Full Paper

Synthesis, *in-vitro* Microbial and Cytotoxic Studies of New Benzimidazole Derivatives

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Several new classes of benzimidazole derivatives were synthesized and evaluated for *in-vitro* antimicrobial and cytotoxic activities. The results showed that all synthesized compounds exhibited moderate antimicrobial activity, and compounds **2**, **4**, and **13** displayed cytotoxic activity (as LD_{50}) at the concentration 1×10^{-3} M against *Artemia salina*.

Keywords: Coumarin / Pyrazole / Thiazolidine / Triazolo-thiadiazole / 2-(Trifluoromethyl)-1*H*-benzimidazole

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Introduction

Recently, fluorinated heterocycles have attracted attention due to the ability of fluorine to act as polar hydrogen or hydroxyl mimic. Substituted 2-(trifluoromethyl)-1*H*-benzimidazoles are known as an important class of compounds due to their wide range of biological activity acting as antiviral, antifungal, antibacterial, and anticancer drugs [1–4]. These compounds also inhibit photosynthesis and, therefore, exhibit appreciable herbicidal activity [5]. Most recently, antiparasitic activities of this class of compounds have been reported [6, 7].

A survey of literature revealed that *S*-triazolo[3,4-*b*]-1,3,4-thiadiazole rings, have received much attention during recent years on possessing a broad spectrum of biological activities [8–13], in addition, thiazolidines also exhibit a broad spectrum of pharmacological properties [14]. Moreover, a triazolo-thiadiazole system may be viewed as a cyclic analogue of two very important components – a thiosemicarbazide [15] and a biguanide [16], which often display diverse biological activities. Also, coumarin derivatives condensed with other heterocycles shows a wide range of pharmacological activities [17–20]. Therefore, prompted by these observations, syntheses and *in-vitro* biological evaluations of the new benzimidazole derivatives were performed and are described in the present study.

The synthesized compounds were tested for their possible *in-vitro* antimicrobial and cytotoxic activities. Most of the tested compounds showed activity against *Vancomycin-resistant Enteroccoccus* (ATCC-51299), *Staphylococcus aureus* (ATCC-29213), *Micrococcus* (natural isolates), *Bacillus subtilis* (natural isolates), *Shigella dysentery* (natural isolates), *Escherichia coli* (ATCC-25922) – as examples for Gram-positive and Gram-negative bacteria, and *Candida albicans*, *Aspergillus niger*, *Penicillium* as representatives of fungi. The minimum inhibitory concentration (MIC) was determined for the test compounds as well as for the reference standards.

Results and discussion

Chemistry

For the synthesis of the target compounds, the reaction sequences outlined in Schemes 1, 2, and 3 were followed. Hydrolysis of 2-(trifluoromethyl)-1*H*-benzimidazole **I** in NaOH / HCl gives the 1*H*-benzimidazole-2-carboxylic acid **II**. Treatment of compound **II** with thionyl chloride followed by hydrazine hydrate gives the desired 1*H*-benzimidazole-2-carboxylic acid hydrazide **III** in 90% yield.

In the present work, compound **III** was used as the key intermediate for the subsequent synthesis. Ethyl acetoacetate, thioglycolic acid, acetyl acetone, and chloroacetyl chloride were reacted with compound **III** resulting in the

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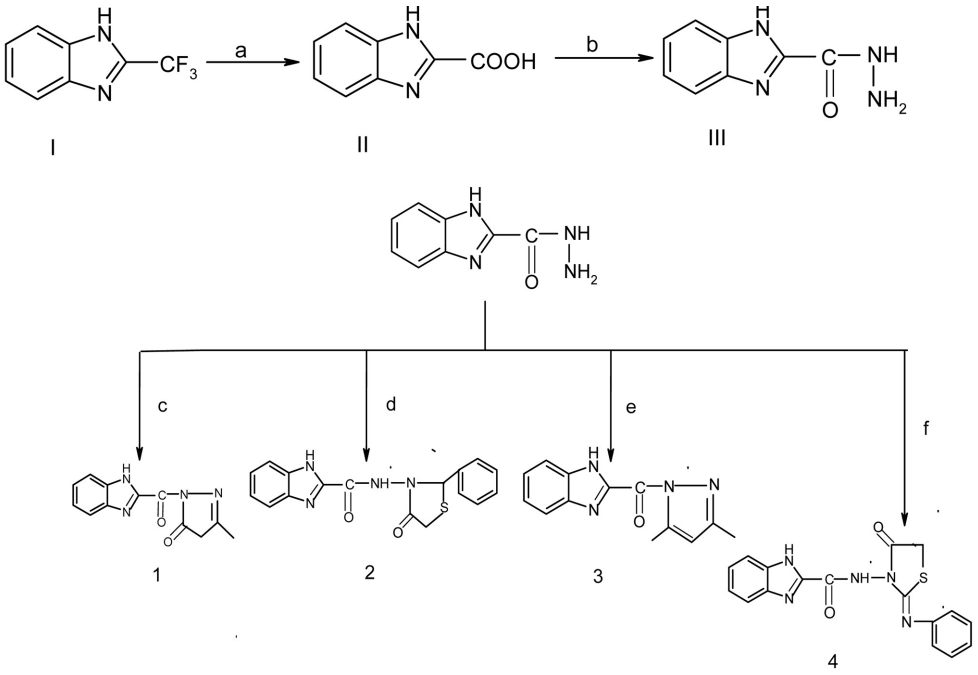


