Synthesis, Antimicrobial, and Antioxidant Screening of Aryl Acetic Acid Incorporated 1,2,4-Triazolo-1,3,4-Thiadiazole Derivatives

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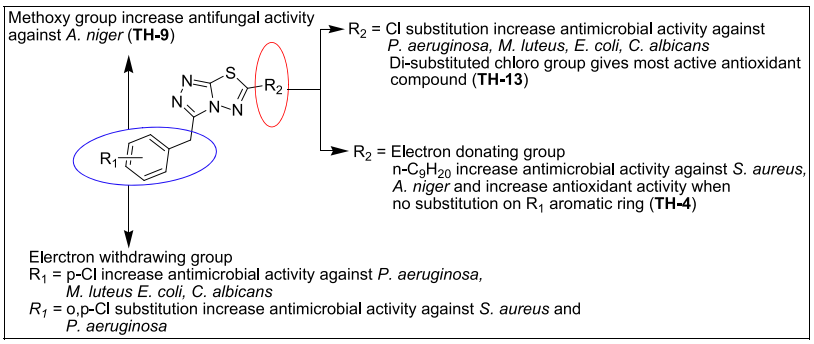


Some novel [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives were synthesized from aryl acetic acids. All the synthesized derivatives were selected for the screening of antibacterial potential against Gram-positive bacteria [*Staphylococcus aureus* (MTCC 3160) and *Micrococcus luteus* (MTCC 1538)] and Gram-negative bacteria [*Escherichia coli* (MTCC 1652) and *Pseudomonas aeruginosa* (MTCC 424)] and antifungal potential against *Aspergillus niger* (MTCC 8652) and *Candida albicans* (MTCC 227), and free radical scavenging activity through 2,2-diphenyl-2-picrylhydrazyl hydrate method. The compounds **TH-4**, **TH-13**, and **TH-19** were found to be more potent antimicrobial agents compared to standard drugs. The compounds **TH-3**, **TH-9**, and **TH-18** also showed significant antimicrobial activity. The compound **TH-13** showed antioxidant activity with IC_{50} value better than the standard compound. The structures of all the synthesized compounds were confirmed by Fourier transform infrared, ^{1}H-NMR, liquid chromatography–mass spectrometry, and CHN analyzer.

INTRODUCTION

1,2,4-Triazole nucleus by virtue of their ambident nucleophilic centers is good starting materials for the synthesis of several interesting N-bridged, and sulfur group containing heterocyclic compounds [1,2]. 1,3,4-Thiadiazole moiety is connected to a broad spectrum of biological activities possibly due to the presence of pharmacophoric (N–C=S) moiety [3]. 1,2,4-Triazole or 1,3,4-thiadiazole moieties has been integrated into a variety of therapeutically important drug candidates including antimicrobial, anti-inflammatories, anticancer, antioxidant, sedatives, antianxiety, and anticonvulsant compounds [4–9]. Fluconazole, itraconazole, voriconazole, triazolam, alprazolam, and etizolam are triazole containing drugs available in the market [10–13]. Acetazolamide, methazolamide, and megazol are thiadiazole containing drugs available in the market [7]. Oxidative stress is one of the major causes of various pathological diseases like diabetics, cancer, atherosclerosis, rheumatoid arthritis, chronic inflammation, cardiovascular diseases, and myocardial infarction [9,14]. The extensive literature survey reveals that the [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole heterocyclic ring has gained much attention during last few years due to their high utilization as antimicrobial, anti-inflammatory, antioxidant, anticancer, antianxiety, sedatives, and anticonvulsant potential.

Based on this concept, we have synthesized [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives from aryl acetic acids with the aim to combine the pharmacological activities of 1,2,4-triazole and 1,3,4-thiadiazole as fused heterocyclic moiety. The substitutions on aryl acetic acid and aromatic carboxylic acids were changed, and the effect of substitution on antimicrobial and antioxidant activities was studied.

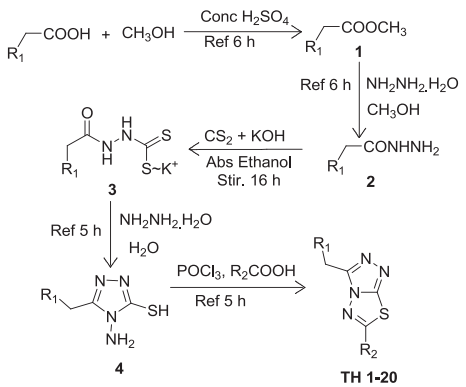
RESULTS AND DISCUSSION

The 4-amino-5-aryl-4*H*-1,2,4-triazole-3-thiol (**4**) was prepared by exploiting intermediates, methyl arylacetate (**1**), 2-arylacetohydrazide (**2**), and potassium dithiocarbazinate (**3**). Intermediate (**1**) was prepared by an esterification reaction between four different substituted phenylacetic acids (**R1**) and methanol. The needle-like

