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Synthesis and Biological evaluation of Novel Triazolo-thiadiazole derivatives

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ABSTRACT

We herein report the synthesis of some novel 4-(3-substituted [1,2,4] triazolo[3,4- b][1,3,4] thiadiazol-6-yl) derivatives. Condensation of the 4-amino-5-substituted-4H-1, 2, 4-triazole-3-thiols with aromatic acids in the presence of polyphosphoric acid (PPA) produced a series of 4-(3-substituted [1,2,4] triazolo[3,4-b][1,3,4]thiadiazol-6-yl) derivatives (TTDZ1-TTDZ27). The structure assigned to compounds was substantiated by their analytical and spectral data. These newly synthesized compounds have been screened for antibacterial, antioxidant and in-vitro anticancer activity.

Key words: Triazolo-Thiadiazole, antibacterial, antioxidant, anticancer

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INTRODUCTION

Heterocyclic compounds represent one of the most active classes of compounds possessing a wide spectrum of biological activities. The recent literature is enriched with progressive findings about the synthesis and pharmacological activity of fused heterocycles. Heterocycles bearing a triazole or 1,3,4-thiadiazole moiety are reported to show wide range of biological properties. The amino and mercapto groups of 1,2,4-triazoles serve as readily accessible nucleophilic centers for the preparation of N-bridged heterocycles. 1,2,4-Triazolederivatives are known to exhibit antimicrobial [1-7], antitubercular [8-11], anticancer [12-16],anticonvulsant [17], anti-inflammatory [18], analgesic [19], molluscicidal properties [20] and antiviral [21] activity. Moreover, synthesis of triazole fused to other heterocycles has attracted attention widely due to their diverse applications.1,3,4-thiadiazoles exhibit wide spectrum of biological activities, possibly due to presence of toxophoric >N – C – S - moiety [22]. They find applications as antibacterial [23,24], antimicrobial [25-29] and anti-inflammatory agents [30] along with anticancer [31,32] and antitubercular [33,34] activity. A triazolothiadiazole system may be viewed as a cyclic analogue of two very important components thiosemicarbazide [35] and biguanide [36] which often display diverse biological activities such as anticancer [37,38], antimicrobial [39], anti-inflammatory analgesic [40-43] activity. We herein report the synthesis of some new4-(3-substituted [1,2,4]triazolo[3,4- b][1,3,4]thiadiazol-6-yl) derivatives. Condensation of the 4-amino-5-substituted-4H-1, 2, 4-triazole-3-thiols with aromatic acids in the presence of PPA produced a series of 4-(3-substituted [1,2,4]triazolo[3,4-b][1,3,4]thiadiazol-6-yl) derivatives (TTDZ1-TTDZ27). The structure assigned to compounds was substantiated by their analytical and spectral data. These newly synthesized compounds have been screened for antibacterial, antioxidant and *in-vitro* anticancer activity.

MATERIALS AND METHODS

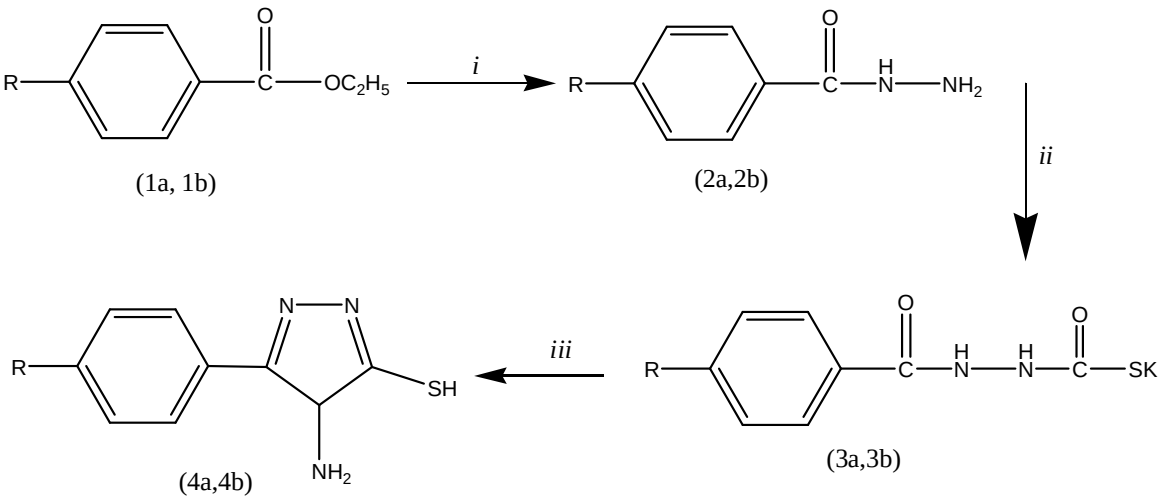
1. Experimental

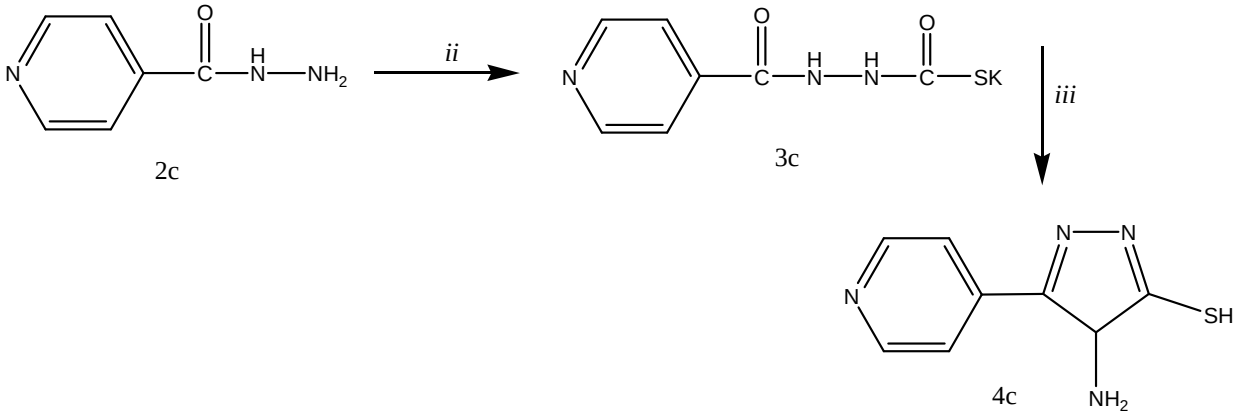
Melting points were determined in open capillaries and were uncorrected. The IR spectra were recorded on Nicolet Impact 410 FT IR spectrophotometer using KBr pellets.^{1}H NMR on Bruker 300-MHz FT NMR spectrometer in $CDCl_3$ and DMSO-d_6 with TMS as internal standard. Mass spectrum was recorded on Finnigan MAT (Model MAT8200) spectrometer. The purity of the compounds was checked by thin layer chromatography (TLC) on silica gel plate using hexane and ethyl acetate.

