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Research Article

A New Series of Bis (Thiosemicarbazone) Derivatives: Synthesis, Spectroscopic Characterization, and Antioxidant Activities

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ABSTRACT

New bis (thiosemicarbazone) derivatives (**1-5**) were obtained from thiophene-2,5-dicarbaldehyde and numerous thiosemicarbazides. The thiosemicarbazides were prepared various isothiocyanates and hydrazine monohydrate. The structure elucidation of all obtained products were determined *via* routine spectroscopic techniques, including proton and carbon NMR (^{1}H, ^{13}C), Fourier-transform infrared spectroscopy (FT-IR), and elemental composition analysis. In this research, the antioxidant activities of the newly synthesized compounds were assessed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Based on the percentage of inhibition, the IC_{50} values indicated the following order of activity: Trolox>**1**>**3**>**4**>**2**>**5**. Additionally, the reducing capabilities of the compounds were determined using the potassium ferricyanide reduction method. The absorbance values obtained from this assay for all the compounds were lower than that of Trolox, suggesting comparatively weaker reducing power. The study also explored how changes in molecular structure influence antioxidant activity, focusing especially on how various functional groups affect radical scavenging efficiency.

1. Introduction

The class of thiosemicarbazones holds substantial importance in synthetic organic chemistry, recognized for their organosulfur structure characterized by the –NH–C(=S)NH–N= functional group. Owing to their structural diversity, they serve as valuable intermediates used in the formation of diverse biologically relevant molecules. These compounds have been extensively studied for their broad spectrum of pharmacological and biological activities, including antimicrobial [1], antibacterial [2], antioxidant [3, 4], antitubercular [5], anticancer [6], antiviral [7], urease inhibitor [8], and anticonvulsant [9]. In recent years, numerous studies have focused on Schiff base-derived thiosemicarbazones due to their significant potential in biological assays conducted *in vitro* and *in vivo*. Such compounds have been reported significant antimicrobial [10], antibacterial and antifungal [11], antioxidant [12], enzyme inhibitory properties [13], anti-HIV [14], anticancer [15], ameliorative effect [16], and anti-inflammatory [17].

Reactive oxygen species (ROS) and free radicals have been extensively implicated in the onset and progression of numerous pathological conditions, including metabolic disorders, ischemia-reperfusion injury, chronic inflammation, aging-related cellular degeneration, and various forms of cancer [18-20]. The accumulation of these reactive species in biological systems disrupts redox homeostasis, contributing significantly to disease development. Consequently, antioxidants have garnered considerable attention due to their protective role in counteracting oxidative stress

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and potentially reducing the risk of severe health conditions [19]. Schiff base derivatives containing thiophene ring and carbothioamide group can show significant antioxidant activity owing to the presence of conjugated π-systems and electron donating/withdrawing groups. In the literature, it has been reported that thiosemicarbazone derivatives, especially those with 2-thiophenylmethylene bond, give significant results in 1,1-diphenyl-2-picrylhydrazyl (DPPH) and (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (ABTS) radical scavenging tests. For example, various aromatic Schiff base derivatives synthesized and showed increased free radical scavenging capacity with the presence of thiophene ring [21-23].

Schiff base derivatives with thiosemicarbazone structure and conjugated with aromatic rings can show significant reductive antioxidant properties due to their capacity to reduce iron (III) ions. It is reported that sulfur and nitrogen atoms, especially in compounds containing thiophene ring, facilitate electron transfer by providing coordination with Fe^{3+} ions and increase the reducing capacity [24-26]. In a study, it was reported that some thiosemicarbazone derivatives carrying thiophene and phenyl groups exhibited high iron reducing power by the FRAP (Ferric Reducing Antioxidant Power) method [26, 27].

In this study, a series of bis (thiosemicarbazone) derivatives were obtained and thoroughly elucidated using spectroscopic methods (FT-IR, ^{1}H-NMR, ^{13}C-NMR), and elemental analysis. Antioxidants play a crucial role in neutralizing free radicals and mitigating their harmful effects in the human body. Therefore, the antioxidant properties of these compounds were assessed *in vitro* using the DPPH· free radical scavenging assay. The effectiveness of each compound was calculated by determining its IC_{50} value. In addition, the potassium ferricyanide reduction technique was employed to determine the reducing potential of these compounds. Moreover, the investigation examined how variations in molecular structure affect antioxidant performance, with particular emphasis on the impact of different groups on the efficiency of radical scavenging.

