

Synthesis, characterization and biological activity potential of some novel thiourea derivatives

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ABSTRACT: Within the scope of this study, a new series of thiourea derivatives were synthesized by refluxing different anthranilic acid derivatives with various isothiocyanates in dry acetone medium. The synthesized compounds were purified by crystallization and their purity was determined by TLC method. The structures of the obtained compounds were elucidated by using different spectroscopic methods such as IR, ¹H-NMR, ¹³C-NMR, besides elemental analysis. The inhibitory effects of the compounds against DPPH radical scavenging, α-glucosidase and acetylcholinesterase were investigated by using spectrophotometric method. The results showed that the compounds had moderate radical scavenging activity according to gallic acid (92.25 ± 0.14% at 100 μM) which was used as a reference compound. **Compound 5** in the presence of trifluoromethyl group demonstrated the highest α-glucosidase inhibitory effect with 52.26 ± 2.35% at 100 μM. On the other hand, the compounds demonstrated low AChE inhibitory effects compared to galantamine (80.33 ± 0.77% at 100 μM) which was used as a reference compound.

KEYWORDS: Thiourea; anthranilic acid; DPPH radical scavenging; α-glucosidase; acetylcholinesterase.

1. INTRODUCTION

Thioureas are important pharmacophoric structures with being responsible for many biological effects. Since before now, they have been important figures of the pharmaceutical industry and have been used successfully at the treatment of many diseases, by reducing or preventing their symptoms [1-3]. Among thioureas, the 1,3-disubstitued ones have always had an important role as active drug substances. For example; Metiamide and Burimamide for the treatment of ulcers [4], Carbimazole and Methimazole for the treatment of thyroid-related diseases [5], Thiocarlide for its antimycobacterial effect, Troviridine for its antiviral effect [6], Loflukarban and Thiambutosine for their antibacterial properties and Tenovin 1 for the treatment of cancer and neurodegenerative diseases [7,8] (Figure 1). In addition, there are recent studies claiming thioureas' intimate pharmacological potential with their diverse biological activities like analgesic [9], antioxidant [10-13], antibacterial [14], anticancer [15-17], antimycobacterial [18-19], antiviral [20], insecticidal [21], etc. Also, they been reported for their various enzyme inhibitory activities as urease [22], carbonic anhydrase [23-24], acetylcholinesterase, butyrylcholinesterase [25-27], α-glucosidase [28-29].

Diabetes mellitus (DM) has become a major public health problem worldwide. According to 2022 data, it has been determined that 422 million people have DM [30]. DM is a metabolic disorder that occurs when blood glucose levels in the body cannot be controlled. Higher than normal blood glucose levels can cause health problems in the long term. There are two main types: Type 1 and Type 2. Type 1 DM defined as a disease that occurs as a result of the insufficient production of beta cells in the pancreas. Type 2 DM occurs when the insulin produced by the body becomes ineffective or insufficient [31-33]. In recent years, in addition to Type 1 and Type 2, Alzheimer's diseases dependent DM has been defined as Type 3. Insulin resistance is an important link in both DM and Alzheimer's disease (AD). Decreased insulin sensitivity causes hyperglycemia. Changes in glucose intake, which is the metabolite that meets the brain's most important energy needs, cause neuronal damage and neurodegenerative diseases [34]. If DM is not controlled, cardiovascular disease, renal problems, eye problems, nerve damage, and other serious health