2. Chemistry

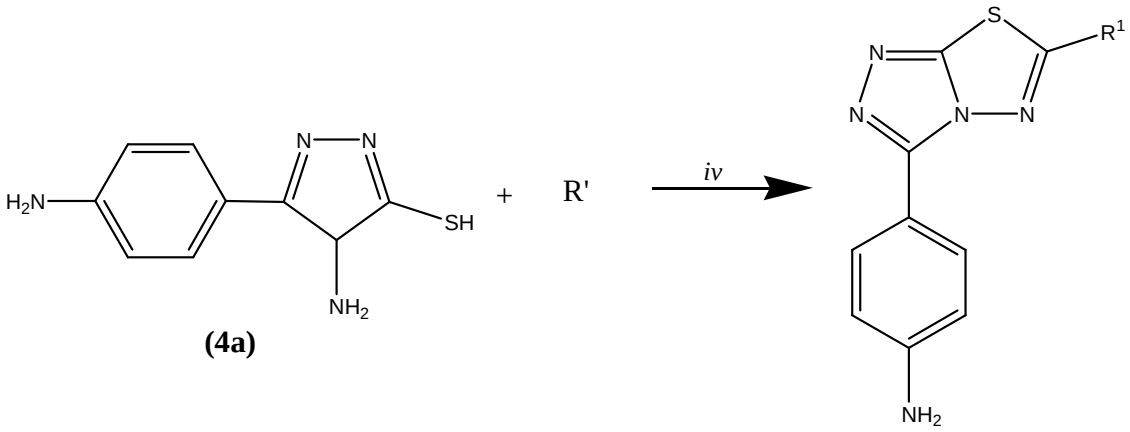
All 4-amino-5-substituted-4H-1,2,4-triazole-3-thiols(4) were prepared as described in Scheme 1. The acid hydrazides (2) were obtained by reaction of the esters(1) with hydrazine in ethanol. Treatment of the hydrazides (2) with CS_2 under a basic condition (KOH/EtOH) gave the corresponding potassium aroyl dithiocarbazates(3). This salt (3) underwent ring closure with an excess of 99% hydrazine hydrate to give the 4-amino-5-substituted-4H-1, 2, 4-triazole-3-thiols.

A one pot synthesis of 4-(3-substituted [1,2,4]triazolo[3,4- b][1,3,4]thiadiazol-6-yl) derivatives (TTDZ) was achieved when 4-amino-5-substituted [1,2,4]triazole-3-thiol (4) and different substituted benzoic acid were heated at 180–200 ^{0}C in polyphosphoric acid (PPA). The reaction temperature appeared to be crucial for this process. When the reaction was carried out below 180^0 C, no intramolecular cyclization was observed, and the starting materials were obtained as the major product (Scheme2).The structures of these new compounds were characterized with IR, 1H NMR and mass spectra.



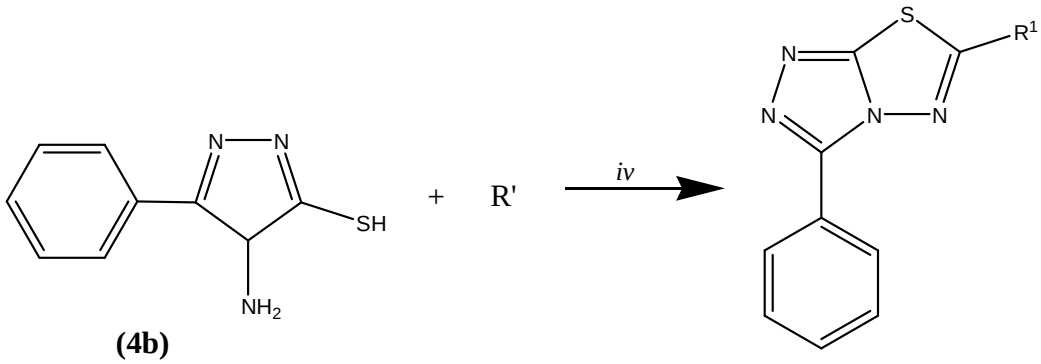


Scheme 1:R= H, NH_2 i) $N_2H_4.H_2O$, Reflux ii) KOH, EtOH, CS_2 iii) $N_2H_4.H_2O$, Reflux 1hr



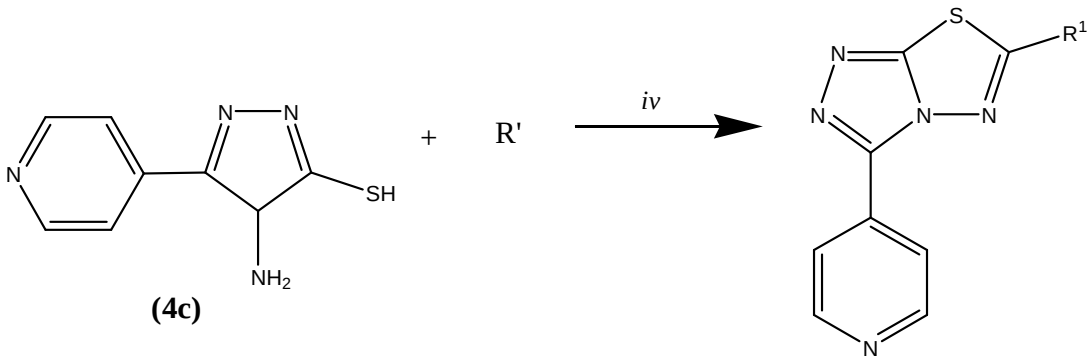
(4a)

TTDZ1-TTDZ9



(4b)

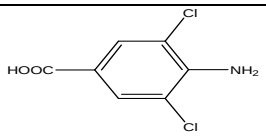
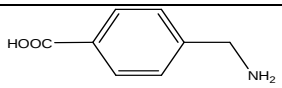
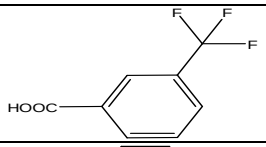
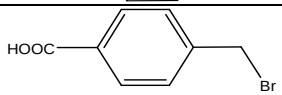
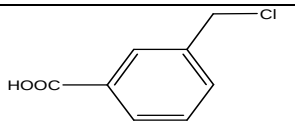
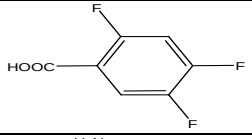
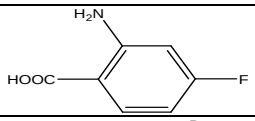
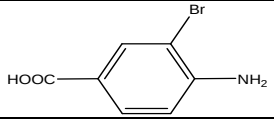
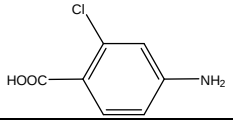
TTDZ10-TTDZ18



