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Synthesis of novel Schiff bases and azol-β-lactam derivatives starting from morpholine and thiomorpholine and investigation of their antitubercular, antiurease activity, acethylcolinesterase inhibition effect and antioxidant capacity

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ABSTRACT

In this study, new Schiff bases and β-lactam derivatives containing morpholine and thio morpholine nuclei were synthesized. Antimicrobial, antioxidant, antimicrobial and antioxidant properties of all synthesized compounds were investigated and highly effective products were obtained. In this context, new effective structures were introduced to the literature.

1. Introduction

The azetidine-2-one ring also known as β-lactam is a four-membered ring that is a class of important bioactive compound. Penicillin, Klavam, Penem, Carbapenem, Cephalosporin, Oxasefem, Izosefem and Izooxasefem are known drugs and contain a β-lactam ring in their molecular structure and are also known β-lactam antibiotics [1]. Recent clinical studies on bacterial infections and the discovery of β-lactam chemistry and biology have led to important advances [2–7].

Tuberculosis (TB) is a devastating disease caused by poverty, and according to World Health Organization (WHO) 2016 data, 95% of 1.4 million people infected with TB in developing countries are killed and 10 million children are orphaned as a result [8]. Moreover, the emergence of a drug-resistant microorganism with the lethal combination of TB, particularly multidrug-resistant TB and HIV-1 infection, makes this disease one of the greatest global health problems of our time [9]. Therefore, new antitubercular drugs and drug targets are urgently needed. The need to identify, detect and synthesize agents that will be effective against rapid action and intracellular *Mycobacterium tuberculosis* by reducing treatment time is of great importance nowadays.

Azole rings are heterocyclic compounds containing nitrogen atoms and constitute a class of compounds with a broad biological effect. Some of these effects are anti-bacterial, anti-malarial, anti-fungal, anti-HIV, anti-inflammatory and anti-TB [8,10]. The antibacterial resistance of pathogenic microorganisms against the drugs used clinically is developing very rapidly all over the world and this threatens public health. As a result of the low number of antimicrobial compounds used and the rapid development of bacterial resistance, the synthesis of new antimicrobial compounds has become an attractive subject for organic medicinal chemists. In last decades in order to obtain antitumor drugs, many compounds with different structure have been synthesized. These include complex molecules containing an azole ring as well as structurally simple azole derivatives [8,11–14]. Some examples of azole drugs are Trazodone, Ribavirin, Rizatriptan, Vorazol, Alprazolam and Nefazonon that contain a 1,2,4-triazole ring in their molecular structure [15–19]. Cefazedone and Sevazolin, which are cephalosporin-derived antibiotics, are important drugs containing 1,3,4-thiadiazole ring [20]. Acetozolamide and methazolamide, which are used in the treatment of glaucoma and epilepsy diseases, are another important molecule in the structure of thiadiazole ring [21,22]. Also, Raltegravir (HIV-infections),

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Furamizol (antibacterial), Zibotentan (anticancer candidate drug) and Tiodazosin (antihypertensive agent) are an important drug or drug candidate containing oxadiazole nucleus [23,24].

In the point of this information, in this study, the effects of antimicrobial, antioxidant properties, antiureas and acetylcholinesterase effects were investigated by the synthesis of new hybrid compounds by combining different active groups in a single molecule.

2. Results and discussion

2.1. Chemistry

In this study there are two starting material thiomorpholine and morpholine. Separately thio/morpholine was reacted with ethyl bromoacetate and hydrazinhydrate to afford the corresponding asetohydrazide compounds **2a** and **2b** and then they were converted to 1,3,4-oxadiazole ring (**3a/3b**) separately with carbondisulfide in basic media. The carbonyl group of compounds **2a/2b** disappeared and the C═S bond was occurred at 1295 cm^{-1} in IR spectrum. And in ^{1}H NMR spectrum the 1,3,4-oxadiazole −NH was resonated at 8.67 ppm of compound **3a** where in compound **3b** −NH was not observed. Compound **4a** and **4b** were synthesized by the reaction of compound **3** with acrylonitrile in basic media, separately. At the N-3 position of 1,3,4-oxadiazole the nitrile moiety was linked by addition reaction. In the ^{13}C NMR spectra the −CN group was resonated at 118.30–118.34 ppm of the target compounds **4a** and **4b**. 1,3,4-Thiadiazole ring were introduced to compound **4** by cycloaddition reaction with thiosemicarbazide in triflouroacetic acid to afford the corresponding compound **5a** and **5b** separately. Compared with the starting material $–NH_2$ protons were resonated at 7.02–7.20 ppm in ^{1}H NMR, and in ^{13}C NMR the carbon atoms of thiadiazole were resonated at 153.88–155.10 ppm as thiadiazole C-3 and 158.30–159.36 ppm as thiadiazole C-5 of compounds **5a** and **5b**. To afford the Schiff bases shown at Fig. 1, compound **5** was firstly reacted with hydrazine hydrate to convert 1,3,4-oxadiazole ring to 1,2,4-triazole-4-amino (**6a, 6b**), then, compound **6** was reacted with different aldehydes to synthesized the corresponding Schiff bases with the value of 8.60–10.89 ppm of −N═CH− (imine) peak of compounds **7a-7f** and **8a-8f**. The beta lactam ring of compounds **9a-9f** and **10a-10f** were occurred by the reaction of Schiff bases with chloroacetyl chloride in dioxane solvent and in IR spectrum the carbonyl group was seen between 1710 and 1730 cm^{-1} and in ^{13}C NMR the signal was resonated between 167.87 and 170.23 ppm respectively.

2.2. Biological activity

2.2.1. Antituberculosis activity

When the antitubercular effect of thiomorpholine and morpholine serie compounds were examined, it was seen that the thiomorpholine derivative **7d** and Schiff base **7e** exhibited very good activity against streptomycin which is the standard drug with the value of 7.81 μg/mL. The compounds of formula **2b** and **3b**, which have the acetohydrazide and oxadiazole ring in the morpholine derivative structure, were equally effective against streptomycin.

Compounds **7c**, **7f** which are Schiff bases of thiomorpholine serie and **9d**, **9e** which are beta-lactam derivatives of thiomorpholine serie exhibit moderate-good activity compared with the standard drug of steptomycin of *Mycobacterium smegmatis*.

The 4-methoxybenzaldehyde group of thiomorpholine serie, compound **7a** and **9a** showed significant antitubercular activity with the value of 31.2 9 μg/mL. β-lactam derivative compounds **9b** containing 4-methylphenyl group and **9f** containing 4-fluorophenyl group, and Schiff base derivatives of compounds **8c** (2-pyridinylcarbaldehyde), **8d** (4-pyridinylcarbaldeyde) and **8e** (2,6-dichlorophenyl) also showed moderate antituberculosis activity.

2.2.2. Anti-urease activity

Nearly all the newly synthesized compounds were found to have highly effective urease inhibition activity.

2.2.3. Acetylcholinesterase inhibition effects

Compounds that exhibit moderate inhibition compared to Donepezil reference drug which is an acetylcholinesterase inhibitor, are **6a, 7b, 7e, 9b, 9c, 10e** and **10f** respectively.

2.2.4. Antioxidant capacity

According to the CUPRAC method, compounds with excellent antioxidant activity results were found to have **8b**, **8c** and **8d** Schiff derivatives containing 4-methylphenyl, 3-pyridine and 4-pyridine ring respectively. The good-moderate characteristics were found to be the β-lactam derivative compounds with **10b-10f** (Table 4). The compounds carrying the phenyl group together with the β-lactam ring of compound **9a, 9b** and **9f** have antioxidant properties because they contain the phenyl ring in the structures of Schiff base **7b**, and have the potential to reduce the Cu (II) complexes.

The compounds exhibiting potent antioxidant properties by DPPH method were **2b, 8b, 8a, 8d, 8e, 10b-10e, 7b, 7c, 7e** and **9a-9c** respectively, and DPPH radical was found to be among the classes that could perform cleansing function. Among all of these compounds, compounds **3b, 7c** and **10e** that are closest to the trolox standard (0.04 ± 0.00) mg/mL have excellent antioxidant capacity with values of 0.06–0.09 mg/mL

The FRAP method is based on the reduction of Fe (III) ion in Fe (III) complex in the presence of an antioxidant. Fe (III) ions form the (Fe (III)-TPTZ-2,4,6-tris(2-pyridyl)-S-triazine complex with the ligand called TPTZ. Evaluation was made by calculating trolox equivalent antioxidant capacity in μmol TE/g. Compounds **2b, 8c, 8d, 10b, 10e, 7c, 7f, 7e, 9a** and **9e** were found to exhibit trolox equivalent antioxidant properties (Table 4).

3. Conclusion

In summary we report here the synthesis of some new kind of Schiff base and of β-lactam derivatives starting from morpholine and thiomorpholine. And all the newly synthesized compounds were screened for their antituberculosis and anti-Urease activity, acetylcholinesterase inhibition effects and antioxidant capacity. Among them compounds **3b, 7d** and **7e** showed very good antituberculosis activity, compounds **6a, 7b, 7e, 9b, 9c, 10e** and **10f** showed Acetylcholinesterase Inhibition effect, compounds **3b, 8b, 8c, 8d, 10a, 10c, 10d, 10e** and **10f** showed antioxidant capacity against standard drugs mentioned at Tables 1, 2 and 4. And also all synthesized compounds showed excellent anti-ureas activity against thiourea (see Table 3).