2. General Methods

2.1. Materials

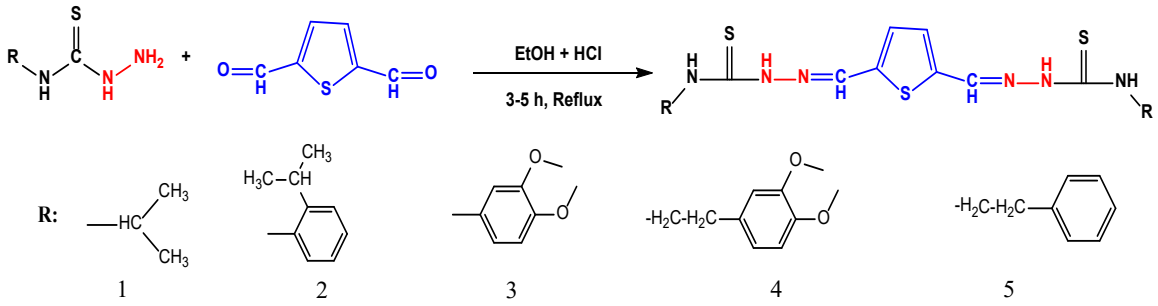
All chemicals were acquired from Merck, Sigma, or Aldrich Chemical Company and used as received without further purification. Elemental analyses were performed using a Eurovector EA3000-Single analyzer. Melting points were determined using a Stuart SMP30 melting point apparatus and are reported without correction.

A Bruker Alpha spectrometer was used to obtain the FT-IR spectra. The ^{1}H and ^{13}C NMR spectra in DMSO-d_6 were recorded on a Bruker Avance DPX-400 MHz spectrometer. UV–Vis absorption spectra were measured using a Shimadzu Pharmaspec 1700 spectrophotometer. NMR signal multiplicities are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), and m (multiplet).

2.2. Synthesis of bis (thiosemicarbazones)

A mixture of various isothiocyanates (7.50 mmol) and hydrazine monohydrate (7.50 mmol) was added dropwise to 20 mL of ethanol under vigorous stirring while maintaining the temperature in an ice bath. Refrigeration of the reaction mixture overnight resulted in the precipitation of the thiosemicarbazide product, which was isolated by filtration, dried, and purified using ethanol.

Subsequently, a few drops of HCl were added to a solution of the thiosemicarbazides (4.00 mmol) and thiophene-2,5-dicarbaldehyde (2.00 mmol) in 20 mL of aqueous ethanol, refluxed at 78 °C for 3 to 5 hours. After completion, the solid product was isolated by filtration, washed, and air-dried. The successful synthesis of the target compounds, obtained in good yields (62–89%), is illustrated in Scheme 1. The procedure followed was adapted from previously reported methods with slight modifications [28, 29].



Scheme 1. Synthesis pathway of bis (thiosemicarbazone) derivatives (**1-5**)

2.3. Antioxidant activity by DPPH assay

A slightly modified version of a previously described method was employed to assess the antioxidant activity of the compounds [30-32]. A 1.0 mL aliquot of DPPH solution (0.1 mM) was mixed with 3.0 mL of the test compound solutions prepared in acetone at various concentrations (4.83–48.33 µM). The samples were kept in the dark at room temperature for 30 minutes, after which their absorbance was recorded at 517 nm using a UV-Vis spectrophotometer [33]. Butylated hydroxyanisole (BHA) was used as a standard antioxidant for comparison.

Lower absorbance values correspond to a higher DPPH· free radical scavenging capacity. The activity of the sample compounds was calculated as percentage inhibition and then compared to the standard (BHA). Percentage inhibition of DPPH radicals by the compounds was computed according to the equation below:

$$\text{Radical scavenging activity (\%)} = [(Ac - A) / Ac \times 100]$$

where A_c is the absorbance of the control (without sample) and A is the absorbance of the test compound or standard [34].

In addition to experimental measurements, the half-maximal inhibitory concentration (IC_{50}) values were determined using calibration curves for each compound. IC_{50} represents the concentration required to inhibit 50% of DPPH radicals under the tested conditions. This value serves as a common quantitative marker of antioxidant capacity, as lower IC_{50} values indicate that less compound is needed to achieve the same inhibitory effect. Thus, a decrease in IC_{50} directly demonstrates an improvement in the antioxidant potential of the tested sample [35].