white crystals of (**2**) were prepared by reacting (**1**) with hydrazine hydrate. Potassium dithiocarbazinate (**3**) was produced quantitatively by reacting carbon disulfide with (**2**) under stirring conditions. The 4-amino-5-aryl-4*H*-1,2,4-triazole-3-thiol (**4**) was obtained by reflexing potassium salt (**3**) with hydrazine hydrate. The unique series of [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives (**TH1**–**20**; Scheme 1) were prepared by reacting (**4**) with different aliphatic/aromatic carboxylic acids (**R2**) in presence of phosphorus oxychloride under reflux conditions. The progress of reaction and purity of compounds were confirmed on every step by thin-layer chromatography (TLC), and all the compounds were obtained in good yield. The physicochemical characteristics of synthesized compounds are presented in Table 1. The structures of all the newly synthesized compounds were confirmed by Fourier transform infrared, ^{1}H-NMR, liquid chromatography–mass spectrometry (LC–MS), and CHN analyzer, which were in full agreement with their structures.

The IR spectra of synthesized compounds (**TH1**–**20**) exhibit the absorption bands for aromatic vibrations in the regions of 3100–3000 cm^{-1}, C=N 1690–1600 cm^{-1}, and C–S–C 704–680 cm^{-1}, respectively. The absence of strong stretching bands of NH/NH$_2$ between 3291 and 3239 cm^{-1} and the presence of C–S–C stretching peaks confirmed [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole formation. Besides the earlier absorption peaks, intense absorption peaks for C–Cl in the region of 760–720 cm^{-1} and for –NO$_2$ functional group, two peaks observed between 1570–1490 cm^{-1} and 1355–1315 cm^{-1} for respective compounds. An aliphatic chain containing compounds **TH-4**, **TH-9**, **TH-14**, and **TH-19** showed absorption bands between 1450 and 1370 cm^{-1} for –CH$_3$ and –CH$_2$ peaks between 1470 and 1465 cm^{-1}, respectively. Fluoro group containing compounds **TH-5**, **TH-10**, **TH-15**, and **TH-20** showed peaks for C–F in the region of 1100–1000 cm^{-1}.

Scheme 1. Synthesis of [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles.



Furthermore, the structure of final compounds was supported by ^{1}H-NMR. The absence of signals of NH$_2$ and SH protons in ^{1}H-NMR spectra of [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (**TH1**–**20**) confirms the synthesis of final compounds. The characteristic signals at δ 7.0–8.0 ppm were observed for (ArH) aromatic rings. The –CH$_2$ of R$_1$ linker showed a signal at δ 4.0–5.0 ppm, whereas for alkyl chain on R$_2$, –CH$_2$ group showed signals in the region of δ 1.1–4.1 ppm and –CH$_3$ groups observed at δ 0.8–0.9 ppm. LC–MS and elemental analysis also supported the structures of final compounds.

In vitro antimicrobial activity. The synthesized [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole compounds (**TH1**–**20**) were evaluated for their *in vitro* antibacterial potential against Gram-positive (*Staphylococcus aureus* and *Micrococcus luteus*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and antifungal activity against *Candida albicans* and *Aspergillus niger* by cup plate and serial dilution method. Ciprofloxacin (antibacterial) and fluconazole (antifungal) were used as standards drugs, and results are summarized in Table 2. The results of antimicrobial activity by serial dilution followed the same pattern of activity as shown by the cup plate method. Antimicrobial activity by cup plate method and pMIC indicated that the compounds **TH-4** and **TH-19** were more potent than standard drug against *S. aureus*; **TH-3** and **TH-13** were most active against *M. luteus*. **TH-13** showed excellent antibacterial activity than the standard drug against *E. coli*. The compound **TH-18** exhibited significant antimicrobial activity against *P. aeruginosa*, and values were comparable to standards drug. Among fungal strains, compounds **TH-3** and **TH-9** were found to be the most potent against *A. niger*, and **TH-13** showed moderate potency against *C. albicans*.

Free radical scavenging activity. Free radical scavenging activity of synthesized [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives was checked by 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) method and results are summarized in Table 3. Overall, 50% inhibition concentration activity (IC$_{50}$) of screened compounds ranging between 8.1 ± 0.325 to 239.6 ± 0.361 μg/mL. The compound **TH-13** was more potent with the IC$_{50}$ value of 8.1 ± 0.325 μg/mL than standard ascorbic acid with IC$_{50}$ value 36.3 ± 0.21 μg/mL, respectively. The compound **TH-18** showed least free radical scavenging activity with IC$_{50}$ value 239.6 ± 0.36 μg/mL, whereas compounds **TH-4**, **TH-6**, and **TH-15** showed moderate IC$_{50}$ value 40.5 ± 0.274, 47.6 ± 0.431, and 43.4 ± 0.338 μg/mL, respectively.

Conclusion. In order to develop new antimicrobial and antioxidant compounds, we have successfully synthesized and reported a series of [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives from different substituted aryl acetic acids. The compounds 3-(4-chlorobenzyl)-6-nonyl-

Table 1

Substitution arrangement and physicochemical properties of final derivatives.