4. Experimental

4.1. Chemistry

All the chemicals were purchased from *FlukaChemie AG Buchs* (Switzerland) and used without further purification. Melting points of the synthesized compounds were determined in open capillaries on a Büchi B-540 melting point apparatus and are uncorrected. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 aluminium sheets. The mobile phase was ethyl acetate:diethyl ether (1:2), and detection was made using UV light. FT-IR spectra were recorded using a *Perkin Elmer* 1600 series FTIR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were registered in DMSO-d_6 on a *BRUKER AVENE II* 400 MHz NMR Spectrometer (400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C). The chemical shifts are given in ppm, *J* values are given in Hz. The Mass spectra were obtained on a *Quattro LC-MS* (70 eV) Instrument.

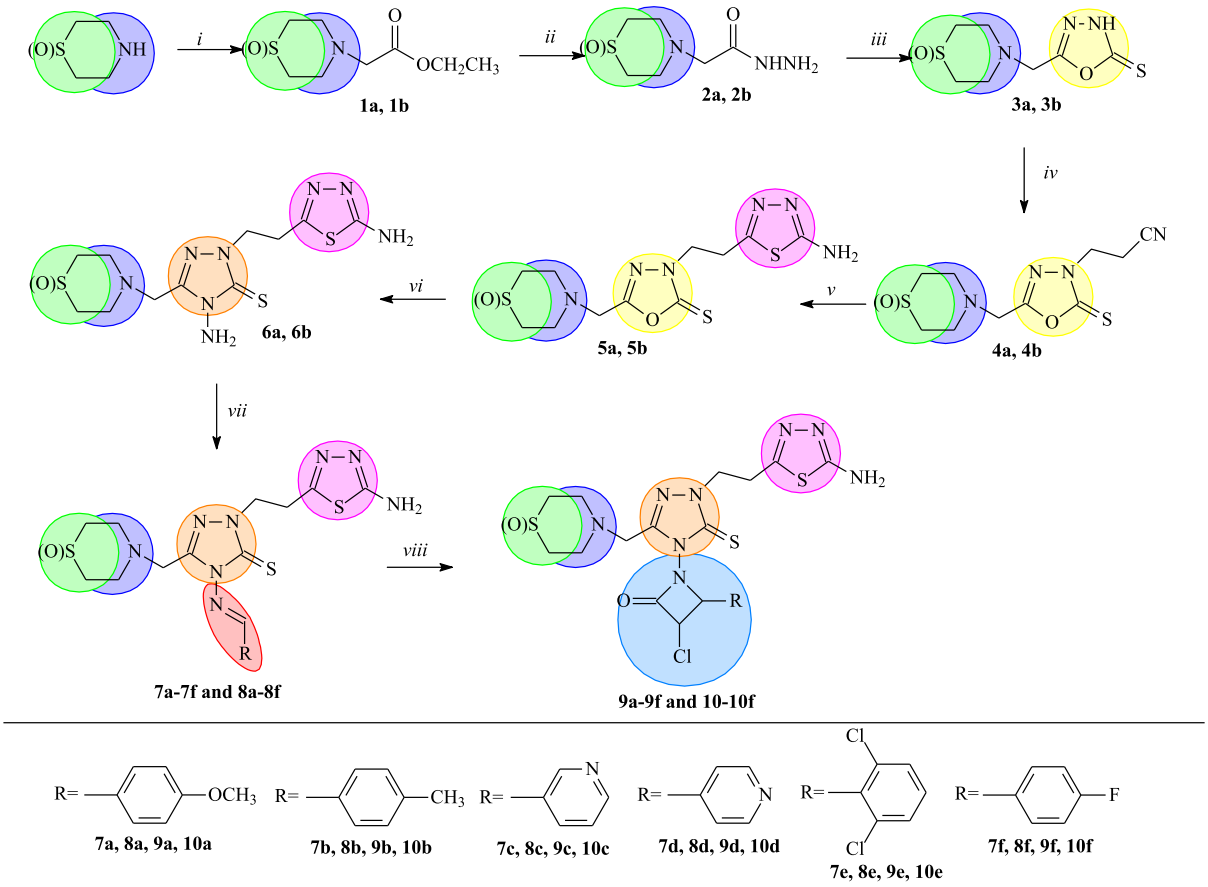

Fig. 1. *i.* $BrCH_2COOEt$, EtOH; *ii.* NH_2NH_2, EtOH; *iii.* CS_2, triethylamine, EtOH; *iv.* CH_2CHCN, EtOH; *v.* Thiosemicarbazide, TFA, NH_3; *vi.* NH_2NH_2, EtOH; *vii.* Aldehyde, MW; *viii.* $ClCH_2COOH$, dioxane, triethylamine.

Table 1
Antitubercular activity of newly synthesized compounds.

Microorganism and Minimal Inhibition Concentrations (MIC) (μg/mL)			
Comp. No	No Gram/*Ms*	Comp. No	No Gram/*Ms*
1b	15.2	**8c**	31.2
2b	7.8	**8d**	31.2
3b	7.8	**8e**	31.2
7a	31.2	**9a**	31.2
7c	15.6	**9b**	31.2
7d	7.8	**9d**	15.6
7e	7.8	**9e**	15.6
7f	15.6	**9f**	31.2
Strep.	4	**Strep.**	4

Ms: *Mycobacterium smegmatis* ATCC607, **Strep.:** Streptomycin.

Table 2
Anti-urease activity results of newly synthesized compounds.

Comp. No	Anti Urease IC_{50} (mg/mL) + SD	Comp. No	Anti Urease IC_{50} (mg/mL) + SD
2a	4.75 ± 0.01	**8c**	1.01 ± 0.02
3a	5.57 ± 0.06	**8d**	1.25 ± 0.05
3b	2.23 ± 0.02	**8e**	2.83 ± 0.06
4a	6.57 ± 0.09	**9a**	1.38 ± 0.05
6a	1.48 ± 0.03	**9b**	1.57 ± 0.09
6b	1.18 ± 0.01	**9c**	0.48 ± 0.00
7b	1.26 ± 0.02	**9e**	0.96 ± 0.00
7c	2.28 ± 0.02	**10b**	1.45 ± 0.02
7d	4.19 ± 0.09	**10c**	1.19 ± 0.06
7e	0.85 ± 0.04	**10d**	0.85 ± 0.05
7f	5.96 ± 0.02	**10f**	1.33 ± 0.03
8a	4.23 ± 0.01	**10e**	2.14 ± 0.09
8b	0.45 ± 0.07		
FP-T58	1.56 ± 0.03	**FP-T58**	1.56 ± 0.03
thiourea	12.02 ± 0.06	**thiourea**	12.02 ± 0.06

*4.2. General synthesis method for compounds **3a** and **3b***

Compound **2a/2b** (10 mmol) and CS_2 (20 mmol) were added to a solution of KOH (10 mmol) in 20 mL H_2O and 20 mL ethanol and the reaction mixture was refluxed for 13 h. Then, the reaction content was acidified with conc. HCl to pH 6. The precipitate formed was filtered off, washed with H_2O and recrystallized from ethanol to afford the desired compound.

*4.2.1. 5-(Morpholin-4-ylmethyl)-1,3,4-oxadiazol-2(3H)-thione (**3a**)*

Yield 73%, m.p: 147–149 °C. FT-IR (υ_{max}, cm^{-1}): 1587 (C=N), 1295 (C=S). ^1H NMR (DMSO-d_6, δ ppm): 3.30 (6H, s, $3CH_2$ + H_2O), 4.48 (4H, s, CH_2), 8.67 (1H, s, NH). ^{13}C NMR (DMSO-d_6, δ ppm): 55.78 (CH_2), 58.36 ($2CH_2$), 63.25 ($2CH_2$), 160.20 (oxadiazole C-2), 168.85 (oxadiazole C-5). EI MS *m/z* (%): 202.25 ($[M + 1]^+$, 100), 170.21 (85), 161.35 (63). Elemental analysis for $C_7H_{11}N_3O_2S$ calculated (%), C, 41.78; H, 5.51; N, 20.88; O, 15.90, found (%), C, 41.72; H, 5.49; N, 20.83; O, 15.86.

*4.2.2. 5-(Thiomorpholin-4-ylmethyl)-1,3,4-oxadiazol-2(3H)-thione (**3b**)*

Yield 76%, m.p: 133–135 °C. FT-IR (υ_{max}, cm^{-1}): 3318 (NH), 1570 (C=N), 1458 (C=S).

^1H NMR (DMSO-d_6, δ ppm): 2.57–2.60 (4H, m, $2CH_2$), 2.69 (4H, d, J = 4.0 Hz, $2CH_2$), 3.65 (2H, s, CH_2). NH was not observed. ^{13}C NMR (DMSO-d_6, δ ppm): 27.52 (CH_2), 52.55 ($2CH_2$), 54.18 ($2CH_2$), 161.09

Table 3
Acetylcholinesterase Inhibition results of newly synthesized compounds.