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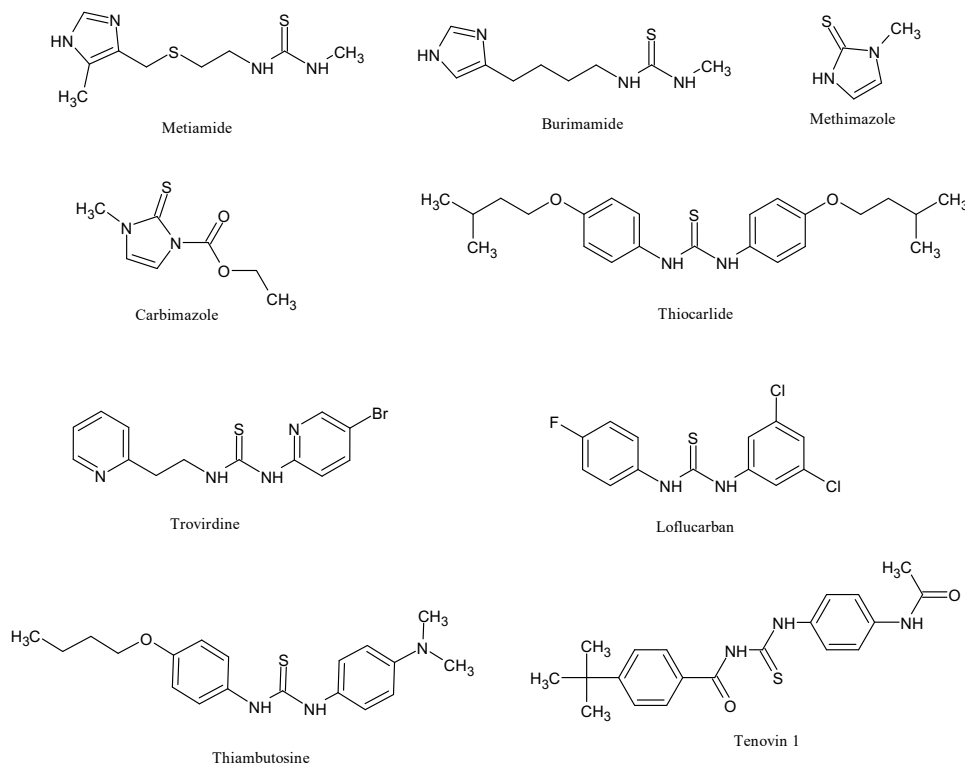


Figure 1. Thiourea compounds used as active drug substances.

problems can develop [35-37]. Therefore, early diagnosis and effective management of DM is crucial. Metformin, sulphonureas (glipizide), dipeptidyl peptidase-4 (DPP-4) inhibitors (sitagliptin, etc.), and α -glucosidase inhibitors (acarbose, etc.) are used in the treatment of DM (Figure 2). However, these drugs have various side effects on some patients, such as gastrointestinal disorders, weight loss, urinary tract infection, allergic reactions, and hypoglycemia [38]. Therefore, the search for alternative medicine continues.

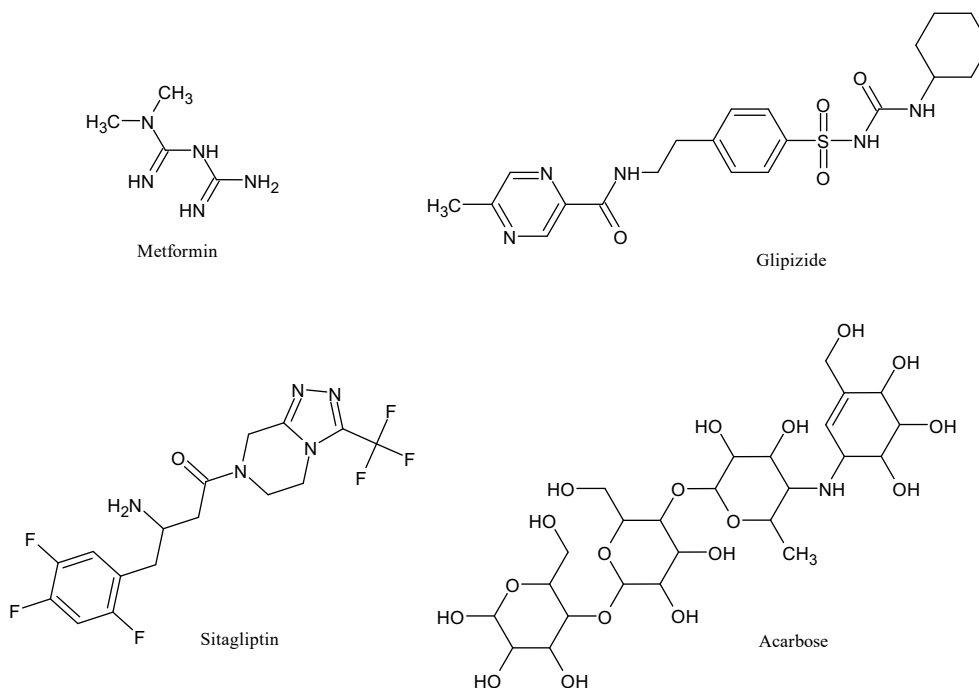


Figure 2. The compounds used in the treatment of DM.