2.4. Potassium ferricyanide reduction method

In an effort to determine the antioxidant capabilities of the compounds, the potassium ferricyanide reduction method was applied. This method is based on the reduction of $[Fe(CN)_6]^{3-}$ ions to $[Fe(CN)_6]^{4-}$ form and the subsequent formation of Prussian blue ($Fe[Fe(CN)_6]^{-}$) complexes in the presence of Fe(III) ions in the medium. The absorbances of the formed complexes are usually measured at a wavelength of 700 nm and evaluated [25, 36].

In this study, firstly, 1 mL of 58×10^{-5} M DMSO solution of each compound was taken as a sample to determine the reducing capacity of the compounds. Then, 1.25 mL of 0.2 M pH 6.5 phosphate buffer and 1.25 mL of potassium ferricyanide (1 g/100 mL) solution were added, respectively. Following a 20 minutes incubation at 50 °C, 1.25 mL of 10% trichloroacetic acid was incorporated into the mixture, which was then centrifuged at 3000 rpm for 10 minutes at room temperature.

The supernatant obtained after centrifugation was first diluted with 2.5 mL of distilled water, then 0.25 mL of 1% ferric chloride solution was added. After this new mixture was incubated at 37 °C for 10 minutes, the iron (Fe^{3+}) reducing powers of the compounds were determined by measuring the absorbance values at 700 nm wavelength [37, 38].

3. Results and Discussion

3.1. Physicochemical data

Presented in Tables 1 and 2 are the experimental findings related to physicochemical properties, yields, melting points, and elemental analyses.

Table 1. The physicochemical data of the products

	M.P. (°C)	Yields (%)	Colour	Mol. Formula	M.W. (g/mol)
1	245-246	70	Dark Yellow	$C_{14}H_{22}N_6S_3$	370
2	213-214	89	Yellow	$C_{26}H_{30}N_6S_3$	522
3	186-187	86	Orange	$C_{24}H_{26}N_6O_4S_3$	558
4	212-213	88	Yellow	$C_{28}H_{34}N_6O_4S_3$	614
5	247-248	62	Dark Yellow	$C_{24}H_{26}N_6S_3$	494

Table 2. Elemental analysis results of the products

	Calculated			Experimental		
Comp.	**C%**	**H%**	**N%**	**C%**	**H%**	**N%**
1	45.38	5.98	22.68	45.42	6.00	22.71
2	59.74	5.78	16.08	59.81	5.79	16.06
3	51.59	4.69	15.04	51.57	4.68	15.02
4	54.70	5.57	13.67	54.78	5.57	13.63
5	58.27	5.30	16.99	58.22	5.29	17.03

3.2. IR spectral analysis

Analysis of the FT-IR spectra of the synthesized compounds revealed that the characteristic asymmetric and symmetric stretching vibrations of the amino group (–NH_2), typically observed as a doublet peak between 3500 and 3350 cm^{-1}, were absent. Additionally, the aldehyde stretching bands (–CHO) of thiophene-2,5-dicarbaldehyde, normally found between 2820 and 2700 cm^{-1}, were not detected. Instead, a new vibration corresponding to the imine group (CH=N) appeared prominently in the range of 1538–1506 cm^{-1}, confirming the successful formation of the target products (see Figures S1–S5 in the Supplementary Information).

Table 3. IR frequencies of the compounds (cm^{-1})

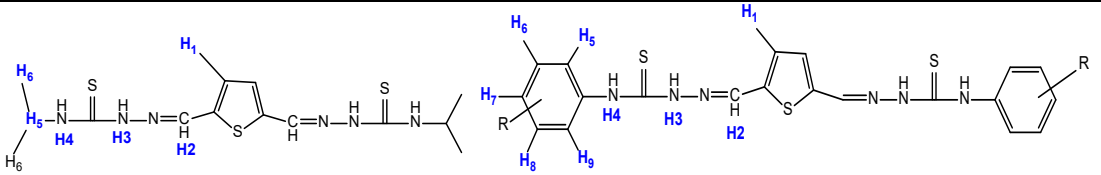
Comp.	**υ_{NH}**	**$\upsilon_{C=N}$**	**$\upsilon_{C=S}$**	**υ_{C-N}**	**υ_{C-S}**
1	3333, 3114	1536	1384	1211	774
2	3350, 3133	1532-1506	1381	1249	701
3	3339, 3116	1534-1509	1382	1219	718
4	3336, 3104	1529-1510	1355	1209	726
5	3346, 3136	1538-1512	1452	1213	804