Comp. No	EeAChE Activity IC_{50} (mg/mL) + SD	Comp. No	EeAChE Activity IC_{50} (mg/mL) + SD
3b	2.32 ± 0.03	**8e**	1.13 ± 0.05
6a	0.95 ± 0.00	**9a**	5.18 ± 0.02
6b	2.45 ± 0.04	**9b**	0.87 ± 0.02
7b	0.46 ± 0.02	**9c**	0.97 ± 0.02
7c	1.28 ± 0.03	**9e**	1.19 ± 0.01
7d	4.19 ± 0.06	**10b**	2.01 ± 0.00
7e	0.75 ± 0.04	**10c**	1.25 ± 0.01
8a	6.21 ± 0.01	**10d**	1.13 ± 0.01
8b	1.73 ± 0.02	**10e**	0.95 ± 0.01
8c	2.12 ± 0.03	**10f**	0.88 ± 0.01
8d	2.86 ± 0.03		
Donepezil	0.03 ± 0.00	**Donepezil**	0.03 ± 0.00

Table 4
Comparing Antioxidant capacity of newly synthesized compounds with CUPRAC/DPPH and FRAP Methods.

Comp. No	CUPRAC (μmol TE/g)	DPPH (mg/mL) SC_{50}	FRAP (μmol TE/g)
1b	65.29 ± 2.00	–	0.473 ± 0.004
2b	4578.11 ± 29.45	0.13 ± 0.00	4.228 ± 0.014
3b	3048.61 ± 9.48	0.06 ± 0.00	4.649 ± 0.021
4b	106.18 ± 5.64	–	0.044 ± 0.003
5b	65.74 ± 5.00	–	0.096 ± 0.008
6b	2484.80 ± 17.00	1.11 ± 0.01	2.908 ± 0.012
8b	7178.35 ± 4.00	0.48 ± 0.01	3.187 ± 0.001
8a	1978.35 ± 14.00	4.29 ± 0.01	1.329 ± 0.016
8c	7265.29 ± 12.00	0.17 ± 0.01	4.878 ± 0.030
8d	8048.11 ± 16.00	0.26 ± 0.01	3.830 ± 0.080
8f	45.12 ± 1.00	–	0.386 ± 0.020
8e	678.35 ± 4.00	0.23 ± 0.01	2.478 ± 0.040
10b	4765.74 ± 5.00	0.32 ± 0.01	4.076 ± 0.008
10a	6048.68 ± 36.42	0.27 ± 0.01	2.648 ± 0.046
10c	7024.24 ± 22.10	0.52 ± 0.03	3.368 ± 0.060
10d	5106.18 ± 5.64	0.33 ± 0.02	3.044 ± 0.002
10e	6879.54 ± 23.00	0.09 ± 0.00	4.911 ± 0.007
10f	6004.45 ± 10.16	0.37 ± 0.01	2.977 ± 2.638
FP-T58	7986.12 ± 23.42	0.29 ± 0.03	4.119 ± 0.030
TROLOX		0.04 ± 0.00	

(oxadiazole C-2), 178.60 (oxadiazole C-5). Elemental analysis for $C_7H_{11}N_3OS_2$ calculated (%), C, 38.69; H, 5.10; N, 19.34; O, 7.36, found (%), C, 38.72; H, 5.15; N, 19.37; O, 7.41.

*4.3. General synthesis method for compounds **4a** and **4b***

To a solution of the compound **3a/3b** (10 mmol) in ethanol was added acrylonitrile (50 mmol) in the presence of triethylamine (50 mmol) and reaction mixture was refluxed for 6 h. After evaporating the solvent under reduced pressure, a solid obtained. The crude product was recrystallized from ethanol to afford the desired product.

*4.3.1. 3-[5-(Morpholin-4-ylmethyl)-2-thioxo-1,3,4-oxadizol-3(2H)-yl] propan nitrile (**4a**)*

Yield 78%, m.p: 115–117 °C. FT-IR (υ_{max}, cm^{-1}): 2250 (C═N), 1252 (C═S). ^{1}H NMR (DMSO-d_6, δ ppm): 1.98 (4H, s, $2CH_2$), 3.57–3.60 (6H, m, $3CH_2$), 3.68 (4H, s, $2CH_2$). ^{13}C NMR (DMSO-d_6, δ ppm): 16.12 (CH_2), 44.58 (CH_2), 51.58 (CH_2), 52.81 ($2CH_2$), 66.44 ($2CH_2$), 118.34 (C≡N), 159.76 (oxadiazole C-2), 176.61 (oxadiazole C-5). EI MS *m/z* (%): 277.13 (89), 255.17 ($[M + 1]^+$, 40), 148.99 (71), 136.04 (22), 102.13 (100). Elemental analysis for $C_{10}H_{14}N_4O_2S$ calculated (%), C, 47.23; H, 5.55; N, 22.03; O, 12.58, found (%), C, 47.17; H, 5.47; N, 21.98; O, 12.51.

*4.3.2. 3-[5-(Thiomorpholin-4-ylmethyl)-2-thioxo-1,3,4-oxadizol-3(2H)-yl]propan nitrile (**4b**)*

Yield 71%, m.p: 114–116 °C. FT-IR (υ_{max}, cm^{-1}): 2251 (C═N), 1462 (C═S). ^{1}H NMR (DMSO-d_6, δ ppm): 2.60 (4H, d, J = 4.0 Hz, $2CH_2$), 2.73 (4H, d, J = 4.0 Hz, $2CH_2$), 3.03–3.06 (2H, m, CH_2), 3.74 (2H, s, CH_2), 4.26–4.29 (2H, m, CH_2). ^{13}C NMR (DMSO-d_6, δ ppm): 16.17 (CH_2), 27.46 (CH_2), 44.61 (CH_2), 52.43 ($2CH_2$), 54.96 ($2CH_2$), 118.30 (C≡N), 159.89 (oxadiazole C-2), 176.73 (oxadiazole C-5). EI MS *m/z* (%): 271.01 ($[M + 1]^+$, 154.23 (100). Elemental analysis for $C_{10}H_{14}N_4OS_2$ calculated (%), C, 44.42; H, 5.22; N, 20.72; O, 5.92, found (%), C, 44.47; H, 5.25; N, 20.74; O, 5.94.

*4.4. General synthesis method for compounds **5a** and **5b***

A mixture of the compound **4a/4b** (10 mmol) and thiosemicarbazide (10 mmol) were combined in ethanol in the presence of trifluoroacetic acid (5 mL) in an oil bath and then was refluxed for 9 h. Then the reaction content was neutralized with diluted NH_3 to pH 6. Water was decanted from the mixture and the remaining fatty fraction was treated with ether to solidify the crude product. The resulting solid was filtered and purified by crystallization from acetone.

*4.4.1. 3-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(morpholin-4-ylmethyl)-1,3,4-oxadiazol-2(3H)-thione (**5a**)*

Yield 53%, m.p: 121–123 °C. FT-IR (υ_{max}, cm^{-1}): 3280 (NH_2), 3092 (NH), 1527(C═N), 1298 (C═S). ^{1}H NMR (DMSO-d_6, δ ppm): 2.42 (2H, s, CH_2), 2.48 (2H, s, CH_2), 3.27–3.30 (2H, m, CH_2), 3.55 (4H, s, $H_2O + 2CH_2$), 3.63 (2H, s, CH_2), 4.27–4.30 (2H, s, CH_2), 7.06 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, δ ppm): 27.57 (CH_2), 47.92 (CH_2), 51.81 (CH_2), 52.82 ($2CH_2$), 66.43 ($2CH_2$), 153.88 (thiadiazole C-3), 159.36 (thiadiazole C-5), 169.28 (oxadiazole C-2), 176.54 (oxadiazole C-5). EI MS *m/z* (%): 329.25 ($[M + 1]^+$, 100), 245.22 (22), 171.13 (31), 128.05 (42). Elemental analysis for $C_{11}H_{16}N_6O_2S_2$ calculated (%), C, 40.23; H, 4.91; N, 25.59; O, 9.74, found (%), C, 40.29; H, 4.95; N, 25.57; O, 9.78.

*4.4.2. 3-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(thiomorpholin-4-ylmethyl)-1,3,4-oxadiazol-2(3H)-thione (**5b**)*

Yield 62%, m.p: 146–148 °C. FT-IR (υ_{max}, cm^{-1}): 3368 (NH_2), 1465 (C═S). ^{1}H NMR (DMSO-d_6, δ ppm): 2.02 (2H, s, CH_2), 2.78 (4H, s, $2CH_2$), 3.00 (4H, s, $2CH_2$), 3.18 (2H, s, CH_2), 3.78 (2H, s, CH_2), 7.20 (2H, brs, NH_2). ^{13}C NMR (DMSO-d_6, δ ppm): 28.12 (CH_2), 48.12 ($2CH_2$), 50.12 ($2CH_2$), 53.96 (CH_2), 55.30 (CH_2), 155.10 (thiadiazole C-3), 158.30 (thiadiazole C-5), 168.52 (oxadiazole C-2), 177.18 (oxadiazole C-5). EI MS *m/z* (%): 345.20 ($[M + 1]^+$, 100), 278.12 (85), 260.10 (76), 178.96 (55). Elemental analysis for $C_{11}H_{16}N_6OS_3$ calculated (%), C, 38.36; H, 4.68; N, 24.40; O, 4.64, found (%), C, 38.39; H, 4.73; N, 24.34; O, 4.69.

*4.5. Genaral synthesis method for compounds **6a** and **6b***

To the solution of compound **5a/5b** (10 mmol) in absolute ethanol hydrazinehydrate (25 mmol) was added and the reaction was refluxed for 10 h. Evaporation of the solvent under reduced pressure gave the oily substance solidified by treatment with *n*-butylacetate/diethylether. After standing overnight in cold, the precipitated solid was filtered off and purified by crystallization from ethanol.