AD is a brain disease that is generally defined as a progressive neurodegenerative disorder, usually occurs in old age, and is characterized by memory loss, cognitive impairment, language problems, temporal problems, and decreases in planning ability [39]. AD is the most common neurodegenerative disease worldwide and the most common cause of dementia [40]. The prevalence of Alzheimer's disease increases significantly with age. The prevalence of the disease is between 5% and 10% in individuals over the age of 65, but can be as high as 30% in those 85 and older. Approximately 50 million people worldwide are living with dementia, of whom 60%-70% are estimated to have Alzheimer's disease [41]. Treatment of AD is usually aimed at relieving symptoms or slowing its progression, but so far no completely curative treatment has been found. Cholinesterase inhibitors (galantamine, donepezil, and rivastigmine (Figure 3) are the most commonly used drugs in the treatment of this disease. It has also been reported that these drugs have side effects such as gastrointestinal problems, fatigue, liver diseases, vomiting, and dizziness etc [42-43].

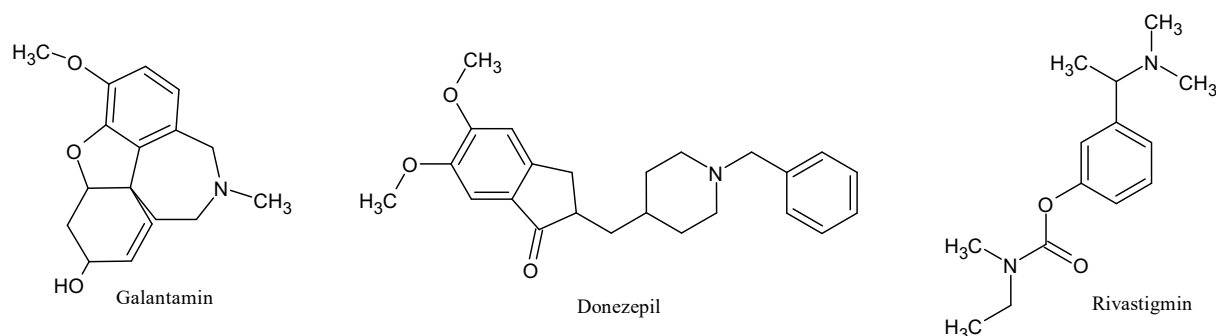


Figure 3. The compounds used as cholinesterase inhibitors.

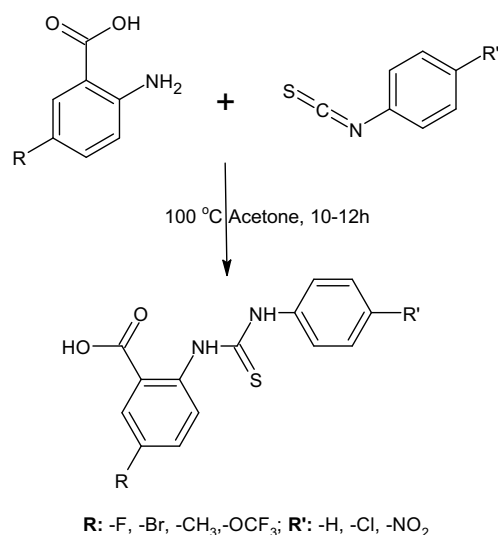
Antioxidant activity plays a crucial role in the management and prevention of both DM and AD. In diabetes, particularly Type 2 diabetes, chronic hyperglycemia (high blood sugar) leads to increased production of free radicals, which contributes to oxidative stress. This oxidative stress damages cells and tissues, particularly the blood vessels, leading to complications [44]. On the other hand, AD is associated with significant oxidative stress, which is the result of an imbalance between the production of free radicals and the body's ability to neutralize them with antioxidants. This stress contributes to the damage of neurons and the accumulation of amyloid-beta plaques and tau tangles, which are characteristic features of Alzheimer's disease [45].