For all compounds (**1–5**), the presence of a novel amine group (–NH) stretching vibration was observed between 3350 and 3104 cm^{-1}. Aromatic proton vibrations appeared in the 2999–2931 cm^{-1} range, while aliphatic proton vibrations were detected between 2924 and 2812 cm^{-1}. The characteristic –C=S stretching bands of the thiosemicarbazone moiety were found at 1452–1355 cm^{-1}, and the –C–N stretching vibrations appeared in the range of 1249–1209 cm^{-1}. Additionally, the –C–S vibrations were observed between 804 and 701 cm^{-1}. Notably, compounds **3** and **4** exhibited C–O stretching signals at 1022 and 1023 cm^{-1}, respectively. These spectral data are consistent with previously reported values for structurally related compounds [39-41]. Key FT-IR vibration frequencies for all compounds are summarized in Table 4.

3.3. ^{1}H NMR analysis

Chemical shifts from the ^{1}H NMR spectra of the compounds, recorded in DMSO-d_6 are summarized in Table 4.

Table 4. ^{1}H NMR data of the compounds, (δ/ppm)



Comp.	**H1**	**H2**	**H3**	**H4**	**H5-H9**
1	7.43 (s,1H)	8.22 (s, 1H)	11.55 (s, 1H)	7.79-7.77 (d, 1H)	4.55 – 4.45 (m, 1H, H5), 1.23-1.22 (d, J = 6.6 Hz, 6H, H6)
2	7.53 (s,1H)	8.29 (s, 1H)	11.96 (s, 1H)	9.57 (s, 1H)	7.37 – 7.16 (m, 4H)
3	7.51 (s,1H)	8.31 (s, 1H)	11.92 (s, 1H)	9.66 (s, 1H)	7.20 (s, 1H), 7.05-7.02 (d, J = 8.6 Hz, 1H), 6.93-6.90 (d, J = 8.7 Hz, 1H)
4	7.41 (s,1H)	8.21 (s, 1H)	11.67 (s, 1H)	8.09-8.07 (t, J = 5.5 Hz, 1H)	6.89 – 6.76 (m, 3H)
5	7.43 (s,1H)	8.22 (s, 1H)	11.69 (s, 1H)	8.24-8.22 (t, 1H)	7.35-7.20 (m, 5H)

For compounds **1-5**, aromatic proton signal (H1) of the thiophene ring was detected as a singlet at 7.53-7.41 ppm, the proton signal of imine (–CH=N, H2) was detected as a singlet in the ranges 8.31-8.21 ppm. When the amino signal (-NH, H3) of thiosemicarbazone moiety was observed as a singlet in the ranges 11.96-11.55 ppm, the amino signal (-NH, H4) was resonated as a doublet, singlet, and triplet at 9.66-7.77 ppm, respectively (see at Figures S6-S10 in Supplementary information).

For compound **1**, the –CH and –$(CH_3)_2$ protons of the isopropyl group were resonated as a multiplet/heptet and doublet in the ranges 4.55-4.45 and 1.23-1.22 ppm, respectively. The each –OCH_3 proton signals of compounds **3** and **4** were observed as a singlet at 3.75, 3.72, 3.73, and 3.69 ppm, respectively. For compounds **4** and **5**, the proton signal of the methylene group (N–$\underline{CH_2}$) was observed as a quartet at 3.82–3.77 and 3.78-3.73 ppm (q, 2H); the –CH_2 proton signal was detected as a triplet at 2.87–2.83 and 2.93-2.89 ppm (t, 2H). For compounds **2-5**, aromatic proton signal (H5-H9) of the phenyl ring was detected at 7.37-6.76 ppm. The chemical shifts observed correspond closely to previously published data for related compounds [39-41]. DMSO-*d*$_6$ and water in DMSO (HOD, H_2O) signals were detected at approximately 2.50 ppm (quintet) and around 3.30 ppm, respectively, with the latter varying depending on solvent conditions and concentration [42].

3.4. ^{13}C NMR interpretations

Table 5 presents the ^{13}C NMR chemical shifts of the synthesized compounds recorded in in DMSO-*d*$_6$. For compounds **1**–**5**, the characteristic –C=S peaks (C4) of the thiosemicarbazone moiety were detected at 177.53–175.90 ppm. The characteristic –CH=N (imine, C3) peaks were observed in the ranges 141.09-140.70 ppm. The aromatic proton signals (C1 and C2) of the thiophene ring were detected at 131.77-131.52 and 137.74-137.40 ppm (figures S11-S15 in supplementary information).