*4.5.1. 4-Amino-2-[2-(5-amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (**6a**)*

Yield 68%, m.p: 168–170 °C. FT-IR (υ_{max}, cm^{-1}): 3239 ve 3133 (NH_2), 1571 (C═N), 1326 (C═S). ^{1}H NMR (DMSO-d_6, δ ppm): 2.40 (4H, s, $2CH_2$), 3.27–3.35 (4H, m, $2CH_2$), 3.54 (4H, d, J = 12.0 Hz, $H_2O + 2CH_2$), 4.34–4.37 (2H, m, CH_2), 5.63 (2H, s, NH_2), 7.02 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, δ ppm): 28.31 (CH_2), 48.29 (CH_2), 50.98 (CH_2), 53.16 ($2CH_2$), 66.46 ($2CH_2$), 147.92 (thiadiazole C-3), 154.47 (thiadiazole C-5), 165.82 (oxadiazole C-2), 169.18 (oxadiazole C-5). EI

MS *m/z* (%): 343.20 ([M + 1]$^+$, 100), 216.06 (32), 200.11 (82), 100.13 (65). Elemental analysis for $C_{11}H_{18}N_8OS_2$ calculated (%), C, 38.58; H, 5.30; N, 32.72; O, 4.67, found (%), C, 38.52; H, 5.22; N, 32.69; O, 4.69.

4.5.2. 4-Amino-2-[2-(5-amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(thiomorpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (6b)

Yield 67%, m.p: 127–129 °C. FT-IR (υ_{max}, cm^{-1}): 3302 (NH_2), 3109 (NH_2), 1494 (C=S). ^{1}H NMR (DMSO-d_6, δ ppm): 2.10 (2H, s, CH_2), 2.65 (4H, s, $2CH_2$), 2.78 (4H, s, $2CH_2$), 3.10 (2H, s, CH_2), 3.28 (2H, s, CH_2), 6.12 (2H, s, NH_2), 7.35 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, δ ppm): 29.10 (CH_2), 49.63 (CH_2), 51.34 (CH_2), 57.30 ($2CH_2$), 65.40 ($2CH_2$), 146.89 (thiadiazole C-3), 153.20 (thiadiazole C-5), 161.20 (oxadiazole C-2), 168.83 (oxadiazole C-5). EI MS *m/z* (%): 359.60 ([M + 1]$^+$, 100), 200.69 (85), 218.54 (62), 107.67 (40). Elemental analysis for $C_{11}H_{18}N_8S_3$ calculated (%), C, 36.85; H, 5.06; N, 31.26, found (%), C, 36.89; H, 5.12; N, 31.32.

4.6. Genaral synthesis method for compounds 7a-7f and 8a-8f

To a solution of compound **6a** (for compounds **7a-7f**) or **6b** (for compounds **8a-8f**) (10 mmol) and corresponding aromatic aldehyde (20 mmol) was refluxed for 2 h at 125 °C in an oil bath. After completion of the reaction, pure water was added to the flask and exracted with dichloromethane (1:3). Organic phase was dried with Na_2SO_4. After filtration off the solvent was evaporated under reduced pressure, a solid obtained. The crude product was recrystallized from an appropriate solvent (Table 1) to afford the desired product.

4.6.1. 2-[(5-Amino-1,3,4-thiadiazol-2-yl)methyl]-4-{(4-metoxyphenyl) methyleneamino}-5-(morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (7a)

Yield 77%. FT-IR (υ_{max}, cm^{-1}): 3221 (NH_2), 3058 (aromatic CH), 1583 (C=N), 1245 (C=S). ^{1}H NMR (DMSO-d_6, δ ppm): 3.08 (4H, s, $2CH_2$), 3.31 (2H, d, J = 8.0 Hz, CH_2), 3.41 (2H, d, J = 4.0 Hz, CH_2), 3.78 (3H, d, J = 12.0 Hz, CH_3), 4.30–4.41 (6H, m, $3CH_2$), 7.03 (2H, d, J = 8.0 Hz, arH), 7.45 (2H, brs, NH_2), 7.79 (2H, d, J = 8.0 Hz, arH), 8.60 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 28.30 (CH_2), 48.48 (CH_2), 48.76 (CH_2), 52.22 (CH_2), 55.84 (CH_3), 56.49 (CH_2), 64.30 ($2CH_2$), 114.84 (CH), arC: [126.96 (C), 130.44 (2CH), 144.12 (C), 160.95 (2CH)], 154.81 (thiadiazole C-3), 162.11 (thiadiazole C-5), 166.72 (triazole C-3), 169.48 (triazole C-5).). EI MS *m/z* (%): 460.60 ([M]$^+$, 95), 169.12 (100), 103.05 (25). Elemental analysis for $C_{19}H_{24}N_8O_2S_2$ calculated (%), C, 49.55; H, 5.25; N, 24.33; O, 6.95, found (%), C, 49.51; H, 5.23; N, 24.29; O, 6.92.

4.6.2. 2-[(5-Amino-1,3,4-thiadiazol-2-yl)methyl]-4-{(4-methylphenyl) methylenamino}-5 (morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (7b)

Yield 68%. FT-IR (υ_{max}, cm^{-1}): 3217 (NH_2), 3053 (aromatic CH), 1582 (C=N), 1284 (C=S). ^{1}H NMR (DMSO-d_6, δ ppm): 2.36 (3H, d, J = 12.0 Hz, CH_3), 2.94 (2H, s, CH_2), 3.07 (2H, s, CH_2), 3.70 (2H, s, CH_2), 3.78 (2H, s, CH_2), 4.20 (2H, s, CH_2), 4.28 (2H, s, CH_2), 4.46 (2H, d, J = 4.0 Hz, CH_2), 7.36–7.42 (4H, m, arH), 7.83 (2H, d, J = 8.0 Hz, NH_2), 9.87 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 21.76 (CH_3), 28.18 (CH_2), 47.98 ($2CH_2$), 52.51 ($2CH_2$), 65.14 ($2CH_2$), 129.36 (CH), arC: [129.62 (2CH), 130.24 (2CH), 131.12 (C), 131.98 (C)], 143.85 (thiadiazole C-3), 157.23 (thiadiazole C-5), 161.53 (triazole C-3), 169.39 (triazole C-5). EI MS *m/z* (%): 470.02 ([M + Na]$^+$, 35), 447.56 ([M + 2]$^+$, 84), 315.12 (100). Elemental analysis for $C_{19}H_{24}N_8OS_2$ calculated (%), C, 51.33; H, 5.44; N, 25.20; O, 3.60, found (%), C, 51.32; H, 5.46; N, 25.22; O, 3.63.

4.6.3. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(morpholin-4-ylmethyl)-4-{[pyridin-3-ylmethylene]amino}-2,4-dihydro-3H-1,2,4-triazol-3-thione (7c)

Yield 71%. FT-IR (υ_{max}, cm^{-1}): 3317 (NH_2), 3060 (aromatic CH), 1578 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.17–1.21 (8H, m, $4CH_2$), 3.05–3.08 (6H, m, $3CH_2$), 6.92 (2H, d, J = 8.0 Hz, NH_2), 7.20–7.25 (2H, m, arH), 7.43 (2H, s, arH), 9.69 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 28.61 (CH_2), 45.99 (CH_2), 47.40 (CH_2), 47.83 (CH_2), 48.20 (CH_2), 51.45 (CH_2), 61.83 (CH_2), 114.90 (CH), arC: [116.20 (CH), 116.44 (CH), 123.26 (C), 124.22 (CH), 124.96 (CH)], 141.95 (thiadiazole C-3), 143.16 (triazole C-3), 161.99 (thiadiazole C-5), 167.09 (triazole C-5). EI MS *m/z* (%): 432.67 ([M + 1]$^+$, 65), 414.32 ([M-H_2O]$^+$, 77), 309.00 (100). Elemental analysis for $C_{17}H_{21}N_9OS_2$ calculated (%), C, 47.32; H, 4.91; N, 29.21; O, 3.71, found (%), C, 47.27; H, 4.88; N, 29.22; O, 3.67.

4.6.4. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(morpholin-4-ylmethyl)-4-{[pyridin-4-ylmethylene]amino}-2,4-dihydro-3H-1,2,4-triazol-3-thione (7d)

Yield 78%. FT-IR (υ_{max}, cm^{-1}): 3331 (NH_2), 3058 (aromatic CH), 1587 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.89–1.93 (8H, m, $4CH_2$), 2.78–2.85 (6H, m, $3CH_2$), 6.07 (2H, s, NH_2), 7.25–7.30 (2H, m, arH), 7.45–7.52 (2H, m, arH), 8.90 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 41.95 (CH_2), 45.97 (CH_2), 47.34 (CH_2), 47.81 (CH_2), 48.20 (CH_2), 51.30 (CH_2), 61.88 (CH_2), 114.88 (CH), arC: [116.03 (CH), 116.24 (CH), 124.02 (CH), 124.90 (CH), 129.28 (C)], 151.47 (thiadiazole C-3), 152.63 (triazole C-3), 154.73 (thiadiazole C-5), 155.49 (triazole C-5). EI MS *m/z* (%): 433.53 ([M + 2]$^+$, 70), 415.72 ([M-H_2O]$^+$, 68), 310.02 (100). Elemental analysis for $C_{17}H_{21}N_9OS_2$ calculated (%), C, 47.32; H, 4.91; N, 29.21; O, 3.71, found (%), C, 47.28; H, 4.88; N, 29.23; O, 3.68.