One of the main objectives of this study was to investigate the potential of using a single drug for many diseases by preventing the use of polypharmacy in elderly patients. In light of all this information, it was aimed to perform analyzes of newly synthesized thiourea-derived compounds to determine their potential for use in DM and AD in this study.

2. RESULTS AND DISCUSSION

2.1. Chemistry

Novel thiourea derivatives were obtained via one step reaction of commercially available 5-substitutedanthranilic acids with different isothiocyanates at 100°C, by stirring 10-12 h in dry acetone(Scheme 1). The completion of the reaction was checked with TLC (Mobile Phase: Chloroform/Methanole 50:50) and the reaction mixture was kept in the fridge overnight. The obtained solid was filtered, washed with water, dried and purified by crystallization with hot absolute ethanol. The obtained compounds' structures was enlightened by elemental analysis, IR and ¹H, ¹³C-NMR spectroscopic methods. According to IR results; the thiourea N-H stretching bands were recorded at 3318-3210 cm⁻¹ and their C=S stretching bands were determined at 1211-1196 cm⁻¹. In ¹H-NMR results; N-H protons belonging to thiourea were mostly replaced with the deuterium in the solvent and could not be determined. Only compounds 1and 8's N-H protons were determined with aromatic protons. In regard to the ¹³C-NMR results, the thiourea C=S carbons were detected at 175.29-176.69 ppm. Based on the elemental and spectral analysis results; it could be said that compounds (1-8) were synthesized and purified properly.



Scheme 1. The synthesis route of compounds 1-8.

2.2. Biological Activity

2.2.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

In this work, DPPH radical scavenging effects of the novel compounds were investigated using spectrophotometric assay. The results of the study are presented as percentage (%) in Table 1. As shown in Table 1, the compounds showed moderate radical scavenging activity according to gallic acid ($92.25 \pm 0.14\%$ at $100 \mu\text{M}$) which was used as a reference compound. Overall, 5-bromo-2-(4-nitrophenyl) containing **Compound 1** had the highest activity with $47.08 \pm 0.40\%$ at $100 \mu\text{M}$ among the synthesized compounds. Furthermore, the DPPH radical scavenging inhibition rate of **Compound 8** (5-trifluoromethoxy-2-[(phenylcarbamothioyl) containing group) was found to be $43.17 \pm 3.22\%$ at $100 \mu\text{M}$. The presence of molecules with high electronegativity in both Compounds 1 and 8 contributed positively to the antioxidant effect. On the other hand, Compounds 5 and 3 showed the lowest DPPH radical scavenging effects due to the presence of a non-electronegative methyl group. The studies have revealed that DPPH radical scavenging properties of the compounds increased with the addition of electronegative groups to the main skeleton.

2.2.2. α -Glucosidase inhibitory effects of the compounds

In this study, α -glucosidase inhibitory properties of the synthesized compounds were examined spectrophotometrically by monitoring the release of para-nitrophenol from para-nitrophenyl- α -D-glucopyranoside. The results were presented in Table 1. The results showed that **Compound 5** exhibited the highest alpha-glucosidase activity with $52.26 \pm 2.35\%$ at $100 \mu\text{M}$. Contrary to antioxidant activity, the presence of methyl group in **Compound 5** may be the reason for the highest activity among the compounds [46]. Similarly, **Compound 6**, which contains a methyl side group, showed second alpha-glucosidase activity after **Compound 5**. The inhibition values of the compounds can be ranked as compounds $5 > 6 > 7 > 8 > 3 > 4 > 1$. On the other hand, **Compound 2** did not show any glucosidase inhibitor activity at $100 \mu\text{M}$.

2.2.3. AChE inhibitory properties of the compounds

In this study, AChE inhibitory properties of the synthesized compounds were examined spectrophotometrically by monitoring 5,5-dithio-bis(2-nitrobenzoic)acid (DTNB) converts the formed thiocholines to nitrobenzoate. The results of obtained from the study are tabulated in Table 1. As presented in Table 1, the compounds showed low AChE inhibitory effects according to galantamine ($80.33 \pm 0.77\%$ at $100 \mu\text{M}$) which was used as a reference compound. Among the compounds, 5-bromo-2-(4-nitrophenyl) containing **compound 1** had the highest inhibitory effects with $28.58 \pm 0.77\%$ at $100 \mu\text{M}$. The results consisted with antioxidant activities. On the other hand, **compounds 2** and **6** showed no inhibitory effects against AChE at studied concentration.