(4c) **TTDZ19-TTDZ27**

Scheme 2: iv) PPA, 180-220 °C, 3 hr

Where

Compound	R^1
TTDZ1, TTDZ10, TTDZ19	
TTDZ2, TTDZ11, TTDZ20	
TTDZ3, TTDZ12, TTDZ21	
TTDZ4, TTDZ13, TTDZ22	
TTDZ5, TTDZ14, TTDZ23	
TTDZ6, TTDZ15, TTDZ24	
TTDZ7, TTDZ16, TTDZ25	
TTDZ8, TTDZ17, TTDZ26	
TTDZ9, TTDZ18, TTDZ27	

3. Antibacterial activity:

The newly synthesized compounds were screened for their antibacterial activity using agar well diffusion method [44].The antibacterial activity of the test compounds was evaluated against two Gram-positive bacteria, *Staphylococcus aureus*-ATCC25923, *Bacillus subtilis*-ATCC 6633 and two Gram-negative bacteria *Pseudomonas aeruginosa*-ATCC 10145 and *Escherichia coli*-ATCC 35218. Ciprofloxacin was used as standard drug.

Most of the compounds showed zone of inhibition at 1µM. Compound such as TTDZ2 ,TTDZ6, TTDZ9, TTDZ13, TTDZ16,TTDZ19,TTDZ26 and TTDZ27 showed good zone of inhibition against *Escherichia coli, Pseudomonas auregnosa, Staphylococcus aureus and Bacillus subtilis*. The compounds were selected for MIC. All the selected compounds were tested for MIC on *Escherichia coli, Pseudomonas auregnosa, Staphylococcus aureus, Bacillus subtilis* bacteria at maximum concentration 1µM to minimum concentration of 0.063µM. None of the selected compounds have shown the MIC in this particular concentration range, compared to standard drug (Table 1)

Table 1: Antibacterial activity of the synthesized compounds against four microorganisms

Compound	Zone of inhibition at 1µM in mm			
	E.coli	***P.aureginosa***	***S.aureus***	***B.subtilis***
TTDZ1	-	12	-	10
TTDZ2	18	15	16	14
TTDZ3	13	14	11	13
TTDZ4	14	-	-	12
TTDZ5	13	10	10	-
TTDZ6	14	16	18	17
TTDZ7	13	-	-	-
TTDZ8	14	15	14	11
TTDZ9	13	14	17	16
TTDZ10	-	-	-	11
TTDZ11	12	-	-	-
TTDZ12	10	12	11	10
TTDZ13	14	15	17	14
TTDZ14	11	10	15	14
TTDZ15	13	-	-	-
TTDZ16	16	18	13	17
TTDZ17	-	-	14	-
TTDZ18	10	12	-	-
TTDZ19	16	18	14	15
TTDZ20	14	13	12	12
TTDZ21	11	10	14	13
TTDZ22	12	10	13	12
TTDZ23	13	14	12	10
TTDZ24	12	14	-	10
TTDZ25	-	-	14	12
TTDZ26	17	14	15	14
TTDZ27	-	-	13	10
Ciprofloxacin	27	24	28	40

E.coli = Escherichia coli, P. aeruginosa = Pseudomonas aeruginosa. S.aureus=Staphylococcus aureus, B. subtilis =Bacillus subtilis.
(-) represent no inhibition growth- Resistant. The zone of inhibition of the various derivatives and the battery of organisms along with the standard drug using diffusion method at 1 µM is given above.

4. Antioxidant Studies

The synthesized compounds were subjected to different *in-vitro* antioxidant models. DPPH radical scavenging assay [45] and Nitric Oxide scavenging assay [45].

4.1. DPPH radical scavenging assay

The assay was carried out in a 96 well microtiter plate. To100µL of DPPH solution, 100 µL of each of the test sample or the standard solution was added separately in wells of the microtiter plate. The final concentrations of the test and standard solutions used were 4 to 0.03µmole. The plates were incubated at 37^0C for 20 minutes and the absorbance of each solution was measured at 540nm, using ELISA micro titer plate reader. The experiment was

performed in triplicate and % scavenging activity was calculated using formula given below. IC_{50} (Inhibitory Concentration) is the concentration of the sample required to scavenge 50% of DPPH free radicals which was calculated and reported in (Table 2).

$$\% \text{ Scavenging} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

Table 2: Free Radical Scavenging Activity by DPPH Method

Compound	Percentage Scavenging (µg/ml)					
	500	250	125	62.5	31.25	IC_{50}Value
TTDZ1	62.35	56.97	48.41	33.25	15.65	274.21
TTDZ2	54.28	44.99	34.47	25.67	13.94	397.35
TTDZ3	46.21	39.85	29.58	22.98	11.00	>500
TTDZ4	33.74	28.36	19.07	8.07	-	>500
TTDZ5	44.99	31.78	26.41	10.76	-	>500
TTDZ6	60.64	51.83	47.43	32.52	25.18	293.13
TTDZ7	64.55	50.86	35.21	27.14	20.54	308.61
TTDZ8	65.28	54.28	39.60	27.14	16.63	293.33
TTDZ9	24.45	14.91	2.69	-	-	>500
TTDZ10	45.95	35.68	24.86	15.14	1.35	>500
TTDZ11	59.73	48.92	43.51	25.41	15.95	330.96
TTDZ12	62.43	48.92	42.97	22.43	18.65	316.62
TTDZ13	60.81	46.49	37.57	19.46	4.32	229.79
TTDZ14	45.97	34.47	27.38	15.65	8.07	>500
TTDZ15	45.14	31.08	22.70	17.84	1.35	>500
TTDZ16	67.30	56.76	46.49	36.76	22.97	244.14
TTDZ17	61.89	48.92	43.51	30.27	19.73	310.37
TTDZ18	33.78	19.46	9.73	-	-	>500
TTDZ19	61.60	49.88	36.66	25.44	11.72	331.47
TTDZ20	45.39	30.67	20.95	13.97	-	>500
TTDZ21	48.66	37.41	22.00	14.91	5.38	>500
TTDZ22	34.59	22.16	15.68	0.81	-	>500
TTDZ23	56.36	38.90	25.69	14.71	10.22	408.35
TTDZ24	46.49	40.27	28.65	19.73	9.46	>500
TTDZ25	67.58	53.37	48.63	33.42	26.93	248.56
TTDZ26	50.62	41.90	25.94	13.97	7.36	499.31
TTDZ27	36.66	20.70	13.97	0.50	-	-
Ascorbic Acid	92.31	89.59	85.67	80.29	79.64	5.54