Reactions and conditions: a) 2 N NaOH / 2 N HCl; b) $SOCl_2$ / $N_2H_4 \times H_2O$; c) $CH_3COOCH_2COC_2H_5$, C_2H_5OH; d) C_6H_5CHO, $SHCH_2COOH$, C_2H_5OH; e) $CH_3COCH_2COCH_3$, C_2H_5OH; f) C_6H_5SCN, $ClCH_2COCl$, C_2H_5OH.

Scheme 1. Schematic representation of compounds **1**–**4**.

Table 1. Molecular formula, yields, and melting points of the newly synthesized compounds.

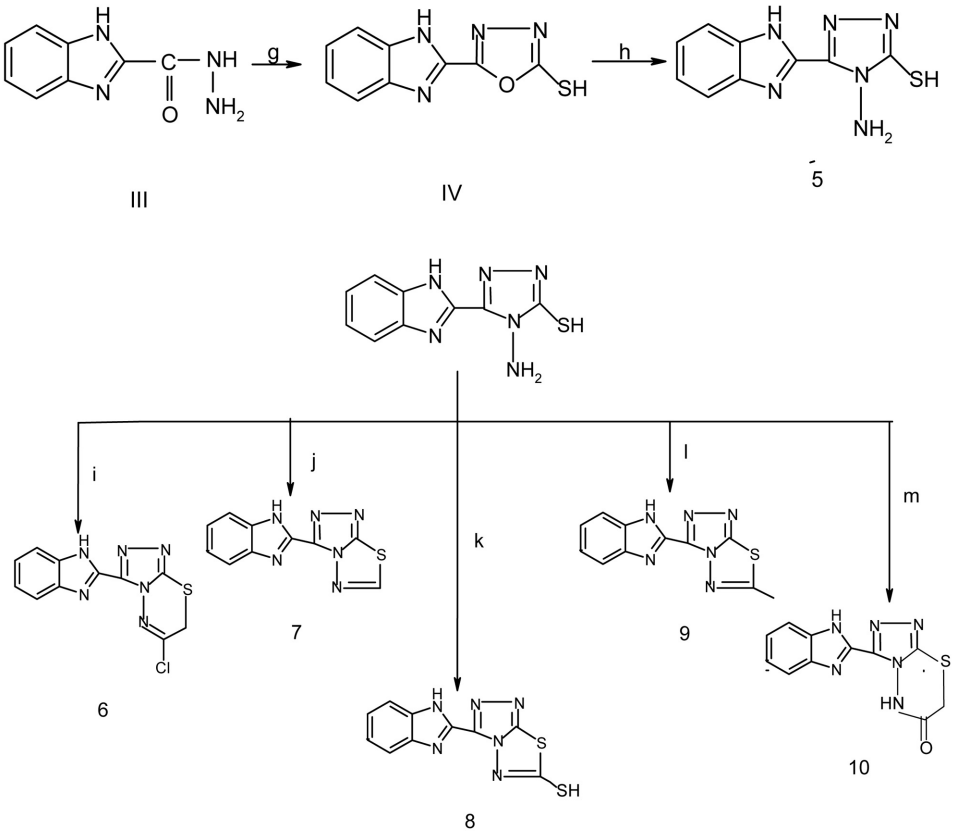
Compound	Molecular Formula	Yield (%)	Melting Point (°C)
1	$C_{12}H_{10}N_4O_2$	73.40	253–255
2	$C_{13}H_{12}N_4O$	78.70	245–247
3	$C_{17}H_{14}N_4O_2S$	71.21	284–286
4	$C_{17}H_{13}N_5O_2S$	85.93	229–231
5	$C_9H_8N_6S$	81.14	266–268
6	$C_{11}H_7ClN_6S$	87.86	254–256
7	$C_{10}H_6N_6S$	65.97	273–275
8	$C_{10}H_6N_6S_2$	77.01	289–291
9	$C_{11}H_8N_6S$	70.35	230–232
10	$C_{11}H_8N_6OS$	73.22	241–243
11	$C_{19}H_{13}F_3N_2O_2$	71.33	180–182
12	$C_{18}H_{10}ClF_3N_2O_2$	69.68	225–227
13	$C_{22}H_{13}F_3N_2O_2$	77.55	204–206