4.6.5. 2-[(5-Amino-1,3,4-thiadiazol-2-yl)methyl]-4-{(2,6-dichlorophenyl) methylene amino}-5-(morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (7e)

Yield 72%. FT-IR (υ_{max}, cm^{-1}): 3178 (NH_2), 3065 (aromatic CH), 1578 (C=N), 1239 (C=S). ^{1}H NMR (DMSO-d_6, δ ppm): 3.09 (2H, s, CH_2), 3.19 (2H, s, CH_2), 3.72–3.79 (4H, m, $2CH_2$), 4.35–4.50 (6H, m, $3CH_2$), 5.92 (2H, s, NH_2), 7.41–7.67 (3H, m, arH), 10.89 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 48.43 (CH_2), 52.13 (CH_2), 52.46 (CH_2), 56.47 (CH_2), 61.71 (CH_2), 64.01 (CH_2), 64.53 (CH_2), 130.30 (CH), arC: [128.47 (C), 133.73 (2CH), 135.25 (C), 143.63 (C), 156.96 (C)], 154.92 (thiadiazole C-3), 161.14 (thiadiazole C-5), 166.93 (triazole C-3), 169.61 (triazole C-5). EI MS *m/z* (%): 517.25 ([M + H_2O]$^+$, 100), 497.23 ([M-2]$^+$, 67). Elemental analysis for $C_{18}H_{20}N_8OS_2Cl_2$ calculated (%), C, 43.29; H, 4.04; N, 22.44; O, 3.20, found (%), C, 43.33; H, 4.00; N, 22.39; O, 3.17.

4.6.6. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-4-{[(4-fluorophenyl) methylene]amino}-5-(morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (7f)

Yield 65%. FT-IR (υ_{max}, cm^{-1}): 3299 (NH_2), 3188 (aromatic CH), 1510 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.42 (2H, s, CH_2), 2.14 (2H, s, CH_2), 2.22 (2H, s, CH_2), 3.24 (4H, s, $2CH_2$), 3.74–3.82 (4H, m, $2CH_2$), 7.28–7.30 (3H, m, arH), 7.50–7.53 (1H, m, arH), 7.78 (2H, s, NH_2), 8.74 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 28.51 (CH_2), 45.21 (CH_2), 48.35 (CH_2), 51.77 (CH_2), 52.15 (CH_2), 56.49 (CH_2), 61.75 (CH_2), 127.50 (CH), arC: [116.18 ve 116.39 (2CH, d, J = 21.0 Hz), 130.88 ve 130.97 (2CH, d, J = 9.0 Hz), 143.49 ve 153.30 (C_F, d, J = 981.0 Hz), 161.70 (C)], 164.21 (triazole C-3), 167.21 (triazole C-5), 169.91 (thiadiazole C-3), 180.17 (thiadiazole C-5). EI MS *m/z* (%): 429.50 [M-F]$^+$, 23), 312.70 (100), 223.12 (67). Elemental analysis for $C_{18}H_{21}N_8OS_2F$ calculated (%), C, 48.20; H, 4.72; N, 24.98; O, 3.57, found (%), C, 48.25; H, 4.74; N, 24.94; O, 3.53.

4.6.7. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-4-{[(4-methoxyphenyl) methylene] amino}-5-(thiomorpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (8a)

Yield 71%. FT-IR (υmax, cm^{-1}): 3298 (NH_2), 3065 (aromatic CH), 1577 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.04–1.07 (2H, m, CH_2), 2.82 (4H, s, $2CH_2$), 3.18 (4H, s, $2CH_2$), 3.44 (3H, s, CH_3), 4.20 (2H, s, CH_2),

4.43 (2H, s, CH_2), 7.05 (2H, d, J = 8.0 Hz, arH), 7.81 (2H, d, J = 8.0 Hz, arH), 7.98 (2H, brs, NH_2), 8.63 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 25.71 (CH_2), 29.33 (CH_2), 48.49 (CH_2), 49.75 (CH_2), 54.16 (CH_2), 55.86 (CH_3), 56.75 (CH_2), 114.86 (CH), arC: [124.73 (C), 127.03 (C), 130.45 (CH), 131.40 (CH), 142.99 (CH), 146.02 (CH)], 154.08 (triazole C-3), 161.62 (triazole C-5), 162.14 (thiadiazole C-3), 163.59 (thiadiazole C-5). EI MS m/z (%): 503.15 (55), 477.02 ([M]$^+$, 63), 402.45 (100). Elemental analysis for $C_{19}H_{24}N_8OS_3$ calculated (%), C, 47.88; H, 5.08; N, 23.51; O, 3.36, found (%), C, 47.92; H, 5.13; N, 23.52; O, 3.37.

*4.6.8. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-4-{[(4-methylphenyl) methylene amino}-5-(thiomorpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (**8b**)*

Yield 80%. FT-IR (υ_{max}, cm^{-1}): 3189 (NH_2), 3063 (aromatic CH), 1580 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.04–1.08 (3H, m, CH_3), 2.28 (6H, s, 3CH_2), 2.36 (4H, s, 2CH_2), 3.45 (4H, s, 2CH_2), 7.14 (2H, d, J = 8.0 Hz, NH_2), 7.28–7.32 (2H, m, arH), 7.38–7.78 (2H, m, arH), 8.65 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 19.01 (CH_3), 27.30 (CH_2), 28.52 (CH_2), 48.31 (CH_2), 54.33 (CH_2), 56.50 (CH_2), 58.29 (CH_2), 61.73 (CH_2), 125.71 (CH), arC: [128.79 (CH), 129.56 (CH), 129.99 (CH), 131.66 (C), 133.98 (C), 139.97 (CH)], 133.37 (triazole C-3), 141.81 (thiadiazole C-3), 161.68 (triazole C-5), 167.78 (thiadiazole C-5). EI MS m/z (%): 462.71 ([M + 2]$^+$, 82), 442.60 (([M-H_2O-1]$^+$, 54), 412.20 (100). Elemental analysis for $C_{19}H_{24}N_8S_3$ calculated (%), C, 49.54; H, 5.25; N, 24.33, found (%), C, 49.50; H, 5.25; N, 24.32.

*4.6.9. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-4-{[pyridin-3-ylmethylene]amino}-5-(thiomorpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (**8c**)*

Yield 73%. FT-IR (υ_{max}, cm^{-1}): 3210 (NH_2), 3051 (aromatic CH), 1583 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 2.63 (2H, d, J = 4.0 Hz, CH_2), 2.79 (2H, s, CH_2), 3.42–3.47 (4H, m, 2CH_2), 3.74–3.80 (4H, m, 2CH_2), 4.41 (2H, d, J = 8.0 Hz, CH_2), 7.35–7.38 (2H, m, NH_2), 7.52–7.55 (1H, m, arH), 7.73 (1H, s, arH), 7.89 (1H, d, J = 8.0 Hz, arH), 8.26 (1H, d, J = 8.0 Hz, arH), 9.03 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 28.33 (CH_2), 48.35 (CH_2), 51.13 (CH_2), 54.39 (CH_2), 56.52 (CH_2), 61.58 (CH_2), 61.84 (CH_2), arC: [124.33 (CH), 124.58 (CH), 129.10 (C), 132.53 (CH), 134.89 (CH), 135.38 (CH)], 146.66 (thiadiazole C-3), 147.96 (triazole C-3), 150.37 (thiadiazole C-5), 152.44 (triazole C-5). EI MS m/z (%): 449.70 ([M + 2]$^+$, 51), 425.32 ([M-Na + 1]$^+$, 34), 400.01 (100). Elemental analysis for $C_{17}H_{21}N_9S_3$ calculated (%), C, 45.62; H, 4.73; N, 28.16, found (%), C, 45.59; H, 4.75; N, 28.09.

*4.6.10. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-5-(morpholin-4-ylmethyl)-4-{[pyridin-4-ylmethylene]amino}-2,4-dihydro-3H-1,2,4-triazol-3-thione (**8d**)*

Yield 81%. FT-IR (υ_{max}, cm^{-1}): 3310 (NH_2), 3067 (aromatic CH), 1582 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.76 (4H, s, 2CH_2), 2.15 (4H, s, 2CH_2), 3.39 (4H, s, 2CH_2), 3.75 (2H, d, J = 8.0 Hz, CH_2), 5.46 (2H, s, NH_2), 7.63–7.65 (2H, m, arH), 7.97–7.99 (2H, m, arH), 10.90 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 19.06 (CH_2), 24.48 (CH_2), 30.63 (CH_2), 40.63 (CH_2), 42.75 (CH_2), 63.05 (CH_2), 65.37 (CH_2), 111.93 (CH), arC: [110.75 (C), 118.94 (CH), 119.04 (CH), 121.57 (CH), 123.80 (CH)], 145.41 (thiadiazole C-3), 153.56 (triazole C-3), 159.64 (thiadiazole C-5), 178.54 (triazole C-5). EI MS m/z (%): 449.53 ([M + 2]$^+$, 49), 423.20 ([M-Na + 3]$^+$, 42), 405.22 (100). Elemental analysis for $C_{17}H_{21}N_9S_3$ calculated (%), C, 45.62; H, 4.73; N, 28.16, found (%), C, 45.60; H, 4.76; N, 28.12.