4.2. Nitric Oxide scavenging assay

The reaction mixture (3mL) containing sodium nitroprusside (10mM, 2mL), phosphate buffer saline (PBS, 0.5mL) and 0.5mL of each test sample in DMSO were incubated separately at 25^{o}C for 150 minutes. After incubation,0.5µL of the reaction mixture containing nitrate was removed and 100µL of sulphanilamide reagent was added, mixed well and allowed to stand for 5minutes for completion of diazotization, then 100 µL of Naphthylethylene diamine dihydro chloride was added, mixed and allowed to stand for 30 minutes in diffused light at room temperature. The absorbance of these solutions was measured at 540 nm using ELISA microtiter plate reader. The experiment was performed in triplicate and % scavenging activity was calculated using formula given below. IC_{50} (Inhibitory Concentration) is the concentration of the sample required to scavenge 50% of nitric acid which was calculated and reportedin (Table 3).

$$\% \text{ Scavenging} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

Table 3: Scavenging activity of selected compounds by Nitric Oxide scavenging assay

Compound	%inhibition (Concentration at µg/ml)					
	500	250	125	62.5	31.25	IC_{50}Value
TTDZ1	56.15	49.64	41.29	34.11	18.64	346.81
TTDZ2	33.26	22.50	11.88	1.62	-	>500
TTDZ3	37.61	29.58	20.38	10.12	0.56	>500
TTDZ4	40.02	31.90	29.61	16.66	7.11	>500
TTDZ5	47.25	35.63	33.95	26.79	23.47	>500
TTDZ6	61.53	62.33	60.56	55.96	51.76	324.32
TTDZ7	61.45	67.94	61.00	59.37	50.35	395.88
TTDZ8	48.54	43.96	37.99	32.95	21.94	>500
TTDZ9	49.51	45.38	47.65	46.16	39.67	>500
TTDZ10	45.18	38.45	285.21	16.43	8.12	>500
TTDZ11	58.66	41.20	23.47	13.52	2.887	398.95
TTDZ12	57.54	49.59	47.17	43.88	42.22	257.41
TTDZ13	46.24	42.46	39.09	32.36	26.05	>500
TTDZ14	47.65	42.86	34.41	24.47	15.61	>500
TTDZ15	40.28	37.65	35.39	26.81	19.74	>500
TTDZ16	53.85	48.49	48.41	44.28	40.48	332.08
TTDZ17	54.15	50.15	45.26	40.88	36.45	>500
TTDZ18	43.46	39.56	29.68	24.91	20.80	>500
TTDZ19	51.84	48.62	41.57	32.46	19.54	445.60
TTDZ20	75.28	56.72	55.66	57.19	44.89	42.30
TTDZ21	48.15	42.07	38.13	30.98	21.26	>500
TTDZ22	37.04	24.78	17.05	6.182	-	>500
TTDZ23	33.067	27.51	13.26	7.876	4.287	>500
TTDZ24	29.65	23.38	15.61	6.20	1.15	>500
TTDZ25	47.95	41.12	42.12	42.42	36.83	>500
TTDZ26	59.61	59.12	54.83	51.16	40.52	92.33
TTDZ27	59.61	51.09	53.23	51.84	47.10	72.01
Ascorbic acid	96.35	89.74	81.47	80.31	79.36	4.12

5. *In–vitro* anticancer screening (MTT method) [46]

All new synthesized compounds were evaluated for their *in-vitro* anticancer activities against $B_{16}F_1$ (Mouse melanoma cells), Hela (cervical cancer) and V79 (Chinese Hamster lung fibroblast cells) from NCCS, Pune, India.

Exponentially growing all three cells (Hela, $B_{16}F_1$ and V79) were harvested from $25cm^2$ tissue culture flasks and a stock cell suspension ($1X10^5$cell/ml) was prepared with respective media. A 96-well flat bottom tissue culture plate was seeded with $1x10^4$cells in 0.1 ml of suitable media supplemented with10% serum and allowed to attach for 24hrs. Test compounds were prepared just prior to the experiment in 0.2% DMSO and serially diluted with suitable medium to get the different concentrations of 0.063, 0.125, 0.25, 0.5, 1µM. After 24 hours of incubation, Cells were treated with 100µl of test compounds from respective top stocks and the plates were again incubated for 24 and 48 hours. The cells in the control group received only the medium containing the 0.2% DMSO (vehicle). Each treatment was performed in triplicates. After the treatment, drug containing media was removed and washed with 200µl of PBS. To each well of the 96 well plate, 100µl of MTT reagent (Stock:1mg/ml in PBS) was added and incubated for 4 hours at 37ºC. After 4 hours of incubation the plate was inverted on tissue paper to remove the MTT reagent. To solubilize formazan crystals in the wells, 100µl of 100% DMSO was added to each well. The optical density (O.D) was measured by an Enzyme Linked immunosorbent Assay (ELISA) plate reader at 540 nm. Percentage cytotoxicity of each compound was calculated and reported (Table 4, Table 5 and Table 6).

$$\% \text{ cytotoxicity} = \frac{(\text{Control-blank}) - (\text{test-blank})}{(\text{Control-blank})} \times 100$$

Table 4: *In-vitro* cytotoxic activity of compounds on Hela cells (Cervical cancer) by MTT assay at 48 hours of exposure