Compound	R_1	R_2	R_f value	% age yield
TH-1	C_6H_5	2-Cl-5-$NO_2C_6H_3$	0.89[a]	75.1
TH-2	C_6H_5	$(C_6H_5)_2CH$	0.90[a]	81.4
TH-3	C_6H_5	2,4-$Cl_2C_6H_3CH_2$	0.82[a]	69.3
TH-4	C_6H_5	n-C_9H_{19}	0.89[a]	76.0
TH-5	C_6H_5	4-F-C_6H_4	0.75[a]	71.2
TH-6	4-$CH_3OC_6H_4$	2-Cl-5-$NO_2C_6H_3$	0.89[a]	71.4
TH-7	4-$CH_3OC_6H_4$	$(C_6H_5)_2CH$	0.90[a]	77.3
TH-8	4-$CH_3OC_6H_4$	2,4-$Cl_2C_6H_3CH_2$	0.87[a]	83.0
TH-9	4-$CH_3OC_6H_4$	n-C_9H_{19}	0.92[a]	75.0
TH-10	4-$CH_3OC_6H_4$	4-F-C_6H_4	0.94[a]	69.0
TH-11	4-ClC_6H_4	2-Cl-5-$NO_2C_6H_3$	0.88[a]	80.0
TH-12	4-ClC_6H_4	$(C_6H_5)_2CH$	0.91[b]	83.0
TH-13	4-ClC_6H_4	2,4-$Cl_2C_6H_3CH_2$	0.83[b]	79.0
TH-14	4-ClC_6H_4	n-C_9H_{19}	0.90[b]	72.0
TH-15	4-ClC_6H_4	4-F-C_6H_4	0.94[a]	81.0
TH-16	2,4-$Cl_2C_6H_3$	2-Cl-5-$NO_2C_6H_3$	0.89[b]	61.0
TH-17	2,4-$Cl_2C_6H_3$	$(C_6H_5)_2CH$	0.92[a]	64.0
TH-18	2,4-$Cl_2C_6H_3$	2,4-$Cl_2C_6H_3CH_2$	0.79[a]	77.0
TH-19	2,4-$Cl_2C_6H_3$	n-C_9H_{19}	0.78[a]	80.0
TH-20	2,4-$Cl_2C_6H_3$	4-F-C_6H_4	0.88[a]	68.3

TLC solvent system.
[a]$CHCl_3$: Methanol (7:3).
[b]Toluene: Ethylacetate: Formic acid (5:4:1).

Table 2

pMIC in μmol/mL and zone of inhibition (mm) of [1,2,4]triazolo[3,4-*b*] [1,3,4]thiadiazole derivatives.

Compound	*Staphylococcus aureus*	*Micrococcus luteus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Aspergillus niger*	*Candida albicans*
TH-1	2.07 (16)	1.47 (9)	2.07 (19)	1.47 (11)	1.47 (10.5)	1.47 (9)
TH-2	2.08 (17)	-	1.18 (11.5)	-	1.48 (11.5)	1.48 (9)
TH-3	2.38 (18)	1.77 (13)	1.47 (10)	-	**2.07** (**15.5**)	1.47 (9)
TH-4	**2.64** (**20**)	-	2.03 (18)	1.73 (12)	1.73 (12)	-
TH-5	1.39 (12)	-	-	-	1.09 (9)	1.69 (10)
TH-6	1.80 (13)	1.50 (9)	-	1.50 (11)	1.80 (11)	-
TH-7	2.42 (18)	1.21 (9)	1.51 (11)	-	1.21 (9)	-
TH-8	2.11 (17)	-	1.51 (12)	1.51 (10)	1.81 (13)	-
TH-9	2.38 (18)	1.47 (9)	2.08 (17)	-	**2.08** (**15.5**)	1.17 (9)
TH-10	1.43 (12)	-	1.43 (10)	1.43 (10)	1.43 (11)	1.13 (8.5)
TH-11	2.41 (19)	-	-	-	1.21 (9)	-
TH-12	1.82 (14)	-	2.12 (15)	-	1.52 (11)	1.22 (8.5)
TH-13	2.11 (17)	**1.81** (**13.5**)	**2.72** (**24**)	1.91 (15)	1.81 (12)	**1.89** (**12**)
TH-14	1.78 (13)	1.17 (9)	1.47 (10)	-	1.47 (11)	1.78 (10)
TH-15	1.44 (12)	-	1.44 (12)	-	1.74 (12.5)	1.13 (8.5)
TH-16	1.54 (11)	-	-	-	1.54 (10.5)	1.54 (9)
TH-17	2.46 (15)	1.55 (8.5)	1.55 (12)	-	1.55 (10)	1.25 (8.5)
TH-18	2.15 (16)	1.24 (8)	-	**2.15** (**15**)	1.55 (10.5)	1.55 (9)
TH-19	**2.72** (**19**)	1.20 (8.5)	1.21 (9)	1.51 (11)	1.21 (9)	-
TH-20	1.18 (9)	1.78 (12)	2.08 (15)	1.78 (14)	1.18 (9)	1.18 (8)
Standard	2.32 (27)[a]	2.32 (20)[a]	2.32 (24)[a]	2.32 (25)[a]	2.29 (17)[b]	1.99 (16)[b]

[a]Ciprofloxacin.
[b]Fluconazole.
Most active to moderately active compounds in series are highlighted in bold.