Table 1. DPPH radical scavenging, α -glucosidase, and AChE inhibitory effects of the compounds at 100 μ M.

Compounds	DPPH % (at 100 μ M)	α -glucosidase % (at 100 μ M)	AChE % (at 100 μ M)
1	47.08 \pm 0.40	7.55 \pm 0.24	28.58 \pm 0.77
2	37.06 \pm 1.25	nd	nd
3	30.52 \pm 0.33	22.95 \pm 0.61	21.34 \pm 0.85
4	38.63 \pm 2.94	11.95 \pm 1.05	18.09 \pm 0.55
5	23.10 \pm 2.36	52.26 \pm 2.35	7.80 \pm 1.37
6	34.22 \pm 2.03	41.43 \pm 1.79	nd
7	36.21 \pm 2.34	38.50 \pm 2.00	14.00 \pm 0.17
8	43.17 \pm 3.22	32.59 \pm 1.09	9.69 \pm 1.11
Reference compound	92.25 \pm 0.14	61.44 \pm 2.48	80.33 \pm 0.77

Reference compound for DPPH: gallic acid, reference compound for α -glucosidase: acarbose, reference compound for AChE: galantamine. nd: no data

3. CONCLUSION

In summary, eight novel thiourea compounds were synthesized by reacting different 5-substituted anthranilic acids bearing fluoro, bromo, methyl or trifluoro methoxy groups with phenyl, 4-chlorophenyl or 4-nitrophenylisothiocyanates at acetone media. The obtained compounds were characterized by IR, 1 H-NMR and 13 C-NMR methods. They were also evaluated for their inhibitory activities against DPPH radical scavenging, α -glucosidase, and acetylcholinesterase. Among them **compound 5** with trifluoromethyl group showed remarkable activity against α -glucosidase and could be used as a lead compound for further studies.

4. MATERIALS AND METHODS

4.1. Chemistry

All of the chemicals, reagents and solvents were purchased from Sigma Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Melting points were determined by CHNS-932 (LECO) apparatus. The IR spectra were recorded on a Shimadzu, IRAffinity-1S Spectrometer. The NMR spectra were recorded (in DMSO- d_6) with a Bruker spectrometer (Billerica, MA, USA) (400 MHz for 1 H-NMR and 100 MHz for 13 C-NMR, decoupled). The chemical shift values are expressed in ppm (δ scale) using tetramethylsilane as an internal standard. Elemental analysis was performed on Leco 215 CHNS-932 analyzer.

4.1.1. General procedure for the synthesis of compounds (1-8).

The compounds (1-8) were synthesized by following the route according to our previous published papers [15, 47, 48].

5-Bromo-2-[[4-nitrophenyl]carbamothioyl]amino}benzoic acid (1)

Yellow solid, yield: 63%, mp: 229–230 $^{\circ}$ C. FT-IR: ν_{\max} = 3318, 3086, 1690, 1605, 1589, 1512, 1474, 1427, 1335, 1204, 826 cm^{-1} . 1 H NMR (400 MHz, DMSO- d_6) δ 7.41 (1H, d, J : 12.00 Hz, C₃-H); 7.65 (2H, d, J = 8.00 Hz, C₈-H, C₁₂-H); 7.97-8.14 (4H, m, C₄-H, C₆-H ve üre -NH-); 8.36 (2H, d, J : 8.00 Hz, C₉-H, C₁₁-H); 13.29 (1H, s, -COOH). 13 C NMR (100 MHz, DMSO- d_6) δ 116.54, 118.34, 118.57, 124.84, 129.70, 131.33, 138.80, 139.26, 142.41, 145.45 (Ar-C); 159.12 (C-14); 175.86 (C-13). Anal. Calcd. for C₁₄H₁₀BrN₃O₄S (396.2159): C, 42.44; H, 2.54; N, 10.61; S, 8.09. Found: C, 42.39; H, 2.61; N, 10.71; S, 8.16.