*4.6.11. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-4-{[(2,6-dichlorophenyl)methylene] amino}-5-(thiomorpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (**8e**)*

Yield 79%, m.p: 152–154 °C. FT-IR (υ_{max}, cm^{-1}): 3215 (NH_2), 3057 (aromatic CH), 1581 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.10–1.14 (2H, m, CH_2), 2.59–2.61 (2H, m, CH_2), 2.70 (2H, d, J = 8.0 Hz, CH_2), 3.32 (2H, s, CH_2), 3.63 (2H, s, CH_2), 3.77 (2H, d, J = 8.0 Hz, CH_2), 4.38–4.41 (2H, m, CH_2), 5.66 (2H, s, NH_2), 7.23–7.45 (3H, m, arH), 8.84 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 48.31 (CH_2), 51.43 (CH_2), 54.46 (CH_2), 58.72 (CH_2), 61.74 (CH_2), 63.68 (CH_2), 66.82 (CH_2), arC: [129.36 (CH), 129.42 (CH), 131.61 (C), 132.29 (C), 132.33 (2CH), 133.61 (C)], 147.98 (thiadiazole C-3), 154.49 (triazole C-3), 165.84 (thiadiazole C-5), 169.21 (triazole C-5). EI MS m/z (%): 533.53 ([M + H_2O]$^+$, 77), 500.11 (100). Elemental analysis for $C_{18}H_{20}N_8S_3Cl_2$ calculated (%), C, 41.94; H, 3.91; N, 21.74, found (%), C, 41.96; H, 3.95; N, 21.71.

*4.6.12. 2-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-4-{[(4-fluorophenyl) methylene]amino}-5-(thiomorpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazol-3-thione (**8f**)*

Yield 85%, m.p: 145–147 °C. FT-IR (υ_{max}, cm^{-1}): 3278 (NH_2), 3065 (aromatic CH), 1578 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 2.72 (6H, s, 3CH_2), 3.65 (4H, s, 2CH_2), 3.72 (4H, s, 2CH_2), 5.64 (2H, s, NH_2), 7.02 (2H, s, arH), 7.39–7.44 (1H, m, arH), 8.00–8.03 (1H, m, arH), 9.82 (1H, s, CH). ^{13}C NMR (DMSO-d_6, δ ppm): 27.61 (CH_2), 28.34 (CH_2), 47.96 (CH_2), 48.31 (CH_2), 51.29 (CH_2), 51.74 (CH_2), 54.15 (CH_2), arC: [116.42 ve 116.64 (CH, d, J = 22.0 Hz), 116.63 ve 117.85 (CH, d, J = 122.0 Hz), 129.11 ve 129.14 (C, d, J = 3.0 Hz), 131.11 ve 131.20 (CH, d, J = 9.0 Hz), 131.72 ve 131.81 (CH, d, J = 9.0 Hz), 154.39 ve 154.48 (C, d, J = 9.0 Hz), 164.24 (CH)], 146.70 (thiadiazole C-3), 147.92 (triazole C-3), 161.49 (thiadiazole C-5), 163.99 (triazole C-5). EI MS m/z (%): 467.52 ([M + 3]$^+$, 100). Elemental analysis for $C_{18}H_{21}N_8S_3F$ calculated (%), C, 46.53; H, 4.56; N, 24.12, found (%), C, 46.56; H, 4.51; N, 24.15.

*4.7. General synthesis method for compounds **9a-9f** and **10a-10f***

A mixture of compound **7** (for compound **9a-9f**) or **8** (for compound **10a-10f**) (10 mmol), triethylamine (5 mmol) and chloroacetyl chloride (5 mmol) were combined in dioxane and stirred in an ice bath (0–5 °C) for 6 h. The reaction mixture was poured into ice-water, the resulting solid was filtered, dried and crystallized from an appropriate solvent.

*4.7.1. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(morpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(4-methoxyphenyl) azetidin-2-one (**9a**)*

Yield 75%. FT-IR (υ_{max}, cm^{-1}): 3317 (NH_2), 1715 (C=O), 1572 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.19–1.23 (8H, m, 4CH_2), 3.03–3.09 (3H, m, 3CH_2), 3.41 (3H, s, −OCH_3), 3.83 (1H, s, CH), 4.27 (1H, s, CH), 7.06 (2H, s, NH_2), 7.22–7.83 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 47.25 (CH_2), 48.96 (CH_2), 49.10 (CH_2), 50.85 (CH_2), 52.63 (CH_2), 51.25 (CH_3), 53.45 (CH_2), 55.21 (CH_2), 68.52 (CH), 69.71 (CH), arC: [125.85 (CH), 127.63 (CH), 128.96 (CH), 130.53 (CH), 139.63 (C), 140.27 (C)], 148.63 (thiadiazole C-3), 150.41 (triazole C-3), 155.32 (thiadiazole C-5), 158.10 (triazole C-5), 169.17 (C=O). EI MS m/z (%): 536.00 ([M − 1]$^+$, 66), 517.28 ([M-H_2O]$^+$, 36), 322.10 (100). Elemental analysis for $C_{21}H_{25}N_8O_3S_2Cl$ calculated (%), C, 46.97; H, 4.69; N, 20.86; O, 8.94, found (%), C, 47.01; H, 4.71; N, 20.83; O, 8.95.

*4.7.2. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(morpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(4-methylphenyl)azetidin-2-one (**9b**)*

Yield 70%. FT-IR (υ_{max}, cm^{-1}): 3210 (NH_2), 1710 (C=O), 1576 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.41 (3H, d, J = 8.0 Hz, CH_3), 2.15 (2H, s, CH_2), 2.49–2.51 (2H, m, CH_2), 2.73 (2H, s, CH_2), 2.89 (4H, s, 2CH_2), 3.35 (6H, s, 3CH_2), 4.59 (1H, s, CH), 5.16 (1H, s, CH), 6.16 (2H, s, NH_2), 7.57–7.59 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 14.45 (CH_3), 30.35 (CH_2), 33.74 (CH_2), 45.99 (CH_2), 50.80 (CH_2), 51.95 (CH_2), 61.27 (CH_3), 66.83 (CH_2), 101.28 (CH), 108.80 (CH), arC: [117.37 (CH), 121.15 (CH), 129.47 (CH), 129.78 (CH), 141.67 (C),

145.65 (C)], 156.15 (thiadiazole C-3), 158.73 (triazole C-3), 160.42 (thiadiazole C-5), 168.82 (triazole C-5), 169.63 (C=O). EI MS *m/z* (%): 521.00 ($[M]^+$, 98), 489.15 (100). Elemental analysis for $C_{21}H_{25}N_8O_2S_2Cl$ calculated (%), C, 48.41; H, 4.84; N, 21.51; O, 6.14, found (%), C, 48.41; H, 4.86; N, 21.48; O, 6.17.

*4.7.3. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(morpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-pyridin-3-ylazetidin-2-one (**9c**)*

Yield 84%. FT-IR (υ_{max}, cm^{-1}): 3218 (NH_2), 1726 (C=O), 1585 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.19–1.22 (8H, m, $4CH_2$), 3.02–3.08 (6H, m, $3CH_2$), 4.27 (1H, s, CH), 4.68 (1H, s, CH), 6.00 (2H, s, NH_2), 7.81–7.84 (1H, m, arH), 8.46 (1H, d, J = 8.0 Hz, arH), 8.94–8.96 (2H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 41.96 (CH_2), 45.79 (CH_2), 56.47 (CH_2), 63.52 ($2CH_2$), 66.81 ($2CH_2$), 87.23 (2CH), arC: [127.97 (CH), 127.41 (CH), 133.54 (C), 139.22 (CH), 144.24 (CH)], 148.77 (thiadiazole C-3), 149.47 (triazole C-3), 152.16 (thiadiazole C-5), 168.99 (triazole C-5), 192.05 (C=O). EI MS *m/z* (%): 508.00 ($[M]^+$, 74), 432.15 (100). Elemental analysis for $C_{19}H_{22}N_9O_2S_2Cl$ calculated (%), C, 44.92; H, 4.36; N, 24.81; O, 6.30, found (%), C, 44.86; H, 4.31; N, 24.83; O, 6.33.

*4.7.4. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(morpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-pyridin-4-ylazetidin-2-one (**9d**)*

Yield 71%. FT-IR (υ_{max}, cm^{-1}): 3320 (NH_2), 1710 (C=O), 1585 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.19–1.22 (8H, m, $4CH_2$), 3.03–3.08 (6H, m, $3CH_2$), 3.95 (1H, s, CH), 4.27 (1H, s, CH), 6.07 (2H, s, NH_2), 7.97–8.95 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 47.20 (CH_2), 48.62 (CH_2), 49.41 (CH_2), 50.78 (CH_2), 52.63 (CH_2), 53.85 (CH_2), 55.67 (CH_2), 68.21 (CH), 70.14 (CH), arC: [127.32 (CH), 129.21 (CH), 130.12 (CH), 131.87 (CH), 139.21 (C)], 149.21 (thiadiazole C-3), 150.17 (triazole C-3), 155.24 (thiadiazole C-5), 157.31 (triazole C-5), 169.21 (C=O). EI MS *m/z* (%): 508.02 ($[M]^+$, 74), 432.07 (100). Elemental analysis for $C_{19}H_{22}N_9O_2S_2Cl$ calculated (%), C, 44.92; H, 4.36; N, 24.81; O, 6.30, found (%), C, 44.84; H, 4.32; N, 24.82; O, 6.35.