[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole and 3-(2,4-dichlorobenzyl)-6-nonyl-[1,2,4]triazolo [3,4-*b*][1,3,4] thiadiazole exhibited higher antibacterial potential than standard drug against *S. aureus* which may be attributed to the presence of long alkyl chain (n-C_9H_{19}) at R_2 position. The fact is further supported by the higher

Table 3

Results of DPPH free radical scavenging activity of [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives.

Compound	IC_{50} ± SD (μg/mL)	Compound	IC_{50} ± SD (μg/mL)
TH-1	68.1 ± 0.342	**TH-11**	65.3 ± 0.448
TH-2	78.1 ± 0.621	**TH-12**	60.8 ± 0.387
TH-3	53.2 ± 0.812	**TH-13**	**8.1 ± 0.325**
TH-4	**40.5 ± 0.274**	**TH-14**	68.2 ± 0.453
TH-5	66.2 ± 0.601	**TH-15**	43.4 ± 0.338
TH-6	47.6 ± 0.431	**TH-16**	54.2 ± 0.491
TH-7	55.6 ± 0.517	**TH-17**	70.2 ± 0.427
TH-8	139 ± 0.457	**TH-18**	239.6 ± 0.361
TH-9	60.4 ± 0.551	**TH-19**	89.1 ± 0.229
TH-10	60.1 ± 0.363	**TH-20**	85.1 ± 0.327
Ascorbic acid	36.3 ± 0.21	Blank	--

Most active to moderately active compounds in series are highlighted in bold.

antifungal activity of the compound 3-(4-methoxybenzyl)-6-nonyl-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole against *A. niger* with the pMIC value of 2.08 μmol/mL evidenced by the presence of the alkyl chain at R_2 position (n-C_9H_{19}). The presence of Di-substituted chloro group on R_2 aromatic ring and chloro substitution on R_1 aromatic ring of compound 6-(2,4-dichlorobenzyl)-3-(4-chlorobenzyl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole showed excellent antioxidant activity as well as the broad spectrum of antimicrobial activity and hence can be selected as lead compound to discover a broad-spectrum antimicrobial drug candidate with antioxidant properties.

EXPERIMENTAL

Chemicals employed for the synthetic work were purchased from S.D. Fine Chem Laboratories, Hi-Media Laboratories, and Sigma Aldrich as "synthesis grade" and were used without further purification. The reaction progress was determined by open capillary tubes on digital auto melting point apparatus. The reaction progress was monitored by TLC on silica gel-G coated plates and spot detected under UV light and iodine chamber. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on Bruker Avance II 400 NMR spectrometer using deuterated $CDCl_3$ as a solvent and were expressed in parts per million (δ, ppm) downfield from tetramethylsilane (internal standard). Infrared (IR) spectra were recorded on Perkin Elmer, BX II spectrophotometer using KBr pellets. LC–MS were recorded on ABI Sciex 5800 TOF/TOF System with LC-MALDI. Elemental analysis was performed on Carlo Erba 1106 CHN analyzer.

General procedure for the synthesis of methyl aryl acetate (1). A mixture of aryl acetic acid (0.1 mol), methanol (1 mol), and 1.1 mL of H_2SO_4 (0.1 mol) was refluxed for 5–6 h at 60–70°C, and completion of the reaction was checked by silica gel G coated TLC plates using ethyl acetate and hexane (3:2) as an eluent, observed under iodine chamber, evaporated the excess of methanol, and extracted the methyl ester with the help of separating funnel, and washed with a strong solution of potassium hydrogen carbonate until all free acid was neutralized and washed with water, dried, and stored in airtight container.

General procedure for the synthesis of 2-arylacetohydrazide (2). A mixture of methyl aryl acetate (0.1 mol) and hydrazine hydrate (0.116 mol) was refluxed with 25 mL of absolute ethanol for 6–7 h, and reaction completion was checked by TLC plates. The excess of ethanol was distilled off; the reaction mixture was cooled and kept overnight. Then, aryl acetohydrazide was recrystallized with ethanol and stored [15].

General procedure for the synthesis of potassium dithiocarbazinate (3). A solution of potassium hydroxide (0.0067 mol) in absolute ethanol (30 mL) was taken in a beaker; aryl acetohydrazide (0.003 mol) and carbon disulfide (0.006 mol) were added dropwise along with stirring, and the mixture was agitated for 16 h. To the resulting solution, anhydrous ether was added, and the precipitated potassium dithiocarbazinate was collected by filtration, washed with diethyl ether, and dried. The potassium salt was obtained in quantitative yield and was used in the next step without further purification [16].