5-Bromo-2-[[4-chlorophenyl]carbamothioyl]amino}benzoic acid (2)

White solid, yield: 61%, mp: 342 $^{\circ}$ C. FT-IR: ν_{\max} = 3233, 3032, 1659, 1612, 1512, 1481, 1420, 1211, 1088, 826 cm^{-1} . 1 H NMR (400 MHz, DMSO- d_6) δ 7.34 (2H, d, J : 8.00 Hz, C₈-H, C₁₂-H); 7.39 (1H, d, J : 8.00 Hz, C₃-H); 7.55 (2H, d, J : 8.00 Hz, C₉-H, C₁₁-H); 7.95-7.98 (1H, dd, J : 8.00 Hz ve J : 4.00 Hz, C₄-H); 8.02 (1H, d, J : 4.00 Hz, C₆-H); 13.20 (1H, s, -COOH). 13 C NMR (100 MHz, DMSO- d_6) δ 116.44, 118.51, 118.60, 129.56, 129.75, 131.41, 133.34, 138.48, 138.68, 139.17 (Ar-C); 159.20 (C-14); 176.36 (C-13). Anal. Calcd. for C₁₄H₁₀BrClN₂O₂S (385.6634): C, 43.60; H, 2.61; N, 7.26; S, 8.31. Found: C, 43.66; H, 2.53; N, 7.19; S, 8.15.

5-Methyl-2-[[4-chlorophenyl]carbamothioyl]amino]benzoic acid (3)

White solid, yield: 67%, mp: 346-349 °C. FT-IR: ν_{\max} = 3248, 3032, 1651, 1628, 1520, 1489, 1427, 1204, 1088, 833 cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ 2.38 (1H, s, $-\text{CH}_3$); 7.33-7.38 (3H, m, C₃-H, C₈-H, C₁₂-H); 7.55 (2H, d, J: 8.00 Hz, C₉-H, C₁₁-H); 7.63 (1H, d, J: 8.00 Hz, C₄-H); 7.77 (1H, s, C₆-H); 13.06 (1H, s, $-\text{COOH}$). ^{13}C NMR (100 MHz, DMSO- d_6) δ 20.91 ($-\text{CH}_3$); 116.20, 116.51, 127.21, 129.47, 131.55, 133.15, 134.48, 137.18, 138.08, 138.80 (Ar-C); 160.26 (C-14); 175.80 (C-13). Anal. Calcd. for C₁₅H₁₃ClN₂O₂S (320.7929): C, 56.16; H, 4.08; N, 8.73; S, 10.00. Found: C, 56.21; H, 4.15; N, 8.71; S, 9.95.

5-Fluoro-2-[[4-chlorophenyl]carbamothioyl]amino]benzoic acid (4)

White solid, yield: 71%, mp: 340 °C. FT-IR: ν_{\max} = 3225, 3040, 1659, 1528, 1489, 1227, 1204, 1088, 833 cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ 7.34 (2H, d, J: 8.00 Hz, C₈-H, C₁₂-H); 7.48-7.51 (1H, dd, J: 8.00 Hz ve J: 4.00 Hz, C₃-H); 7.55 (2H, d, J: 8.00 Hz, C₉-H, C₁₁-H); 7.66-7.74 (2H, m, C₄-H, C₆-H); 13.17 (1H, s, $-\text{COOH}$). ^{13}C NMR (100 MHz, DMSO- d_6) δ 112.91 (d, $^2J_{\text{C-F}}$ =24.00 Hz, C-4); 118.06 (d, $^3J_{\text{C-F}}$ =8.00 Hz, C-3); 118.84 (d, $^3J_{\text{C-F}}$ =8.00 Hz, C-1); 124.26 (d, $^2J_{\text{C-F}}$ =24.00 Hz, C-6); 129.53, 131.46, 133.28, 136.97, 138.58 (Ar-C); 158.72 (d, $^1J_{\text{C-F}}$ =241.00 Hz, C-5); 159.65 (C-14); 175.92 (C-13). Anal. Calcd. for C₁₄H₁₀ClFN₂O₂S (324.7578): C, 51.78; H, 3.10; N, 8.63; S, 9.87. Found: C, 51.66; H, 3.03; N, 8.51; S, 9.83.