Compound	Percentage Scavenging (µM)					
	1	**0.5**	**0.25**	**0.125**	**0.063**	**IC_{50}Value**
TTDZ1	38.97	35.94	13.31	0.00	0.00	--
TTDZ2	54.56	44.48	33.33	20.38	8.03	0.83
TTDZ3	38.56	34.12	25.90	4.44	0.00	--
TTDZ4	39.93	32.49	26.50	9.54	4.12	--
TTDZ5	49.28	34.05	28.31	17.32	8.36	--
TTDZ6	57.19	48.08	41.73	34.17	27.10	0.67
TTDZ7	60.07	57.43	49.04	43.53	29.74	0.46
TTDZ8	38.24	32.13	21.10	11.32	2.32	--
TTDZ9	43.53	34.65	26.38	20.31	9.36	--
TTDZ10	48.86	36.19	26.63	19.04	15.28	--
TTDZ11	59.89	52.21	42.32	30.23	22.79	0.61
TTDZ12	58.58	51.23	36.19	31.05	20.67	0.66
TTDZ13	49.35	31.86	20.92	14.71	9.31	--
TTDZ14	54.56	43.41	35.61	30.22	16.91	0.79
TTDZ15	38.40	14.79	27.45	3.10	1.14	--
TTDZ16	37.09	27.45	17.40	4.74	1.96	--
TTDZ17	40.28	32.43	20.26	10.62	8.17	--
TTDZ18	50.82	42.81	37.58	21.24	15.77	1.0
TTDZ19	39.33	33.69	22.18	17.99	9.34	--
TTDZ20	60.19	52.64	42.93	30.94	17.39	0.48
TTDZ21	41.01	33.21	22.30	6.59	4.80	--
TTDZ22	36.33	17.42	8.46	1.01	--	--
TTDZ23	48.61	39.22	31.86	24.35	15.36	--
TTDZ24	49.28	33.69	25.78	9.23	6.00	--
TTDZ25	54.08	36.45	26.26	8.15	7.67	0.85
TTDZ26	45.80	35.25	24.82	8.27	8.15	--
TTDZ27	52.04	37.53	28.90	15.59	14.87	0.92

Table 5: *In-vitro* cytotoxic activity of compounds on $B_{16}F_1$(Mouse Melanoma cells) by MTT assay at 48 hours of exposure

Compound	Percentage Scavenging (µM)					
	1	**0.5**	**0.25**	**0.125**	**0.063**	**IC_{50}Value**
TTDZ1	47.40	43.74	36.18	33.09	25.04	--
TTDZ2	49.11	43.90	42.76	31.06	22.36	--
TTDZ3	40.98	29.76	20.89	10.08	5.93	--
TTDZ4	40.65	27.72	20.16	16.26	4.39	--
TTDZ5	37.15	29.11	15.77	3.50	--	--
TTDZ6	61.87	59.84	53.74	45.69	41.71	0.23
TTDZ7	71.63	67.40	66.50	62.36	52.60	0.057
TTDZ8	47.24	43.82	37.89	13.50	10.08	--
TTDZ9	53.66	45.04	39.11	31.38	18.86	0.78
TTDZ10	48.37	43.25	35.69	30.49	18.13	--
TTDZ11	34.47	30.73	18.13	5.45	--	--
TTDZ12	54.63	45.77	35.61	20.33	7.24	0.77
TTDZ13	48.86	35.93	29.84	20.00	6.18	--
TTDZ14	49.43	44.47	35.20	25.45	11.38	--
TTDZ15	48.54	43.17	32.85	25.12	10.08	--
TTDZ16	40.12	31.30	27.89	24.55	10.24	--
TTDZ17	44.47	37.97	34.55	12.11	6.91	--
TTDZ18	55.53	52.68	43.82	31.87	25.61	0.47
TTDZ19	53.58	44.96	39.27	29.59	23.90	0.79
TTDZ20	73.98	72.20	70.81	66.83	59.11	0.052
TTDZ21	41.01	33.21	22.30	6.59	4.80	--
TTDZ22	48.61	39.22	31.86	24.35	15.36	--
TTDZ23	46.21	37.56	24.97	10.34	1.03	--
TTDZ24	38.21	23.78	9.64	0.054	--	--
TTDZ25	40.16	36.95	31.95	19.35	16.50	--
TTDZ26	43.58	42.85	41.38	32.11	25.85	--
TTDZ27	46.26	32.60	29.43	24.96	18.05	--

Table 6:*In-vitro* cytotoxic activity of compounds on V79 (Chinese Hamster lunge fibroblast cells) cells by MTT assay at 48 hours of exposure

Compound	Percentage Scavenging (µM)					
	1	0.5	0.25	0.125	0.063	IC_{50}Value
TTDZ1	44.90	39.52	34.53	22.86	12.03	--
TTDZ2	49.37	40.93	27.18	21.41	7.59	--
TTDZ3	23.26	17.89	6.40	--	--	--
TTDZ4	10.02	9.66	10.00	5.67	--	--
TTDZ5	16.58	13.85	5.19	--	--	--
TTDZ6	48.92	43.94	27.71	11.69	10.39	--
TTDZ7	49.13	44.37	38.66	16.45	4.33	--
TTDZ8	53.03	41.59	25.32	11.04	4.55	0.91
TTDZ9	41.78	34.63	28.42	18.14	2.60	--
TTDZ10	49.77	37.79	27.64	21.27	9.99	--
TTDZ11	49.18	39.78	18.61	7.87	4.38	--
TTDZ12	45.65	30.30	23.42	16.36	6.26	--
TTDZ13	6.55	2.50	--	--	--	--
TTDZ14	41.01	38.10	17.10	4.55	--	--
TTDZ15	48.97	39.30	24.25	10.82	0.62	--
TTDZ16	34.21	26.94	19.67	8.36	1.25	--
TTDZ17	43.29	36.53	27.94	16.32	10.27	--
TTDZ18	48.20	25.28	24.78	15.86	5.68	--
TTDZ19	34.62	31.75	14.58	5.35	--	--
TTDZ20	43.50	38.11	28.09	25.84	12.89	--
TTDZ21	47.32	44.21	35.90	23.28	11.99	--
TTDZ22	44.26	40.45	24.93	14.39	0.10	--
TTDZ23	29.72	15.86	1.76	--	--	--
TTDZ24	41.25	36.47	30.29	26.85	9.06	--
TTDZ25	35.36	29.97	8.19	3.12	--	--
TTDZ26	52.86	42.32	29.18	19.75	8.19	0.87
TTDZ27	49.91	43.21	38.58	37.91	31.21	--

CONCLUSION

Twenty seven 4-(3-substituted [1, 2, 4] triazolo [3, 4-b] [1, 3, 4] thiadiazole-6-yl derivatives were synthesized in good yield. All the compounds were characterized by TLC (Acetone:ethylacetate, 3.5:6.5), melting point, IR, ^{1}H-NMR and mass spectral data. All the compounds were evaluated for antibacterial activity, antioxidant activity and anticancer activity.