*4.7.5. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(thiomorpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(2,6-dichlorophenyl)azetidin-2-one (**9e**)*

Yield 79%. FT-IR (υ_{max}, cm^{-1}): 3178 (NH_2), 1723 (C=O), 1586 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.03–1.07 (4H, m, $2CH_2$), 1.18–1.21 (4H, m, $2CH_2$), 3.04–3.07 (4H, m, $2CH_2$), 3.43 (2H, d, J = 4.0 Hz, CH_2), 4.15 (1H, s, CH), 4.58 (1H, s, CH), 7.55 (2H, d, J = 4.0 Hz, NH_2), 8.31–8.34 (3H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 18.93 (CH_2), 41.24 (CH_2), 41.92 (CH_2), 45.94 ($2CH_2$), 56.49 ($2CH_2$), 61.83 (CH), 66.82 (CH), arC: [129.85 (CH), 131.87 (CH), 134.39 (C), 139.68 (CH), 142.31 (2C)], 143.93 (triazole C-3), 165.19 (thiadiazole C-3), 167.20 (triazole C-5), 168.13 (thiadiazole C-5). EI MS *m/z* (%): 594.82 ($[M + K]^+$, 33), 576.14 ($[M + 1]^+$, 73), 521.21 (100). Elemental analysis for $C_{20}H_{21}N_8O_2S_2Cl_3$ calculated (%), C, 41.71; H, 3.68; N, 19.46; O, 5.56, found (%), C, 41.72; H, 3.71; N, 19.41; O, 5.61.

*4.7.6. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(morpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(4-fluorophenyl)azetidin-2-one (**9f**)*

Yield 72%. FT-IR (υ_{max}, cm^{-1}): 3218 (NH_2), 1723 (C=O), 1583 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.18–1.22 (8H, m, $4CH_2$), 3.02–3.09 (6H, m, $3CH_2$), 3.56 (1H, s, CH), 4.27 (1H, s, CH), 6.08 (2H, brs, NH_2), 7.20–7.45 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 41.95 (CH_2), 45.89 (CH_2), 51.74 (CH_2), 59.95 (CH_2), 61.77 (CH_2), 63.51 (CH_2), 66.81 (CH_2), 72.14 (CH), 74.28 (CH), arC: [116.38 ve 116.60 (CH, d, J = 22.0 Hz), 125.98 (C), 127.65 (CH), 131.15 ve 131.31 (CH, d, J = 16.0 Hz), 140.78 (C), 142.08 (C)], 146.12 (thiadiazole C-3), 147.20 (triazole C-3), 150.30 (thiadiazole C-5), 159.79 (triazole C-5), 169.01 (C=O). EI MS *m/z* (%): 523.00 ($[M - 2]^+$, 87), 499.01 (100). Elemental analysis for $C_{20}H_{22}N_8O_2S_2ClF$ calculated (%), C, 45.75; H, 4.22; N, 21.34; O, 6.09, found (%), C, 45.72; H, 4.26; N, 21.39; O, 6.15.

*4.7.7. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(thiomorpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(4-methoxyphenyl)azetidin-2-one (**10a**)*

Yield 75%. FT-IR (υ_{max}, cm^{-1}): 3310 (NH_2), 1712 (C=O), 1582 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.15 (2H, s, CH_2), 1.31 (2H, s, CH_2), 1.77 (2H, s, CH_2), 2.67 (2H, s, CH_2), 2.73 (2H, s, CH_2), 2.82 (2H, s, CH_2), 2.88 (2H, s, CH_2), 2.93 (3H, s, CH_3), 4.90 (1H, s, CH), 5.21 (1H, s, CH), 5.48 (2H, s, NH_2), 7.05–7.48 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 15.54 (CH_3), 43.18 (CH_2), 46.97 (CH_2), 48.15 (CH_2), 51.13 (CH_2), 51.48 (CH_2), 51.95 (CH_2), 6.29 (CH_2), 67.79 (CH), 78.21 (CH), arC: [101.33 (CH), 108.71 (CH), 114.04 (CH), 120.87 (CH), 132.41 (C), 133.08 (C)], 135.83 (triazole C-3), 140.28 (thiadiazole C-3), 151.06 (triazole C-5), 154.04 (thiadiazole C-5), 157.87 (C=O). EI MS *m/z* (%): 553.01 ($[M]^+$, 42), 500.11 (100). Elemental analysis for $C_{21}H_{25}N_8O_2S_3Cl$ calculated (%), C, 45.60; H, 4.56; N, 20.26; O, 5.79, found (%), C, 45.62; H, 4.61; N, 20.21; O, 5.84.

*4.7.8. 1-[1-[2-(5-Amino-1,3,4-tiyadiazol-2-il)etil]-3-(tiyomorfolin-4-ilmetil)-5-tiyokso-1,5-dihidro-4H-1,2,4-triazol-4-il]-3-kloro-4-(4-metilfenil)azetidin-2-on (**10b**)*

Yield 78%. FT-IR (υ_{max}, cm^{-1}): 3278 (NH_2), 1710 (C=O), 1578 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.02–1.06 (3H, m, CH_3), 1.17 (4H, s, $2CH_2$), 2.34 (2H, s, CH_2), 3.05 (4H, s, $2CH_2$), 3.43 (2H, d, J = 8.0 Hz, CH_2), 3.58 (2H, s, CH_2), 4.23 (1H, s, CH), 4.27 (1H, s, CH), 6.00 (1H, s, NH_2), 7.22–7.35 (3H, m, arH), 7.73–7.75 (1H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 18.81 (CH3), 41.87 (CH_2), 46.05 (CH_2), 46.66 (CH_2), 56.54 (CH_2), 62.14 (CH_2), 63.18 (CH_2), 66.79 (CH_2), 126.98 (CH), 128.38 (CH), arC: [128.78 (C), 129.03 (CH), 129.22 (CH), 129.56 (CH), 129.75 (CH), 129.97 (C)], 131.50 (triazole C-3), 133.92 (thiadiazole C-3), 141.92 (triazole C-5), 161.70 (thiadiazole C-5), 169.02 (C=O). EI MS *m/z* (%): 557.10 ($[M]^+$, 65), 538.65 ($[M\text{-}1 - H_2O]^+$, 54), 498.14 (100). Elemental analysis for $C_{21}H_{25}N_8OS_3Cl$ calculated (%), C, 46.96; H, 4.69; N, 20.86; O, 2.98, found (%), C, 46.89; H, 4.74; N, 20.84; O, 3.04.

*4.7.9. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(thiomorpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-pyridin-3yl-azetidin-2-one (**10c**)*

Yield 77%. FT-IR (υ_{max}, cm^{-1}): 3314 (NH_2), 1725 (C=O), 1574 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.18–1.22 (8H, m, $4CH_2$), 3.04–3.07 (6H, m, $3CH_2$), 3.44 (1H, d, J = 8.0 Hz), 3.57 (1H, s, CH), 6.05 (2H, brs, NH_2), 7.23–7.53 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 45.83 (CH_2), 56.47 (CH_2), 57.20 (CH_2), 58.13 (CH_2), 59.32 (CH_2), 61.71 (CH_2), 66.82 (CH_2), 68.90 (CH), 69.37 (CH), arC: [123.87 (CH), 125.63 (CH), 126.98 (CH), 127.37 (CH), 130.12 (C)], 147.85 (triazole C-3), 150.39 (thiadiazole C-3), 163.85 (triazole C-5), 167.89 (thiadiazole C-5), 169.52 (C=O). EI MS *m/z* (%): 547.10 ($[M + Na]^+$, 70), 523.00 ($[M\text{-}1]^+$, 48), 490.35 (100). Elemental analysis for $C_{19}H_{22}N_9OS_3Cl$ calculated (%), C, 43.54; H, 4.23; N, 24.05; O, 3.05, found (%), C, 43.50; H, 4.28; N, 24.06; O, 3.09.

*4.7.10. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(thiomorpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-pyridin-2yl-azetidin-2-one (**10d**)*

Yield 70%. FT-IR (υ_{max}, cm^{-1}): 3314 (NH_2), 1730 (C=O), 1571 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.17–1.20 (8H, m, $4CH_2$), 3.02–3.06 (6H, m, $3CH_2$), 3.42 (1H, d, J = 8.0 Hz, CH), 3.54 (1H, s, CH), 6.26 (2H, brs, NH_2), 7.26–8.00 (4H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 41.23 (CH_2), 41.59 (CH_2), 41.93 (CH_2), 45.98 (CH_2), 53.65 (CH_2), 56.53 (CH_2), 59.88 (CH_2), 62.17 (CH), 66.78 (CH), arC: [123.38 (CH), 125.05 (CH), 126.07 (CH), 128.33 (CH), 138.70 (C)], 146.51 (triazole C-3), 149.89 (thiadiazole C-3), 165.44 (triazole C-5), 169.06 (thiadiazole C-5), 170.01 (C=O). EI MS *m/z* (%): 547.13 ($[M + Na]^+$,

67), 523.05 ([M-1]$^+$, 42), 490.12 (100). Elemental analysis for $C_{19}H_{22}N_9OS_3Cl$ calculated (%), C, 43.54; H, 4.23; N, 24.05; O, 3.05, found (%), C, 43.51; H, 4.25; N, 24.02; O, 3.09.