corresponding derivatives of pyrazoles and thiazolidines **1**–**4**, respectively (Scheme 1, Table 1). Reacting compound **III** with carbon disulphide and then with hydrazine hydrate (99%) gives 4-amino-5-(1*H*-benzimidazole-2-yl)-4*H*-[1,2,4]triazole-3-thiol **5** (Scheme 2, Table 1). Then, compound **5** was treated with chloroacetyl chloride, formic acid, carbon disulfide, acetic acid, and chloroacetic acid to give the corresponding triazolo-thiadiazole derivatives **6**–**10**, respectively. Further condensation of the novel substituted 4-bromomethyl coumarins with compound **I** afforded the corresponding **11**, **12**, and **13**, respectively (Scheme 3, Table 1).

Biological evaluation

Antimicrobial activity

All the test compounds were assayed for *in-vitro* antimicrobial activity, and the strains used in this study were maintained at the Department of Botany, Karnatak University, Dharwad, India. The antimicrobial activity was determined by using disk-diffusion method [21–23] and MIC (twofold serial dilution method). Streptomycin and Nystatin were used as reference standards to compare antibacterial and antifungal activities, respectively. For determining the antimicrobial activity, the synthesized compounds were dissolved in dimethyl sulphoxide (stock solution 1 mg/mL). Further dilutions were prepared at the required quantities of 100, 50, and 25 μg/mL concentrations. In order to ensure that the solvent had no effect on bacterial growth, a control test was also performed containing a disc loaded only with DMSO at the same dilutions used in our experiment. For determining the MIC of the synthesized compounds, they were diluted at 100, 50, 25, and 12 μg/mL concentrations and expressed in μM/mL. The MIC of the compounds was defined as the lowest concentration at which there was 100% inhibition



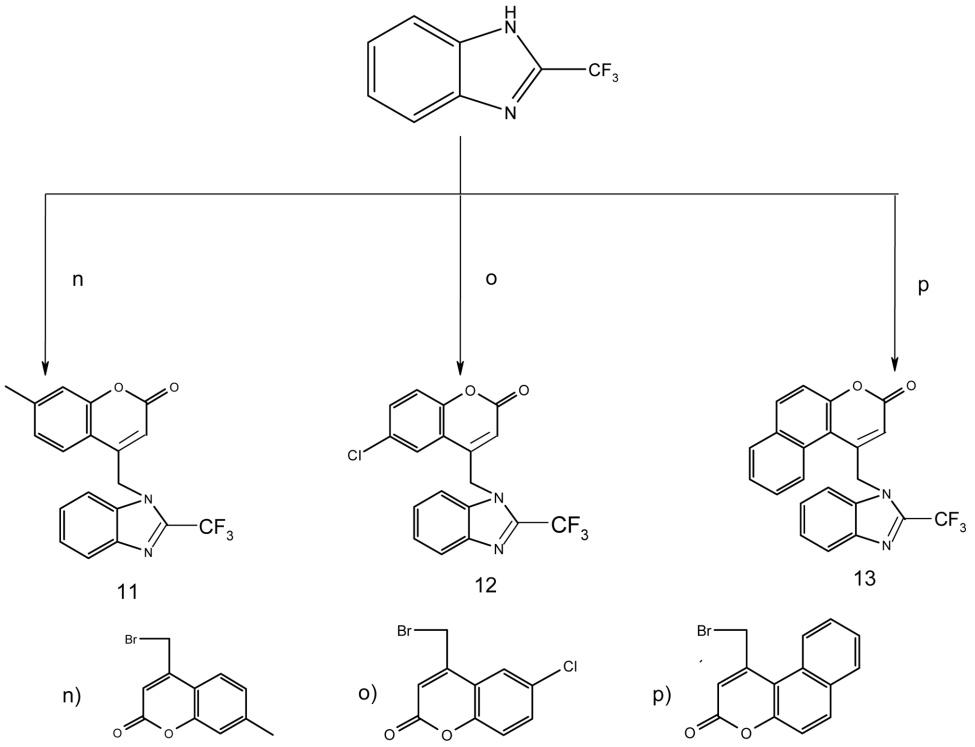
Reactions and conditions: g) CS_2 / KOH, C_2H_5OH; h) $N_2H_4 \times H_2O$, C_2H_5OH; i) $ClCH_2COCl$, C_2H_5OH; j) HCOOH / H_2SO_4; k) CS_2, / KOH, C_2H_5OH; l) CH_3COOH / $POCl_3$; m) $ClCH_2COOH$ / NaOAc, C_2H_5OH.