In antibacterial activity most of the compounds showed zone of inhibition at concentration of 1µM. Few compounds were selected for MIC(minimium inhibitory concentration) determination. None of the compounds exhibited the MIC at this particular concentration range, compared to standard drug. Compound TTDZ20 showed good *in-vitro* antioxidant activity. Compound TTDZ1, TTDZ2, TTDZ6, TTDZ7, TTDZ8, TTDZ11, TTDZ12, TTDZ13, TTDZ16,TTDZ17,TTDZ19,TTDZ23,TTDZ25,TTDZ26 and TTDZ27 had IC_{50} values ranging from 42-500µg/ml. The IC_{50} value of all other synthesized compounds was above 500µg/ml. MTT assay was performed in cultured Hela Cells(cervical cancer),$B_{16}F_1$ (Mouse melanoma cells) to conform their *in-vivo* cytotoxicity potency. The compounds TTDZ6, TTDZ7, TTDZ18 and TTDZ20 were found promising. MTT assay was also performed in cultured normal Human Lung cells as V79 to confirm their *in-vitro* cytotoxic potency. Here the compound TTDZ8 and TTDZ26 were found to possess cytotoxicity. Based on the *in-vitro* results TTDZ6, TTDZ7, TTDZ18 and TTDZ20 were selected for *in-vivo* anticancer activity. In EAC the TTDZ6,TTDZ7,TTDZ18 and TTDZ20 compounds enhanced the life span of tumor bearing mice at both doses (75mg/kg and150mg/kg) indicating them to be promising anticancer agents because prolongation of life span is a liable criteria for judging the value of any anticancer agent.

Method of synthesis

General methods of synthesis of synthesis of 4-(3-substituted [1, 2, 4]triazolo [3, 4-b] [1, 3, 4] thiadiazol-6- yl)

(TTDZ1-TTDZ27): 0.1mole of substituted ester, in 30ml of ethanol was added to 0.1mole equivalents of hydrazine hydrate .The solution was stirred under reflux for 6 hours. After the completion of the reaction (monitored by TLC) and cooling to room temperature, 200 ml of ice water was added to the solution and the precipitate was formed. The precipitate was isolated by filtration and purified by re-crystallization in absolute ethanol to get pure acid hydrazide (1a, 1b). Potassium hydroxide (0.15mol) in 100 ml of absolute ethanol and 0.1 mole of acid hydrazide were mixed together until the solution became clear. To the clear solution was added 0.15mole of carbon disulfide. The solution was stirred for 3 hours at 25^{O}C and then 100ml diethyl ether was added to form a precipitate (2a, 2b). The precipitates were filtered and washed with ethyl ether several times. The precipitate was mixed with160 mmoles of hydrazine hydrate and 2ml of water. The solution was refluxed for 1 hour until the color of the solution become clear green. After cooling to room temperature, 100ml of ice water was added to the solution and neutralized with 3N hydrochloric acid to form precipitate. The precipitate was isolated by filtration and purified by recrystallization from absolute ethanol to give pure 4-amino-5-substituted-4H-1, 2, 4-triazole-3-thiols (3a, 3b). A mixture of 4-amino-5-substituted-4H-1,2,4-triazole-3-thiol(6.81mmol) and polyphosphoric acid (20ml) was heated to 50-60^{0}C with stirring, then substituted aromatic acid was added (6.9 mmol) portion wise. The mixture was heated at 180-220^{O}C for 4 hours with stirring and poured in ice water. After neutralizing with concentrated aqueous ammonia solution, the crude product was collected by filtration, and recrystallized from absolute ethanol to get pure 4-(3-substituted [1, 2, 4]triazolo [3, 4-b] [1, 3, 4] thiadiazol-6- yl) (TTDZ1-TTDZ18). Similarly 0.1mole of Isonicotinic acid hydrazide was converted to (3-substituted [1, 2, 4]triazolo [3, 4-b] [1, 3, 4] thiadiazol-6-yl)(TTDZ19- TTDZ27) by the same procedure as mentioned above.

Physical and Spectal data of the compounds

2,6-dichloro-4-(3-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline(TTDZ1):

Mol. Formula: $C_{15}H_{10}C_{l2}N_6S$; Mol. Weight: 377.24; Yield: 84%;mp 290-293^{0}C; R_f value: 0.62; IR: (KBr) 3367 cm-1 (-NH2), 3192 cm-1 (C-H), 1467 cm-1 (C=N), 1228 cm-1 (C-Cl), 677cm-1 (C-S-C).

1-[4-(3-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)phenyl]methanamine(TTDZ2):

Mol. Formula: $C_{16}H_{14}N_6S$; Mol. Weight: 322.37; Yield: 81%; mp 276-279^{0}C; R_f value: 0.42; IR: (KBr) 3317 cm-1 (-NH2), 3084 cm-1 (C-H), 1496 cm-1 (C=N), 680 cm-1 (C-S-C).

3-phenyl-6-[3-(trifluoromethyl)phenyl][1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (TTDZ3):

Mol. Formula: $C_{16}H_{10}F_3N_5S$; Mol. Weight: 361.33; Yield: 64%; mp 286-291^{0}C; R_f value: 0.76; IR: (KBr) 3346 cm-1 (-NH2), 3061 cm-1 (C-H), 1471 cm-1 (C=N), 1178 cm-1 (C-F), 680 cm-1 (C-S)

6-[4-(bromomethyl)phenyl]-3-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole(TTDZ4):

Mol. Formula: $C_{16}H_{12}BrN_5S$; Mol. Weight: 386.25; Yield: 60%; mp 245-248^{0}C; R_f value: 0.53; IR: (KBr) 3344 cm-1 (-NH2), 3061 cm-1 (C-H), 1471 cm-1 (C=N), 1178 cm-1 (C-Br), 680 cm-1 (C-S).

6-[3-(chloromethyl)phenyl]-3-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (TTDZ5):

Mol. Formula: $C_{16}H_{12}ClN_5S$; Mol. Weight: 341.80; Yield: 75%; mp 238-240^{0}C; R_f value: 0.74; IR: (KBr) 3342 cm-1 (-NH2), 3049 cm-1 (C-H), 1475 cm-1 (C=N), 682 cm-1 (C-S), 574 cm-1 (C-Cl)

3-phenyl-6-(2,4,5-trifluorophenyl) [1,2,4] triazolo[3,4-*b*] [1,3,4]thiadiazole (TTDZ6):

Mol. Formula: $C_{15}H_8F_3N_5S$; Mol. Weight: 347.19; Yield: 63%; mp 254-256^{0}C; R_f value: 0.67; IR: (KBr) 3464 cm-1 (-NH2), 3113 cm-1 (C-H), 1468 cm-1 (C=N), 1301 cm-1 (C-F), 684 cm-1 (C-S) NMR: (DMSO) δ 3.2 (s, 2H, H-NH2), 6.73-7.82 (m, 2H,Ar-H), 7.6-8.7(m, 4H, Ar-H). Mass: 347.92(m/z)