4.7.11. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(thiomorpholin-4-ylmethyl)-5-thioxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(2,6-dichlorophenyl)azetidin-2-one (10e)

Yield 79%. FT-IR (υ_{max}, cm^{-1}): 3178 (NH_2), 1723 (C=O), 1586 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 1.03–1.07 (4H, m, $2CH_2$), 1.18–1.21 (4H, m, $2CH_2$), 3.04–3.07 (4H, m, $2CH_2$), 3.43 (2H, d, J = 4.0 Hz, CH_2), 4.15 (1H, s, CH), 4.58 (1H, s, CH), 7.55 (2H, d, J = 4.0 Hz, NH_2), 8.31–8.34 (3H, m, arH). ^{13}C NMR (DMSO-d_6, δ ppm): 18.93 (CH_2), 41.24 (CH_2), 41.92 (CH_2), 45.94 ($2CH_2$), 56.49 ($2CH_2$), 61.83 (CH), 66.82 (CH), arC: [129.85 (CH), 131.87 (CH), 134.39 (C), 139.68 (CH), 142.31 (2C)], 143.93 (triazole C-3), 165.19 (thiadiazole C-3), 167.20 (triazole C-5), 168.13 (thiadiazole C-5). EI MS m/z (%): 592.00 ([M]$^+$, 100). Elemental analysis for $C_{20}H_{21}N_8OS_3Cl_3$ calculated (%), C, 40.58; H, 3.58; N, 18.93; O, 2.70, found (%), C, 40.55; H, 3.61; N, 18.97; O, 3.65.

4.7.12. 1-[1-[2-(5-Amino-1,3,4-thiadiazol-2-yl)ethyl]-3-(thiomorpholin-4-ylmethyl)-5-thipxo-1,5-dihydro-4H-1,2,4-triazol-4-yl]-3-chloro-4-(4-fluorophenyl)azetidin-2-one (10f)

Yield 72%. FT-IR (υ_{max}, cm^{-1}): 3312 (NH_2), 1693 (C=O), 1582 (C=N). ^{1}H NMR (DMSO-d_6, δ ppm): 0.1.16–1.20 (8H, m, $4CH_2$), 3.03–3.06 (6H, m, $3CH_2$), 4.25 (1H, s, CH), 4.58 (1H, s, CH), 6.16 (2H, s, NH_2), 7.26 (1H, s, arH), 7.38 (1H, s, arH), 7.51 (1H, s, arH), 10.08 (2H, s, 2OH). ^{13}C NMR (DMSO-d_6, δ ppm): 41.93 (CH_2), 45.94 (CH_2), 59.99 (CH_2), 60.02 (CH_2), 61.20 (CH_2), 61.78 (CH_2), 62.32 (CH_2), 67.10 (CH), 68.63 (CH), arC: [122.10 (CH), 123.75 (CH), 126.85 (CH), 139.02 (C), 140.85 (C)], 147.85 (triazole C-3), 150.10 (thiadiazole C-3), 163.78 (triazole C-5), 168.52 (thiadiazole C-5), 170.23 (C=O). EI MS m/z (%): 560.11 ([M + K]$^+$, 35), 543.22 ([M + 2]$^+$, 77), 525.12 ([M + 2-H_2O]$^+$, 42), 512.98 (100). Elemental analysis for $C_{20}H_{22}N_8OS_3ClF$ calculated (%), C, 44.40; H, 4.10; N, 20.71; O, 2.96, found (%), C, 44.38; H, 4.04; N, 20.72; O, 2.96.

4.8. Antimicrobial activity/sensitivity studies

Antimicrobial activity or susceptibility tests were performed to determine the in-vitro activity of the antimicrobial agent against a particular bacterial species. Two techniques are used to measure susceptibility testing of microorganisms, including “diffusion” and “dilution”. Disc diffusion technique is a frequently used technique and the sensitivity of the antibiotic absorbed into paper discs (the solution to be measured by antibacterial activity) is based on the diffusion of the organism to the medium in which the organism is inoculated. After the impregnated paper discs are placed on solid media in which the microorganism is inoculated, the discs dissolve and diffuse into the agar, while the inoculated microorganism begins to multiply. There is no growth in the vicinity of the disc where the inhibitor concentrations of the drug are achieved after a certain incubation period. The more sensitive the microorganism is to the drug, the larger the zone of inhibition around the disc. The diameter of the zone of inhibition is measured in mm and evaluations are made according to standard zone tables and the susceptibility status of the microorganism to the antimicrobial agents used is determined.

Minimal Inhibition Test measurements are tested by applying the microdilution technique to determine the dose value of the effectiveness of the substances determined to be effective in the agar well. The quantity of the material is diluted with serial dilutions to the lowest doses and the same amount of microorganism is added to each diluent. With this test, the efficacy dose of the lowest amount of substance is determined.

4.8.1. Minimal Inhibition Concentration (MIC) method

In order to determine the minimum amount of substance that shows antimicrobial activity, in other words, to determine the quantitative effect value, the micro-dilution liquid method is applied in the liquid medium and the minimal inhibition concentration (MIC) is determined as microgram/milliliter (μg/mL) [25]. For the determination of antimicrobial activity, liquid media were used for determining the antifungal activity of Mueller-Hinton liquid (MHB, pH.7.3) (Difco, Detroit, MI) and yeast extract liquid medium (YEG, pH 7.0) (Difco, Detroit, MI). ELISA plates were used for micro-dilution tests and serial dilutions were made with 0.1 mL of dissolved chemicals. McFarland 0.5 turbidity (1×10^8 cfu/mL) from overnight cultures of inoculated microorganisms was prepared for reconstitution and diluted 1:10 and 0.005 mL of microorganism (final assay concentration 5×10^4 cfu/well) was added to each well. Plates were incubated at 35 °C for 16–24 h under aerobic conditions. The MIC value was completely inhibited by the growth of the microorganism in the micro-dilution wells and was determined as the lowest antimicrobial concentration that could be determined by the naked eye. Streptomycin (4 μg) and standard solvent control were used as standard control drugs.

4.9. Urease inhibition assay [26]

Urease activity was defined by using Weatherburn process. Reaction mixtures including 25 μL of Jack Bean urease, 55 μL of buffer (0.01 mol/L K_2HPO_4, 1 mmol/L EDTA and 0.01 mol/L LiCl, pH 8.2) and 10 mmol/L urea were incubated with 5 μL of the test compounds at room temperature for 15 min in microtiter plates. The production of ammonia was measured following the indophenol method and was used to determine the urease inhibitory activity. The phenol reagent (45 μL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkali reagent (70 μL, 0.5% w/v sodium hydroxide and 0.1% v/v NaOCl) were added to each well. This mixture was incubated for 15 min more at 35 °C and optical density was measured at 625 nm against a blank solution including distilled water instead of enzyme. For the determination of the IC_{50} value of the extracts, activity assays were conducted at five different extract concentration and dose response curve was generated. Thiourea was used as standard inhibitor.

4.10. Determination of acetylcholinesterase activity

The AChE inhibitory activity of the compounds was determined using the ELHMAN method [27] using AChE from E. electricus (Sigma) and acetylthiocololine iodide (0.35 mM) as a substrate. The reaction was carried out in the final volume of 3 mL of phosphate buffered solution containing 0.35 mM 5,50-dithiobis-2-nitrobenzoic acid (DTNB), produced from 5-thio-2-nitrobenzoic acid, 0.035 U/mL EeAChE and a yellow anion. Inhibition curves were made by incubating with different compounds for 15 min; A sample without any compound was always used to determine 100% of the enzymatic activity. After a 15 min incubation period, color production as an indicator of enzymatic activity was assessed by measuring the absorbance at 412 nm in a spectrophotometer plate reader.

4.11. Antioxidant activity

DPPH (2.2-diphenyl-1-picrylhydrazyl) radical scavenging activity was performed the synthesized compounds with different chemicals was determined using the free radical DPPH (2.2-diphenyl-1-picrylhydrazyl), as described by Blois [28]. A 100-μL: chemical solution was mixed with 1 mL of freshly prepared methanolic DPPH solution. The reaction mixture was incubated for 30 min at room temperature in the dark and was then measured at 520 nm. The activity was expressed as μmol Trolox equivalent. FRAP (the ferric reducing ability of plasma) was measured using the method described by Benzie & Strain [29] with some modification. To 100 μL of each sample was added 2900 μL

freshly prepared FRAP reagent containing 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ (2,4,6-tripyridyle-s-triazine) and 20 mmol/L $FeCl{\cdot}6H_2O$ in proportions of 10:1:1 (v/v/v). The mixture was incubated for 30 min at 37 °C and measured at 593 nm. The values were given as μmol of Trolox/g. CUPRAC (cupric ion reducing antioxidant capacity) was measured following the procedure described by Apak et al. [30] with some modification. Briefly, 100 μL of each chemical solution was mixed with 900 μL bidistilled water, 1 mL acetate buffer solution (1 mmol/L, pH: 7.0), 1 mL $CuCl_2$ (10 mmol/L) and 1 mL 7.5 mmol/L neocuproine to a final volume of 4 mL. The reaction mixture was then incubated in the dark for 30 min at room temperature and the absorbance of the reaction mixture was measured at 450 nm against a water blank. Trolox was used as the standard calibration curves and the results were expressed as μmol Trolox equivalent per g.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of the paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102928.

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