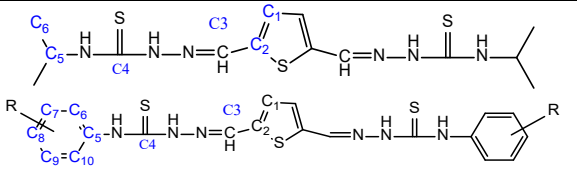
For compound **1**, the –CH and –$(CH_3)_2$ carbon atoms of the isopropyl group were observed at 46.09 and 22.36 ppm, respectively.

For compounds **2-5**, aromatic carbon atoms (C5-C10) of the phenyl ring was detected at 149.19-110.85 ppm. The C5 carbon atom of the compounds **2-5** was detected at 139.61-131.94 ppm.

For compounds **3** and **4**, the C7 and C8 carbon atoms (149.19-147.02 ppm) were shifted down-field (high values of δ) relative to the signal of phenyl carbon (128.5 ppm) due to the presence of methoxy (–OCH_3) group. The each –OCH_3 carbon atom signals of compounds **3** and **4** were observed at 56.12, 56.02, 55.93, and 55.79 ppm, respectively.

For compounds **4** and **5**, the carbon atom signal of the methylene group (N–$\underline{CH_2}$) was detected at 45.54 and 45.64 ppm; the –CH_2 proton signal was observed at 34.64 and 35.28 ppm. The values observed align with those found in earlier studies on similar compounds [39-41].

Table 5. ^{13}C NMR data of the compounds, (δ/ppm)



	C1	C2	C3	C4	C5-C10	R
1	131.5	137.4	140.7	175.9	46.09 22.36	-
2	131.6	137.6	141.1	177.5	136.9 145.7 129.4 126.1 127.7 126.0	28.2, 23.6
3	131.8	137.7	141.1	176.1	132.3 110.9 148.5 147.0 111.6 118.2	56.1, 56.0
4	131.7	137.5	140.8	177.0	131.9 112.5 149.2 147.8 112.8 120.9	55.9, 55.8, 45.5, 34.6
5	131.5	137.5	140.9	177.1	139.6 128.9 129.1 126.7 129.1 128.9	45.6, 35.3

3.5. Evaluation of antioxidant activity

The molecules synthesized in the study were studied against different concentrations using the DPPH radical quenching method. The antioxidant activities were compared with the help of the obtained (%) inhibition data in Figure 1. In the study where Trolox was used as the standard antioxidant, it was determined that the (%) inhibition values of the molecules increased regularly with the increase in concentration. As understood from the data in Figure 1, it can be said that especially compound **1** and **3** exhibited a significant increase in inhibition.

Additionally, IC_{50} (mg/mL) values and linear regression equations used to compare the antioxidant activities of the synthesized compounds and Trolox are given in Table 6. When Table 6 is examined, we can say that some synthesized molecules show IC_{50} values almost close to Trolox. In particular, it is seen that the values found for the DPPH radical reduction capacity of Trolox (16.62±0.15 µM) and for compound **1** (23.38±0.17) are close. IC_{50} data show that compound **1** exhibits the highest antioxidant activity, while compound **5** exhibits the lowest antioxidant activity.

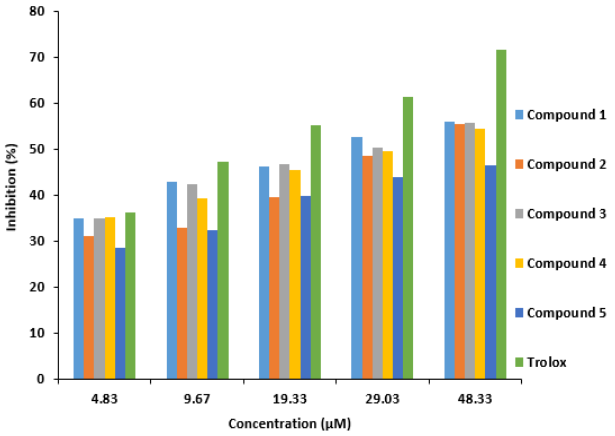


Figure 1. % Inhibition graph of all compounds (**1-5**)

Table 6. IC_{50} values of Trolox and all compounds

Comp.	Linear Concentration equation (y=ax±b)	IC_{50} (µM)	R^2
1	y=0.45x+39.39	23.38±0.17	0.879
2	y=0.59x+28.35	36.70±0.25	0.970
3	y=0.44x+36.31	31.44±0.21	0.902
4	y=0.43x+35.07	34.53±0.23	0.938
5	y=0.41x+28.97	50.76±0.37	0.874
Trolox	y=0.76x+37.41	16.62±0.15	0.934