5-fluoro-2-(3-phenyl[1,2,4] triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline (TTDZ7):

Mol. Formula: $C_{15}H_{11}FN_6S$; Mol. Weight: 326.34; Yield: 60%; mp 246-249^{0}C; R_f value: 0.64; IR: (KBr) 3336 cm-1 (-NH2), 3064 cm-1 (C-H), 1465 cm-1 (C=N), 1300 cm-1 (C-F), 684cm-1 (C-S) NMR: (DMSO) δ 3.29 (s, 2H, H-NH2), 6.87-7.72 (m, 3H, Ar-H),7.52- 7.82 (m, 4H, H-Ar). Mass: 327.004 (M+1)

2-bromo-4-(3-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline (TTDZ8):

Mol. Formula: $C_{15}H_{11}BrN_6S$; Mol. Weight: 387.24; Yield: 71%; mp 204-207^{0}C; R_f value: 0.42; IR: (KBr) 3416 cm-1 (-NH2), 3184 cm-1 (C-H), 1228 cm-1 (C-Br), 1280 cm-1 (C=N), 680 cm-1 (C-S) NMR: (DMSO) δ 3.29, (s, 2H, H-NH2), 6.71-7.62 (m, 4H,Ar- H), 6.94-7.61 (m, 3H, Ar-H).

3-chloro-4-(3-phenyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline (TTDZ9):

Mol. Formula: $C_{15}H_{11}ClN_6S$; Mol. Weight: 342.79; Yield: 74%; mp 268-272^{0}C; R_f value: 0.72; IR: (KBr) 3319 cm-1 (-NH2), 3037 cm-1 (C-H), 1228 cm-1 (C=N), 1172 cm-1 (C-Cl), 677 cm-1 (C-S) NMR: (DMSO) 3.30 (s, 2H, H-NH2), 6.34 (m, 1H, J=2.4 Hz,Ar- 2H), 6.36 (d, 1H, J=3.6 Hz, Ar-4H'), 6.70 (d, 1H, J=2.00 Hz,Ar- 5H), 6.71 (d, 1H, J=3.2 Hz,Ar- 5H'), 6.78 (d, 1H, J=2.00 Hz, Ar-3H), 7.84 (s, 1H, Ar-2H'), 7.95 (d, 1H, J=2.4 Hz, Ar-2H)

4-[3-(4-aminophenyl)[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl]-2,6-dichloroaniline (TTDZ10):

Mol. Formula: $C_{15}H_9Cl_2N_5S$; Mol. Weight: 362.23; Yield: 76%; mp 293-295^{0}C; R_f value: 0.73; IR: (KBr) 3321 cm-1 (-NH2), 3063 cm-1 (C-H), 1600 cm-1 (Ar), 1456 cm-1 (C=N), 1253cm-1 (C-Cl), 678 cm-1 (C-S).NMR: (CDCl3) δ 5.04(d, 1H, H-NH2), 7.30 (dd, 1H, j=6.8Hz, Ar-2H'), 7.82 (dd, 1H, J=6.8 Hz, Ar-6H'), 7.74-7.79 (m, 5H, Ar-H)

4-{6-[4-(aminomethyl)phenyl][1,2,4] triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl}aniline (TTDZ11):

Mol. Formula: $C_{16}H_{13}N_5S$; Mol. Weight: 307.37; Yield: 63%; mp 188-191^{0}C; R_f value: 0.74; IR: (KBr) 3308 cm-1 (-NH2), 3182 cm-1 (C-H), 1614 cm-1 (Ar), 1464 cm-1 (C=N), 678 cm-1 (C-S). NMR: (CDCl3) δ 4.34(s, 1H, H-NH2), 2.94 (s, 2h, H-CH2), 7.50 (d, 1H, J=14.0Hz, Ar-2H'), 7.56 (dd, 1H, J=14.8Hz, Ar-3H'), 8.40 (dd, 1H, J=15.6Hz, Ar-5H'), 8.42(d, 1H, J=15.6Hz,Ar- 6H'), 7.72-7.75 (m, 5H, H-Ar). Mass: 308.98 (M+2)

4-{6-[3-(trifluoromethyl)phenyl][1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl}aniline (TTDZ12):

Mol. Formula: $C_{16}H_9F_3N_4S$; Mol. Weight: 346.29; Yield: 49%; mp 270-274^{0}C; R_f value: 0.49; IR: (KBr) 3284 cm-1 (C-H), 1496 cm-1 (Ar), 1462 cm-1 (C=N), 1174 cm-1 (C-F), 682 cm-1 (C-S). NMR: (CDCl3) δ 7.49-7.55 (m, 5H, H-Ar), 7.57 (d, 1H, J=8.00Hz, Ar-5H'), 7.83 (d, 1H, J=8.00Hz,Ar- 6H'), 8.17 (d, 1H, J=2.00Hz, Ar-2H'), 8.35 (d, 1H, J=2.00,Ar- 4H'). Mass: 345.965 (m/z)

4-{6-[4-(bromomethyl)phenyl][1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl}aniline (TTDZ13):

Mol. Formula: $C_{16}H_{11}BrN_4S$; Mol. Weight: 371.25; Yield: 78%; mp 235-239^{0}C; R_f value: 0.69; IR: (KBr) 3182 cm-1 (C-H), 1554 cm-1 (Ar), 1469 cm-1 (C=N), 1246 cm-1 (C-Br), 680 cm-1(C-S)

4-{6-[3-(chloromethyl)phenyl][1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl}aniline (TTDZ14):

Mol. Formula: $C_{16}H_{11}ClN_4S$; Mol. Weight: 326.80; Yield: 73%; mp 257-260^{0}C; R_f value: 0.74; I.R: (KBr) 3314 cm-1(-NH2), 3049 cm-1 (C-H), 1472 cm-1 (C=N), 672 cm-1 (C-S)

4-[6-(2,4,5-trifluorophenyl)[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl]aniline (TTDZ15):

Mol. Formula: $C_{15}H_7F_3N_4S$; Mol. Weight: 332.11; Yield: 47%; mp 160-164^{0}C; R_f value: 0.41;IR: (KBr) 3182 cm-1 (C-H), 1602 cm-1 (Ar), 1464 cm-1 (C=N), 1298 cm-1 (C-F), 688 cm-1 (C-S).