General procedure for the synthesis of 4-amino-5-aryl-4*H*-1,2,4-triazole-3-thiol (4). The earlier potassium salt, hydrazine hydrate (1.5 mL), and water (1.0 mL) were heated under reflux for 5 h; hydrogen sulfide gas evolved, and homogeneous solution was obtained, which was diluted with 50 mL of water, and subsequent acidification with dilute acetic acid gives white precipitate. The precipitates were washed with water and recrystallized from methanol. The purity was checked by silica gel G coated TLC plates using toluene: ethyl acetate: formic acid (5:4:1) as a solvent system [16].

General procedure for the synthesis of 3-aryl-6-aryl/alkyl[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole (TH1–20). An equimolar mixture of 4-amino-5-aryl-4*H*-1,2,4-triazole-3-thiol (0.1 mol) and appropriate aromatic/aliphatic acids in 10 mL of phosphorus oxychloride were refluxed for 5 h. The reaction mixture was cooled to room temperature and poured onto crushed ice with continuous stirring. The mixture was allowed to stand overnight, and the solid separated out was filtered, treated with dilute sodium hydroxide solution, and washed thoroughly with cold water. The compound was dried and recrystallized from methanol [2].

3-Benzyl-6-(2-chloro-5-nitrophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-1). Yield 75.1%; mp: 138–140°C, IR (KBr, cm^{-1}): 3060 (C-H str. Ar), 1609 (C=N str), 1578 (C=C str, Ar), 1534, 1345 (C-NO_2 str), 739 (C-Cl

str), 692 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.5 (s, 2H, CH_2), 7.2–7.4 (m, 5H, ArH), 7.6–7.8 (m, 3H, ArH); MS (*m/z*): 371.9933 (M+), 373.9939 (M + 2); *Anal*. Calcd for $C_{16}H_{10}ClN_5O_2S$ (371.80): C, 51.69; H, 2.71; N, 18.84. Found: C, 51.64; H, 2.68; N, 18.80.

6-Benzhydryl-3-benzyl-[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole (TH-2). Yield 81.4%; mp: 140–142°C; IR (KBr, cm^{-1}): 3019 (C-H str, Ar), 1629 (C=N str) 1598 (C=C str, Ar), 1511 (C-N str), 695 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.6 (s, 2H, CH_2), 5.8 (s, 1H, CH), 7.2–7.5 (s, 15H, ArH); MS (*m/z*): 382.9834 (M+); *Anal*. Calcd for $C_{23}H_{18}N_4S$ (382.48): C, 72.22; H, 4.74; N, 14.65. Found: C, 72.18, H, 4.76, N, 14.61.

6-(2,4-Dichlorobenzyl)-3-benzyl-[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole (TH-3). Yield 69.3%; mp: 162–164°C; IR (KBr, cm^{-1}): 3020 (C-H str, Ar), 1618 (C=N str), 1576 (C=C str, Ar), 733 (C-Cl str), 704 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.6 (s, 2H, CH_2), 4.1–4.3 (t, 2H, CH_2), 6.9–7.1 (m, 5H, ArH), 7.2 (m, 3H, ArH); MS (*m/z*): 374.9303 (M+). *Anal*. Calcd for $C_{17}H_{12}Cl_2N_4S$ (375.27): C, 54.41; H, 3.22; N, 14. Found: C, 54.45; H, 3.20; N, 14.90.

3-Benzyl-6-nonyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-4). Yield 76%; mp: 58–60°C; IR (KBr, cm^{-1}): 3025 (C-H str, Ar), 1655 (C=N str), 1646 (C-N str), 1560 (C=C band, Ar), 1465 (C-H bend, CH_2), 1375 (C-H bend, CH_3), 696 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.8–0.9 (t, 3H, CH_3), 1.27–1.40 (t, 8H, CH_2), 1.8 (t, 2H, CH_2), 2.17 (s, 2H, CH_2), 3.0 (s, 2H, CH_2), 4.1 (s, 2H, CH_2), 4.5 (s, 2H, CH_2), 7.2–7.4 (m, 5H, ArH); MS (*m/z*): 342.9183 (M +); *Anal*. Calcd for $C_{19}H_{26}N_4S$ (342.50): C, 66.63; H, 7.65; N, 16.36. Found: C, 66.60; H, 7.61; N, 16.41.

3-Benzyl-6-(4-fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole (TH-5). Yield 71.2%; mp: 164–166°C; IR (KBr, cm^{-1}): 3081 (C-H str, Ar), 1656 (C=N band), 1562 (C=C str, Ar), 1528 (C-N str), 693 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.4–4.5 (d, 2H, CH_2), 7.4–7.3 (m, 5H, ArH), 7.4 (m, 4H, ArH); MS (*m/z*): 309.9961 (M +), 311.9922 (M + 2); *Anal*. Calcd for $C_{16}H_{11}FN_4S$ (310.34): C, 61.92; H, 3.57; N, 18.05. Found: C, 61.87; H, 3.55; N, 18.11.