Scheme 2. Schematic representation of compounds **5**–**10**.

Table 2. Antibacterial activity: The MIC values of compounds **1**–**13** (in μM/mL).

Compound	V. r. e.[a)]	S. a.[b)]	M.[c)]	B. s.[d)]	S. d.[e)]	E. c.[f)]
1	1.27–2.17	0.37–0.84	0.59–1.31	0.88–2.32	1.27–2.27	≥2.73
2	≥2.76	0.72–1.65	0.75–1.41	≥2.82	0.75–1.49	1.85–2.87
3	≥2.52	0.34–0.89	≥2.21	0.27–0.77	1.36–2.21	≥2.15
4	≥2.82	1.65–2.37	0.41–0.78	1.71–2.37	0.34–0.78	1.67–2.35
5	≥2.43	0.88–1.51	0.55–1.39	≥2.12	0.65–1.39	≥2.29
6	0.97–2.92	0.34–0.88	≥2.32	≥2.31	1.43–2.78	1.66–2.91
7	≥2.83	≤0.28	1.56–2.37	≥2.41	1.56–2.35	≥2.73
8	≥2.33	0.37–0.91	0.21–0.79	1.31–2.57	1.56–2.35	0.95–1.72
9	≥2.14	≥2.12	0.94–2.29	≥2.82	0.23–0.72	0.23–0.68
10	0.85–2.35	≥2.49	0.33–0.81	≥2.82	1.33–2.53	1.56–3.13
11	0.23–0.82	1.43–2.22	≥2.31	≥2.12	≥2.14	≥2.29
12	0.65–1.42	0.39–1.71	≥3.33	1.31–2.57	≥2.33	≥2.15
13	≥2.82	0.78–1.67	1.62–2.63	≥2.87	1.58–2.33	1.45–2.72
SD[g)]	0.88–1.81	≤0.88	≤0.88	0.88–1.81	1.81–3.36	≤0.88

[a)] *V. r. e.*: *Vancomycin-resistant enteroccoccus*.
[b)] *S. a.*: *Staphylococcus aureus*.
[c)] *M.*: *Micrococcus*.
[d)] *B. s.*: *Bacillus subtilis*.
[e)] *S. d.*: *Shigella dysentery*.
[f)] *E. c.*: *Escheria coli*.
[g)] SD: Streptomycin (Standard).



Scheme 3. Schematic representation of compounds **11** – **13**.

of growth compared with the growth for a drug-free control. Every experiment in the antibacterial assay and MIC was replicated thrice.

Cytotoxic activity

Brine-shrimp (*Artemia salina* leach) eggs were hatched in a shallow rectangular plastic dish (22 × 32 cm) filled with artificial seawater, which was prepared with a commercial salt mixture and double-distilled water. An unequal partition was made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs were sprinkled into the large compartment, which was darkened while the other compartment was opened to ordinary light. After two days, nauplii were collected by pipette from the lighted side. A sample of the test compound was prepared by dissolving 20 mg of each compound in 2 mL of DMF. From these stock solutions, 100, 50, and 25 μg/mL were transferred to nine vials (three for each dilution were used for each test sample, and LD_{50} is the mean of three values) and one vial was kept as control having 2 mL of DMF only. The solvent was allowed to evaporate overnight. After two days, when the shrimp larvae were ready, 1 mL of seawater and ten shrimps were added to each vial (30 shrimps/dilution) and the volume was adjusted with seawater to 5 mL per vial. After 24 h, the numbers of survivors were counted [24]. Data were analyzed by a Finney computer program to determine the LD_{50} values [25].