5-Methyl-2-[[4-nitrophenyl]carbamothioyl]amino]benzoic acid (5)

White solid, yield: 66%, mp: 333 °C. FT-IR: ν_{\max} = 3256, 3085, 1728, 1651, 1620, 1566, 1520, 1420, 1204, 833. ^1H NMR (400 MHz, DMSO- d_6) δ 2.46 (3H, s, $-\text{CH}_3$); 7.37-8.42 (7H, m, Ar-H); 13.15 (1H, s, $-\text{COOH}$). ^{13}C NMR (100 MHz, DMSO- d_6) δ 20.90 ($-\text{CH}_3$); 116.30, 116.54, 124.75, 127.17, 131.46, 134.61, 137.29, 138.17, 145.81, 147.55 (Ar-C); 160.18 (C=O); 175.29 (C=S). Anal. Calcd. for C₁₅H₁₃N₃O₄S (331.3464): C, 54.37; H, 3.95; N, 12.68; S, 9.68. Found: C, 54.45; H, 3.91; N, 12.75; S, 9.65.

5-Methyl-2-[(phenylcarbamothioyl)amino]benzoic acid (6)

White solid, yield: 67%, mp: 344-347 °C. FT-IR: ν_{\max} = 3248, 3031, 2987, 1651, 1620, 1520, 1204 cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ 2.38 (3H, s, $-\text{CH}_3$); 7.05-7.81 (8H, m, Ar-H); 13.01 (1H, s, $-\text{COOH}$). ^{13}C NMR (100 MHz, DMSO- d_6) δ 20.92 ($-\text{CH}_3$); 116.16, 116.49, 127.21, 128.55, 129.37, 129.48, 134.43, 137.14, 138.08, 139.83 (Ar-C); 160.28 (C=O); 176.00 (C=S). Anal. Calcd. for C₁₅H₁₄N₂O₂S (286.3489): C, 62.93; H, 4.89; N, 9.79; S, 11.18. Found: C, 62.87; H, 4.85; N, 9.83; S, 11.09.

5-Fluoro-2-[(phenylcarbamothioyl)amino]benzoic acid (7)

White solid, yield: 71%, mp: 359-362 °C. FT-IR: ν_{\max} = 3210, 3040, 1659, 1528, 1489, 1458, 1196, 1227 cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ 7.22-7.78 (8H, m, Ar-H); 13.13 (1H, s, $-\text{COOH}$). ^{13}C NMR (100 MHz, DMSO- d_6) δ 112.77, 113.01, 117.98, 118.06, 118.77, 118.85, 124.10, 124.34, 128.68, 129.39, 129.42, 136.99, 139.62, 157.50, 159.91 (Ar-C); 159.66 (C=O); 176.10 (C=S). Anal. Calcd. for C₁₄H₁₁FN₂O₂S (290.3127): C, 57.93; H, 3.79; N, 9.65; S, 11.03. Found: C, 57.95; H, 3.75; N, 9.55; S, 11.15.

5-Trifluoromethoxy-2-[(phenylcarbamothioyl)amino]benzoic acid (8)

White solid, yield: 64%, mp: 303 °C. FT-IR: ν_{\max} = 3233, 3040, 1659, 1528, 1489, 1204, 1258 cm^{-1} . ^1H NMR (400 MHz, DMSO- d_6) δ 7.23-7.87 (10H, m, Ar-H and thiourea N-H); 13.20 (1H, s, $-\text{COOH}$). ^{13}C NMR (100 MHz, DMSO- d_6) δ 117.90, 118.70, 119.64, 126.43, 128.49, 128.73, 129.35, 129.44, 129.56, 130.39, 139.10, 139.55, 144.40, 144.41 (Ar-C); 159.52 (C=O); 176.69 (C=S). Anal. Calcd. for C₁₅H₁₁F₃N₂O₃S (356.3196): C, 50.56; H, 3.11; N, 7.86; S, 9.00. Found: C, 50.47; H, 3.03; N, 7.91; S, 9.02.