3-(4-Methoxybenzyl)-6-(2-chloro-5-nitrophenyl)-[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole (TH-6). Yield 71.4%; mp: 164–166°C; IR (KBr, cm^{-1}): 3065 (C-H str, Ar), 1608 (C=N band), 1569, 1343 (-NO_2 str), 1243, 1031 (C-O-C str), 744 (C-Cl str), 695 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.78 (s, 3H, CH_3), 4.5 (s, 2H, CH_2), 6.88–6.90 (m, 3H, ArH), 7.2–7.4 (m, 4H, ArH); MS (*m/z*): 401.9901 (M+); *Anal*. Calcd for $C_{17}H_{12}ClN_5O_3S$ (401.82): C, 50.81; H, 3.01; N, 17.43; O, 11.94. Found: C, 50.78; H, 2.97; N, 17.49; O, 11.90.

3-(4-Methoxybenzyl)-6-benzhydryl-[1,2,4]triazolo[3,4-b] [1,3,4]thiadiazole (TH-7). Yield 77.3%; mp: 128–130°C; IR (KBr, cm^{-1}): 3028 (C-H str, Ar), 1610 (C=N str), 1590 (C=C str, Ar), 1249, 1030 (C-O-C str), 698 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.7 (d, 3H, CH_3), 4.45 (s, 2H, CH_2), 5.0 (s, 1H, CH), 7.2–7.4 (m, 10H, ArH), 7.49–7.56 (m, 4H, ArH); MS (*m/z*): 412.9383 (M+), 414.8918 (M + 2); *Anal*. Calcd for $C_{24}H_{20}N_4OS$ (412.50): C, 69.88; H, 4.89; N, 13.58; O, 3.88. Found: C, 69.82; H, 4.85; N, 13.63; O, 3.93.

6-(2,4-Dichlorobenzyl)-3-(4-methoxybenzyl)-[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole (TH-8). Yield 83%; mp: 168–170°C; IR (KBr, cm^{-1}): 3064, 3002 (C-H str, Ar), 1611 (C=N str), 1585 (C=C str, Ar), 1255, 1036 (C-O-C str), 739 (C-Cl str), 684 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.9–3.0 (s, 2H, CH_2), 3.7 (s, 3H, CH_3), 4.3–4.5 (d, 2H, CH_2), 6.8 (m, 3H, ArH), 7.2–7.3 (m, 4H, ArH); MS (*m/z*): 404.9604 (M+); *Anal*. Calcd for $C_{18}H_{14}Cl_2N_4OS$ (405.30): C, 53.34; H, 3.48; N, 13.82; O, 3.95. Found: C, 53.29; H, 3.43; N, 13.87; O, 3.99.

3-(4-Methoxybenzyl)-6-nonyl-[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole (TH-9). Yield 75%; mp: 56–58°C; IR (KBr, cm^{-1}): 3002 (C-H str, Ar), 1611 (C=N str), 1580 (C=C str, Ar), 1465 (C-H bend, CH_2), 1375 (C-H bend, CH_3), 1251, 1031 (C-O-C str), 692 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.86–0.89 (d, 3H, CH_3), 1.2 (s, 2H, CH_2), 1.3–1.4 (t, 4H, CH_2), 1.6 (t, 2H, CH_2), 1.7–1.8 (t, 2H, CH_2), 2.1 (s, 2H, CH_2), 2.3 (t, 2H, CH_2), 2.9 (t, 2H, CH_2), 3.77–3.83 (q, 3H, CH_3), 4.3 (s, 2H, CH_2), 7.2–7.3 (m, 4H, ArH); MS (*m/z*): 372.9270 (M+); *Anal*. Calcd for $C_{20}H_{28}N_4OS$ (372.52): C, 64.48; H, 7.58; N, 15.04; O, 4.29. Found: C, 64.44; H, 7.62; N, 15.09; O, 4.33.

3-(4-Methoxybenzyl)-6-(4-fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-10). Yield 69%; mp: 130–132°C, IR (KBr, cm^{-1}): 3014 (C-H str, Ar), 1604 (C=N str), 1546 (C=C str, Ar), 1515 (C-N str), 1251, 1032 (C-O-C str), 1104 (C-F str), 692 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.77 (s, 3H, CH_3), 4.49 (s, 2H, CH_2), 6.8 (m, 4H, ArH), 7.1–7.2 (m, 4H, ArH); MS (*m/z*): 340.9981 (M+); *Anal*. Calcd for $C_{17}H_{13}FN_4OS$ (340.37): C, 59.99; H, 3.85; N, 16.46; O, 4.70. Found: C, 59.94; H, 3.82; N, 16.49; O, 4.73.

3-(4-Chlorobenzyl)-6-(2-chloro-5-nitrophenyl)-[1,2,4] triazolo[3,4-b][1,3,4]thiadiazole (TH-11). Yield 80%; mp: 142–144°C; IR (KBr, cm^{-1}): 3067 (C-H str, Ar), 1608 (C=N str), 1568, 1344 (-NO_2 str), 1525 (C-N str), 742 (C-Cl str), 689 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.5 (d, 2H, CH_2), 7.3 (m, 3H, ArH), 7.4 (m, 4H, ArH); MS (*m/z*): 406.9921 (M+); *Anal*. Calcd for $C_{16}H_9Cl_2N_5O_2S$ (406.24): C, 47.30; H, 2.23; N, 17.24; O, 7.88. Found: C, 47.34; H, 2.21; N, 17.19; O, 7.91.