2-[3-(4-aminophenyl) [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl]-5-fluoroaniline (TTDZ16):

Mol. Formula: $C_{15}H_{10}FN_5S$; Mol. Weight: 311.30; Yield: 62%; mp 208-210^{0}C; R_f value: 0.57; IR: (KBr) 2918 cm-1(C-H), 1620 cm-1 (Ar), 1462 cm-1(C=N), 1290 cm-1 (C-F), 3066 cm-1 (-NH2), 684 cm-1 (C-S). NMR: (DMSO) δ 3.29 (s, 2H, H-NH2), 6.71 (d, 1H, J=2.8 Hz, Ar-5H'), 6.74 (d, 1H, J=2.8 Hz, Ar-6H'), 6.86 (s, 1H,Ar- 3H'), 7.53 (s, 1H,Ar- 4H), 7.60 (d, 1H, J=2.00Hz, Ar- 5H), 7.73 (d, 1H, J=2.00Hz,Ar- 6H), 7.83 (d, 1H, J=2.0Hz, Ar-2H), 7.63 (d, 1H, J=2.4 Hz, Ar-3H). Mass-: 311 (M+1)

4-[3-(4-aminophenyl) [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl]-2-bromoaniline (TTDZ17):

Mol. Formula: $C_{15}H_{10}BrN_5S$; Mol. Weight: 372.24; Yield: 81%; mp 141-145^{0}C; R_f value: 0.48; IR: (KBr) 3454 cm-1 (-NH2), 3342 cm-1 (C-H), 1560 cm-1 (Ar), 1469 cm-1 (C=N), 1288 cm-1 (C-Br), 682 cm-1 (C-S).

4-[3-(4-aminophenyl)[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl]-3-chloroaniline (TTDZ18):

Mol. Formula: $C_{15}H_{10}ClN_5S$; Mol. Weight: 327; Yield: 63%; mp 245-248^{0}C; R_f value: 0.61; IR: (KBr) 3317 cm-1 (-NH2), 3180 cm-1 (C-H), 1600 cm-1 (Ar), 1465 cm-1 (C=N), 682 cm-1 (C-S).

2,6-dichloro-4-(3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline(TTDZ19):

Mol. Formula: $C_{14}H_8Cl_2N_6S$; Mol. Weight: 365.21; Yield: 64%; mp 260-264^{0}C; R_f value: 0.63; IR: (KBr) 3462 cm-1, (C=N) 3373-3174 cm-1 (-CH==CH), 1608 cm-1 (N=H), 684 cm-1 (-C-S-C), 1226 cm-1 (C-Cl), 1456 cm 1(C=N).

1-[4-(3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)phenyl]methanamine (TTDZ20):

Mol. Formula: $C_{15}H_{12}N_6S$; Mol. Weight: 308.34; Yield: 77%; mp 225-228^{0}C; R_f value: 0.52; IR: (KBr) 3277 cm-1 (C-H), 2926 cm-1 (N=H), 1604 cm-1 (C=N), 678 cm-1 (C-S-C). Mass: 308.455 (m/z)

3-pyridin-4-yl-6-[3-(trifluoromethyl)phenyl][1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (TTDZ21):

Mol. Formula: $C_{15}H_8F_3N_5S$; Mol. Weight: 347.30; Yield: 49%; mp 247-251^{0}C; R_f value: 0.41; IR: (KBr) 3327 cm-1 (C-H), 2948 cm-1 (N-H), 1596 cm-1 (C=N), 1143 cm-1 (C-F), 615 cm-1 (C-S)

6-[3-(bromomethyl)phenyl]-3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (TTDZ22):

Mol. Formula: $C_{15}H_{10}BrN_5S$; Mol. Weight: 372.24; Yield: 51%; mp 274-276^{0}C; R_f value: 0.52; IR: (KBr) 3327 cm-1 (-NH2), 3054 cm-1 (C-H), 1589 cm-1 (C=N), 1342 cm-1 (C-F), 684 cm-1 (C-S-C),

6-[3-(chloromethyl)phenyl]-3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (TTDZ23):

Mol. Formula: $C_{15}H_{10}ClN_5S$; Mol. Weight: 327.78;Yield: 45%; mp 243-248^{0}C; R_f value: 0.49; IR: (KBr) 3049 cm-1 (C-H), 1600 cm-1 (C=N), 690 cm-1 (C-S-C), 518 cm-1 (C-Cl).

3-pyridin-4-yl-6-(2,4,5-trifluorophenyl)[1,2,4] triazolo[3,4-*b*][1,3,4] thiadiazole (TTDZ24):

Mol. Formula: $C_{14}H_6F_3N_5S$; Mol. Weight: 333.29; Yield: 42%; mp 261-264^{0}C; R_f value: 0.71; IR: (KBr) 3462 cm-1 (-NH2), 3112 cm-1 (C-H), 1464 cm-1 (C=N), 1301 cm-1 (C-F), 684 cm-1 (C-S-C).

5-fluoro-2-(3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline (TTDZ25):

Mol. Formula: $C_{14}H_9FN_6S$; Mol. Weight: 312.31; Yield: 78%; mp 296-298^{0}C; R_f value: 0.49; IR: (KBr) 3435 cm-1 (-NH2), 3309 cm-1 (C-H), 1610 cm-1 (C-N), 1224 cm-1 (C-F), 680 cm-1 (C-S-C).

2-bromo-4-(3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline (TTDZ26):

Mol. Formula: $C_{14}H_9BrN_6S$; Mol. Weight: 373.22; Yield: 72%; mp 135-140 ^{0}C; R_f value: 0.71; IR: (KBr) 3437 cm-1 (-NH2), 3137 cm-1 (C-H), 1600 cm-1 (C=N), 1226 cm-1 (C-Br), 680 cm-1 (C-S-C).

3-chloro-4-(3-pyridin-4-yl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-yl)aniline (TTDZ27):

Mol. Formula: $C_{14}H_9ClN_6S$; Mol. Weight: 328.71; Yield: 76%; mp 252-256^{0}C; R_f value: 0.64; IR: (KBr) 3329 cm-1 (-NH2), 3203 cm-1 (C-H), 1600 cm-1 (C=N), 1147 cm-1 (C-Cl). NMR: (DMSO) δ 3.29 (s, 2H, H-NH2), 6.45 (s, 1H, 2H'), 6.73 (dd, 1H, J=2.4 Hz, 5H'), 6.78 (d, 1H, J=2.4, 6H'), 8.21 (d, 1H, J=1.6Hz, 3H), 8.14 (d, 1H, J=1.6Hz, 5H), 8.23 (d, 1H, J=1.6Hz, 2H), 8.33 (d, 1h, J=1.6Hz, 2H). Mass: 329 (M+1).

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