4.2. Biological Activity

4.2.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging ability of the compounds was examined according to the our previously method with minor modifications [49]. The synthesized compounds in DMSO were prepared as 25 mM,

separately. The working solution was diluted with methanol at 1 mM. Then, DPPH solution (0.2 mM) (Sigma–Aldrich, D9132) were prepared in methanol. Afterward, the compounds (25 µL/well, 100 µM) were added to the DPPH solution (225 µL/well) and incubated at room temperature in the dark for 30 min. Afterward, the extracts (25–200 µg/mL) were added to the DPPH solution (0.2 mM) (Sigma–Aldrich, D9132) in methanol and mixed vigorously. The mixtures were incubated at room temperature in the dark for 30 min. After 30 min, the DPPH scavenging ability of the compounds was monitored spectrophotometrically by absorbance at 517 nm. Gallic acid (Sigma–Aldrich, G7384) was used as a reference compound. The inhibition (%) was obtained using equation. % Inhibition = $(A_{\text{control}} - A_{\text{compound}}) / A_{\text{control}} \times 100$ A_{control} : Absorbance of DPPH solution; A_{compound} : Absorbance of DPPH solution after addition of compound.

4.2.2. α -Glucosidase from *Saccharomyces cerevisiae* inhibitory assay

The α -glucosidase from *Saccharomyces cerevisiae* inhibitory ability of the compounds was examined according to the our previously method with minor modifications [50]. The synthesized compounds in DMSO were prepared as 25 mM, separately. The working solution was diluted with 100 mM sodium phosphate buffer pH 6.9 at 1 mM. Then, the synthesized compounds (50 µL/well, 100 µM) in sodium phosphate buffer (100 mM, pH 6.9) were added to α -glucosidase from *Saccharomyces cerevisiae* (Sigma–Aldrich, G5003) (100 µL/well, 0.5 U/mL) and incubated for 15 min. Afterward, 4-p-nitrophenyl- α -glucopyranoside (Sigma–Aldrich, 487506) (50 µL/well, 5 mM) was added to mixture and allowed to react at room temperature for 15 min. After 15 min, the α -glucosidase inhibitory ability of the compounds was monitored spectrophotometrically by absorbance at 405 nm. Acarbose (Sigma–Aldrich, A8980) was used as a reference compound, while DMSO (0.4%) was used as a negative control. The inhibition (%) was obtained using equation. % Inhibition = $(A_{\text{control}} - A_{\text{compound}}) / A_{\text{control}} \times 100$ A_{control} : buffer, enzyme and substrate; A_{compound} : buffer, compounds, enzyme and substrate.

4.2.3. AChE from *Electrophorus electricus* inhibitory assay

The AChE from *Electrophorus electricus* inhibitory ability of the compounds was examined according to the our previously method with minor modifications [51]. The synthesized compounds in DMSO were prepared as 25 mM, separately. The working solution was diluted with 50 mM Tris-HCl buffer pH 8.0 at 1 mM. Then, Tris-HCl buffer (50 µL/well), DTNB (5,5-dithio-bis(2-nitrobenzoic)acid) (Sigma–Aldrich, D8130) (125 µL/well, 3 mM) as chromatographic reagent, AChE from *Electrophorus electricus* (Sigma–Aldrich, C3389) (25 µL/well, 0.2 U/mL) and the compounds (25 µL/well) were added into the plate and incubated at room temperature for 15 min. After incubation, acetylthiocholine iodide (ATChI) (25 µL/well, 15 mM) as substrate was (Sigma–Aldrich A5751) was added to enzyme-inhibitor mixtures and incubated for 15 min. After 15 min, the AChE inhibitory ability of the compounds was monitored spectrophotometrically by absorbance at 405 nm. Galantamine (Sigma–Aldrich, 1287755) was used as a reference compound, while DMSO (0.4%) was used as a negative control. The inhibition (%) was obtained using equation. % Inhibition = $(A_{\text{control}} - A_{\text{compound}}) / A_{\text{control}} \times 100$ A_{control} : buffer, enzyme and substrate; A_{compound} : buffer, compounds, enzyme and substrate.

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