3-(4-Chlorobenzyl)-6-benzhydryl-[1,2,4]triazolo[3,4-b][1,3,4] thiadiazole (TH-12). Yield 83%; mp: 64–66°C; IR (KBr, cm^{-1}): 3058, 3025 (C-H str, Ar), 1615 (C=N str), 1580 (C=C str, Ar), 1516 (C-N str), 747 (C-Cl str), 699 (C-S str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.6 (d, 2H, CH_2), 5.7 (s, 1H, CH), 7.1–7.4 (m, 14H, ArH); MS (*m/z*): 418.9903 (M + 2); *Anal*. Calcd for $C_{23}H_{17}ClN_4S$

(416.92): C, 66.26; H, 4.11; N. 13.44. Found: C, 66.21; H, 4.14; N, 13.41.

6-(2,4-Dichlorobenzyl)-3-(4-chlorobenzyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-13). Yield 79%; mp: 84–86°C; IR (KBr, cm^{-1}): 3070, 3025 (C-H str, Ar), 1610 (C=N str), 1584 (C=C str, Ar), 759 (C-Cl str), 685 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.6 (s, 2H, CH_2), 4.1–4.3 (t, 2H, CH_2), 6.9–7.1 (m, 4H, ArH), 7.2 (m, 3H, ArH); MS (*m/z*): 407.9929 (M+); *Anal*. Calcd for $C_{17}H_{11}Cl_3N_4S$ (409.72): C, 49.83; H, 2.71; N, 13.67. Found: C, 49.80; H, 2.73; N, 13.64.

3-(4-Chlorobenzyl)-6-nonyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-14). Yield 72%; mp: 110–112°C; IR (KBr, cm^{-1}): 3010 (C-H str, Ar), 1610 (C=N str), 1580 (C=C str, Ar), 1517 (C-N str), 1467 (C-H bend, CH_2), 1376 (C-H bend, CH_3), 721 (C-Cl str), 697 (C-S str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.8–0.9 (q, 3H, CH_3), 1.2–1.4 (t, 2H, CH_2), 1.6 (q, 2H, CH_2), 1.8 (t, 2H, CH_2), 2.1 (s, 2H, CH_2), 2.3 (s, 2H, CH_2), 2.99 (s, 2H, CH_2), 3.49 (s, 2H, CH_2), 4.5 (s, 2H, CH_2), 7.2–7.3 (m, 4H, ArH); MS (*m/z*): 376.9735 (M+); *Anal*. Calcd for $C_{19}H_{25}ClN_4S$ (376.94): C, 60.54; H, 6.68; N, 14.86. Found: C, 60.51; H, 6.71; N, 14.83.

3-(4-Chlorobenzyl)-6-(4-fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-15). Yield 81%; mp: 158–160°C; IR (KBr, cm^{-1}): 3042 (C-H str, Ar), 1602 (C=N str), 1523 (C-N str), 1092 (C-F str), 758 (C-Cl str), 686 (C-S str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.4–4.5 (d, 2H, CH_2), 7.4–7.3 (m, 4H, ArH), 7.4 (m, 4H, ArH); MS (*m/z*): 344.9116 (M+); *Anal*. Calcd for $C_{16}H_{10}ClFN_4S$ (344.79): C, 55.74; H, 2.92; N; 16.25. Found: C, 55.70; H, 2.87; N, 16.29.

3-(2,4-Dichlorobenzyl)-6-(2-chloro-5-nitrophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-16). Yield 61%; mp: 142–144°C; IR (KBr, cm^{-1}): 3067 (C-H str, Ar), 1602 (C=N str), 1566, 1350 (-NO_2 str), 1525 (C-N str), 743 (C-Cl str), 684 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.6–4.7 (d, 2H, CH_2), 7.2–7.3 (m, 3H, ArH), 7.4 (m, 3H, ArH); MS (*m/z*): 438.9981 (M+). *Anal*. Calcd for $C_{16}H_8Cl_3N_5O_2S$ (440.69): C, 43.61; H, 1.83; N, 15.89. Found: C, 43.65; H, 1.86; N, 15.85.

3-(2,4-Dichlorobenzyl)-6-benzhydryl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-17). Yield 64%; mp: 60–62°C; IR (KBr, cm^{-1}): 3058, 3028 (C-H str, Ar), 1586 (C=N str), 1514 (C-N str), 745 (C-Cl str), 699 (C-S str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.4–4.6 (t, 2H, CH_2), 5.7 (d, 2H, CH_2), 6.99–7.5 (m, 13H, ArH); MS (*m/z*): 452.9912(M + 2); *Anal*. Calcd for $C_{23}H_{16}Cl_2N_4S$ (451.37): C, 61.20; H, 3.57; N, 12.41. Found: C, 61.25; H, 3.53; N; 12.39.