In the compounds we synthesized in this study, the redox properties of the thiocarbamoyl group (–NH–C(=S)–NH–) are thought to play an important role in the antioxidant mechanism. In particular, the 2-ethylidene-*N*-isopropylhydrazine-1-carbothioamide group in the position attached to the thiophene structure is responsible for the effective antioxidant activity of compound **1**. This situation has been emphasized in previous studies and it has been stated that aliphatic chains such as the isopropyl group increase the lipophilicity of the molecule, facilitate its interaction with biological membrane surfaces and thus contribute to antioxidant activity [43-45].

3.6. Potassium ferricyanide total reduction method evaluation

In this study, the compounds synthesized were compared in terms of their reducing power using the potassium ferricyanide reduction method. The measured absorbance values are given in Figure 2 against concentration in comparison with Trolox. Absorbances for all compounds increased linearly with concentration.

Accordingly, all compounds showed low reducing power compared to Trolox. As in the IC_{50} values we used for DPPH radical quenching activities for the compounds, it was seen that the reducing powers of the compounds gave similar results. Among these, compound **3** and **4**, which contain methoxy substituent groups, showed similar reducing power, while compound **5**, which does not contain any substituent attached to the aromatic ring, showed the lowest reducing power.

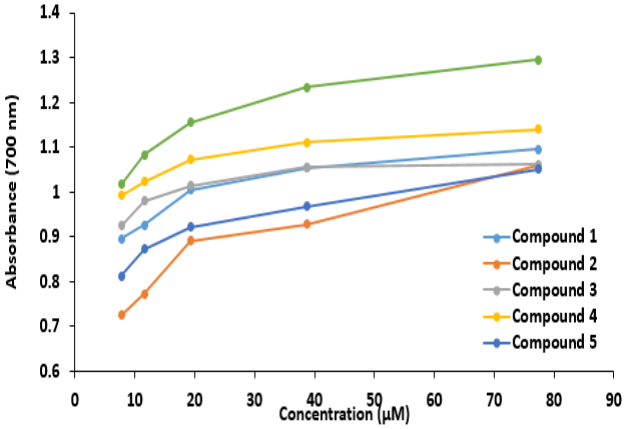


Figure 2. Total reducing power of the synthesized compounds and Trolox for potassium ferricyanide

In a study, alkyl side chains such as isopropyl increase the overall lipophilicity of the molecule, facilitate interaction with metal ions and support reducing behavior [27]. In such compounds, as in this study, C=N and C=S groups create electron donor sites with high redox potential, effectively reducing Fe^{3+} ions to the Fe^{2+} form.

4. Conclusion

A series of new bis (thiosemicarbazone) derivatives) were successfully synthesized and isolated in acceptable yields ranging from 62% to 89%. All synthesized compounds were structurally characterized by IR, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Their antioxidant properties were tested *in vitro* using the DPPH assay, revealing IC_{50} values in the range of 23.38±0.17 to 50.76±0.37 µM. The comparative analysis of IC_{50} values, calculated from inhibition data, indicated that the antioxidant potential decreased in the order: Trolox > **1** > **3** > **4** > **2** > **5**. Among the tested derivatives, compound **1** exhibited the most potent antioxidant activity against the DPPH radical.

The molecules in this study exhibited free radical scavenging ability similar to Trolox, a standard industrial antioxidant, even at low concentrations. In conclusion, *N*-isopropyl-2-(thiophen-2-ylmethylene)hydrazine-1-carbothioamide-like structures are among the promising antioxidant agents due to both their structural and electronic properties. In addition, compound **1**-like structures seem to have remarkable potential in terms of antioxidant mechanisms based on metal reduction. Overall, these results provide valuable insight into the design of more effective antioxidant agents within the bis (thiosemicarbazone) structural framework and highlight the importance of electronic effects in modulating biological activity.

Article Information Form

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Authors' Contribution
H.Y.; Formal analysis, investigation, writing – original draft, data curation, concept/design, data analysis, validation, writing – review & editing, visualization. H.M.; Investigation, methodology, data analysis, validation, writing – original draft, data curation. All authors reviewed the manuscript.

The Declaration of Conflict of Interest/ Common Interest
No conflict of interest or common interest has been declared by authors.

Artificial Intelligence Statement
No artificial intelligence tools were used while writing this article.

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