58], 2-hydroxy-1-naphthaldehyde[59], respectively, according to the previously reported methods (Figure 1). As proof of the formation and the purity of the synthesized compounds, melting points, elemental analyses, IR, 1H-NMR spectra were recorded for them and compared with those reported previously[3,58,59].

1,5-bis(2-Oxoindolin-3-ylidene)thiocarbohydrazone, 1: Yield: 78%. M.p.:284-285 °C. Color: Orange. Anal. calc. for $C_{17}H_{12}N_6O_2S$ (364.38 g/mol), found (calc.): 55.91 (56.04) C%, 3.25 (3.32) H%, 23.00 (23.06) N%, 8.72 (8.80) S%. ^{1}H NMR (DMSO-d_6, δ, ppm): 13.02 (s, 2H, NH), 11.36 (s, 2H, NH_{isatin}), 6.93-7.01 (d, 2H, H_{arom}), 7.14 (t, 2H, H_{arom}), 7.43 (t, 2H, H_{arom}), 7.56-7.63 (d, 2H, H_{arom}). IR (cm^{-1}): *ν*(NH) 3173 and 3140, *ν*(C=O) 1684, *ν*(C=N) 1618 and 1597, *ν*(C=S) 1237.

1,5-bis(2-Hydroxybenzylidene)thiocarbohydrazone, 2: Yield: 85%. M.p.: 190-191 °C. Color: Pale yellow. Anal. calc. for $C_{15}H_{14}N_4O_2S$ (314.36 g/mol), found (calc.): 57.50 (57.31) C%, 4.46 (4.49) H%, 17.84 (17.82) N%, 10.28 (10.20) S%. ^{1}H NMR (DMSO-d_6, δ, ppm): 11.70 (s, 2H, OH), 11.62 (s, 1H, NH), 10.62 (s, 1H, NH), 8.67 (s, 2H, CH=N), 6.89-7.35 (m, 8H, $H_{arom.}$). IR (cm^{-1}): *ν*(OH) 3286 and 3250, *ν*(NH) 3173 and 3135, *ν*(C=N) 1615 and 1595, *ν*(C=S) 1238, *ν*(C-O)1203.

1,5-bis(2-Hydroxy-1-naphthylidene)thiocarbohydrazone, 3: Yield: 83%. M.p.: 260-261 °C. Color: Yellow.Anal. calc. for $C_{23}H_{18}N_4O_2S$ (414.48 g/mol), found (calc.): 66.71 (66.65) C%, 4.44 (4.38) H%, 13.45 (13.52) N%, 7.66 (7.74) S%. ^{1}H NMR (DMSO-d_6, δ, ppm): 11.37 (s, 2H, OH), 10.47 (s, 1H, NH), 9.04 (s, 1H, NH), 8.48 (s, 1H, CH=N), 8.18 (s, 1H, CH=N), 7.12-7.86 (m, 12H, $H_{arom.}$). IR (cm^{-1}): *ν*(OH) 3323 and 3249, *ν*(NH) 3226 and 3180, *ν*(C=N) 1620 and 1591, *ν*(C=S) 1252, *ν*(C-O)1182.

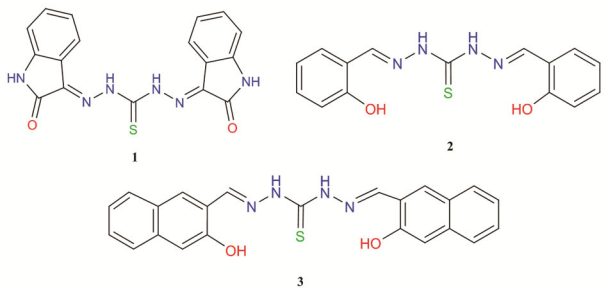

Figure 1 — Structure of compounds **1**, **2** and **3**

Enzyme inhibitory activity assay

Tyrosinase inhibitory activity of the bisthiocarbohydrazones was determined spectrophotometrically[60]. Briefly, test reaction mixtures were prepared by adding mushroom tyrosinase, sample, L-tyrosine, and sodium phosphate buffer (pH 6.5). The resulting mixture was incubated for 10 mins at 37° C and the absorption value was measured at 475 nm. The percentage of the inhibition of tyrosinase activity was calculated according to the following equation:

$$\text{Tyrosinase Inhibitory Activity (\%)} = [(A-B)/A\,] \times 100,$$

where, A represents the activity of the enzyme without sample and B is the activity of tyrosinase in the presence of the sample. Kojic acid was used as a standard compound as control.

Elastase inhibitory activity of the bisthiocarbohydrazones was also estimated according to the method of Kraunsoe et al.[61]. N-Succinyl-AlaAla-Ala-paranitroanalide (STANA) was used as a substrate in this method. The absorbance change was measured at 410 nm using a spectrophotometer. The percent inhibition of elastase was calculated as follows:

$$\text{Elastase Inhibitory Activity (\%)} = [(A-B)/A\,] \times 100,$$

where A is the enzyme activity without inhibitor and B is the activity in the presence of inhibitor. Ursolic acid was used as a standard compound.

The IC_{50} values were determined as the concentration of some symmetric bisthiocarbohydrazone compounds required to inhibit tyrosinase and elastase activities by 50%. Percentage enzyme inhibition activities of the inhibitors were used to calculate half maximum inhibitions (IC_{50}) for individual enzymes, via regression analysis data.Lower IC_{50} values indicate the higher inhibitory potential of the tested compounds.

Determination of DPPH radical scavenging activity of the compounds

The antioxidant activity test was conducted according to the method described by Brand-Williams *et al*.[62] with slight modifications. The method is based on the usage of 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is a nitrogen centered stable radical that gives a specific absorption with a maximum at 515 nm. 2 mL of 10^{-4} M methanolic DPPH solution and 1 mL of 10^{-4} M methanolic sample solution were

added to a test tube and the total volume was adjusted to 4 mL with methanol. The tubes were stoppered, and after incubation for 30 min at room temperature and in the dark, the absorbance at 515 nm was recorded against methanol. The absorbance of the DPPH radical, which did not contain an antioxidant as a control solution, was also measured. 10^{-4} M methanolic Trolox solution was used as the reference standard. The DPPH radical scavenging activity of each sample was calculated according to the formula:

DPPH Radical Scavenging Activity (%) = $[(A_o - A_s) / (A_o)] \times 100$

where A_o represents the absorbance of the control (DPPH radical) and A_s refers to the absorbance of the mixture DPPH-antioxidant. All determinations were performed in triplicate.

Conclusions

In this study, all of the tested bisthiocarbohydrazone compounds exhibited anti-tyrosinase and anti-elastase activities. The results showed that compound **1**had the most effective anti-tyrosinase and anti-elastase activities. This can be explained as the contribution of the bioactive isatin molecule in the structure. On the other hand, the bisthiocarbohydrazones (compounds **2** and **3)** derived from hydroxy aldehydes showed higher radical scavenging activity. It can be said that the phenolic hydroxyl groups in the structure increase the antioxidant activity. The results will guide new studies for the determination of similar activities of other thiocarbohydrazone compounds. In addition, these compounds can be used in the cosmetic, insecticide and pharmaceutical industries as a source of anti-tyrosinase, anti-elastase, and antioxidant activity.

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