3,6-Bis(2,4-dichlorobenzyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-18). Yield 77%; mp: 138–142°C; IR (KBr, cm^{-1}): 3010 (C-H str, Ar), 1653 (C=N str), 1586 (C=C str, Ar), 1512 (C-N str), 758 (C-Cl str), 676 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.3 (d, 2H, CH_2), 4.5 (d, 2H, CH_2), 7.1 (m, 3H, ArH), 7.2 (m, 3H, ArH); MS (*m/z*): 443.9751 (M+); *Anal*. Calcd for $C_{17}H_{10}Cl_4N_4S$ (444.16): C, 45.97; H, 2.27; N, 12.61. Found: C, 45.94; H, 2.30; N, 12.57.

3-(2,4-Dichlorobenzyl)-6-nonyl-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-19). Yield 80%; mp: 134–136°C; IR (KBr, cm^{-1}): 3010 (C-H str, Ar), 1660 (C=N str), 1582 (C=C str, Ar), 1522 (C-N str), 1470 (C-H bend, CH_2), 1385 (C-H bend, CH_3), 721 (C-Cl str), 695 (C-S-C str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 0.8–0.9 (t, 3H, CH_3), 1.2–1.4 (t, 8H, CH_2), 1.81 (s, 2H, CH_2), 2.17 (s, 2H, CH_2), 2.99–3.0 (s, 2H, CH_2), 3.4 (s, 2H, CH_2), 4.6 (s, 2H, CH_2), 7.23–7.40 (m, 3H, ArH); MS (*m/z*): 412.9015 (M + 2); *Anal*. Calcd for $C_{19}H_{24}Cl_2N_4S$ (411.39): C, 55.47; H, 5.88; N, 13.62. Found: C, 55.44; H, 5.86; N, 13.65.

3-(2,4-Dichlorobenzyl)-6-(4-fluorophenyl)-[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole (TH-20). Yield 68.3%; mp: 124–126°C; IR (KBr, cm^{-1}): 3114 (C-H str, Ar), 1600 (C=N str), 1520 (C-N str), 1043 (C-F str), 693 (C-S str); ^{1}H-NMR ($CDCl_3$) δ (ppm): 4.5–4.6 (t, 2H, CH_2), 7.3 (m, 4H, ArH), 7.2 (m, 3H, ArH); MS (*m/z*): 378.9764 (M+); *Anal*. Calcd for $C_{16}H_9Cl_2FN_4S$ (379.23): C, 50.67; H, 2.39; N; 14.77. Found: C, 50.62; H, 2.36; N, 14.79.

Biological evaluation of synthesized 1,2,4-triazolo-1,3,4-thiadiazole derivatives: Evaluation of antimicrobial activity. The antibacterial activity was performed by cup plate and serial dilution methods against Gram-positive bacteria [*S. aureus* (MTCC 3160) and *M. luteus* (MTCC 1538)] and Gram-negative bacteria [*E. coli* (MTCC 1652) and *P. aeruginosa* (MTCC 424)] and antifungal activity against *A. niger* (MTCC 8652) and *C. albicans* (MTCC 227) [15,17].

Cup-plate method. The 10 μg/mL of dilutions of standard drugs (ciprofloxacin and fluconazole) and test compounds were prepared from 100 μg/mL of stock solutions and used for cup plate method. After the incubation period, the diameter of the zone of inhibition in plates was recorded.

Serial dilution method. On the confirmation of the antimicrobial activity of compounds by the cup-plate method, serial dilution method was performed. Different seven concentrations (50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 μg/mL) of each test compound and standard drugs were prepared by serial dilution method and used for the antimicrobial activity. The minimum inhibitory concentration was observed by the turbidity of assay tubes, and pMIC values were calculated by the following formula:

$$\text{pMIC} = -\log\left(\frac{\text{MIC}}{\text{Molecular weight}}\right) \quad (1)$$

Free radical scavenging activity. Free radical scavenging activity of synthesized derivatives was screened by using 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH). On reduction, the deep violet color of DPPH in

methanol changes into yellow color, and a decrease in absorption was observed at 517 nm. Five concentrations (25, 50, 75, 100, and 125 μg/mL) was prepared in methanol and 2 mL of each concentration of test was mixed with equal volume of 3 μg/mL of concentration of DPPH in methanol solution. The absorption was determined by UV–Vis spectrophotometer against blank, and screening was performed in triplicate. Ascorbic acid was used as positive control. The percentage of free radical scavenging was calculated by equation 2. IC_{50} values of the test compounds were calculated from percentage scavenging activity (Table 3) [18,19].

$$\text{Scavenging activity } (\%) = \left[\frac{A - B}{A}\right] \times 100 \qquad (2)$$

where A is the absorbance of the control (containing methanolic DPPH and ascorbic acid) and B is the absorbance of the test compound (containing methanolic DPPH and test compound).

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