Table 3. Antifungal activity: The MIC values of compounds **1** – **13** (in μM/mL).

Compound	C. a.[a)]	A. n.[b)]	P.[c)]
1	0.69 – 1.33	1.33 – 2.45	>2.63
2	0.28 – 0.85	0.89 – 1.85	1.51 – 3.47
3	1.44 – 3.11	1.67 – 2.71	1.65 – 2.33
4	0.34 – 0.78	0.81 – 1.56	1.56 – 3.13
5	0.31 – 0.75	≤0.27	0.78 – 1.46
6	1.72 – 2.82	>2.08	1.51 – 2.73
7	≤0.33	0.37 – 0.78	0.78 – 1.65
8	0.73 – 1.46	1.43 – 2.71	>2.71
9	0.33 – 0.72	>2.97	>2.78
10	0.32 – 0.79	0.39 – 0.91	1.61 – 3.12
11	≤0.37	0.84 – 1.56	1.66 – 2.21
12	0.82 – 1.56	1.52 – 2.84	0.63 – 1.39
13	≤0.35	≤0.29	1.39 – 3.18
SD[d)]	≤1.82	≤3.54	≤3.54

[a)] *C. a.*: *Candida albicans*.
[b)] *A. n.*: *Aspergillus niger*.
[c)] *P.*: *Penicillium*.
[d)] SD: Nystatin (Standard).

Table 4. Brine-shrimp bioassay data for compounds **1**–**13**.

Compound	LD_{50} (mol/mL)
1	4.531×10^{-3}
2	8.742×10^{-4}
3	6.398×10^{-3}
4	8.324×10^{-4}
5	4.054×10^{-3}
6	5.728×10^{-3}
7	7.750×10^{-3}
8	5.431×10^{-3}
9	6.839×10^{-3}
10	4.241×10^{-3}
11	4.733×10^{-3}
12	5.135×10^{-3}
13	7.517×10^{-4}

Conclusion

This synthetic approach provides convenient access to new structurally complex, pharmacologically interesting five- and six-member benzimidazole derivatives in good purities. The bioassays indicated that all the compounds showed *in-vitro* antimicrobial activity against six strains of bacteria and three strains of fungi. Compounds **2**, **4**, **7**, and **13** displayed potent cytotoxic activity against *Artemia salina*.

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The authors have declared no conflict of interest.

Experimental

Materials and methods

Thin layer chromatography was used to confirm the completion of the reaction and the purity of the compounds synthesized. Melting points were determined with open capillary method and are uncorrected. IR spectra were recorded on a Nicolet 5700 FT-IR instrument (Nicolet, Madison, WI, USA) as potassium bromide discs. The ^{1}H- and ^{13}C-NMR spectra were measured with a Bruker-300 (Bruker Bioscience, USA), 300 MHz instrument using TMS as internal standard and DMSO-d_6. All chemical shifts were reported as δ values (in ppm). Mass spectra with ionization energy maintained at 70 eV using were taken on a Shimadzu spectrometer (GC-MS) (Shimadzu, Tokyo, Japan).

Chemistry

General procedure for the preparation of 1H-benzimidazole-2-carboxylic acid II

1.861 g (0.01 mol) 2-(trifluoromethyl)-1*H*-benzimidazole was added to a cooled solution of sodium hydroxide (30 mL, 1 N). The resulting solution was kept for 1 h and the solution was filtered. The filtrate was acidified with hydrochloric acid (1 N) to pH 4. The precipitate was filtered off, washed twice with cold water, and recrystallized from DMSO solvent.

IR (KBr): ν (cm^{-1}) 3340 br (ArNH), 1665 (C=O), 1592 (C=O), 1580 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz,) δ (ppm): 4.16 (br, s, 1H, NH-benzimidazole), 7.16 (m, 2H, Ar-H), 7.59 (s, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 75 MHz,) δ (ppm): 111.97 (s, C, Ar-C), 137.1 (s, C, Ar-C), 145.03 (s, C, C of imidazole), 171.2 (s, C, C of carboxylic acid); CIMS *m*/*z*: 162.14: Anal. Calcd. for $C_8H_6N_2O_2$: C, 59.26; H, 3.73; N, 17.28; O, 19.73. Found: C, 59.25; H, 3.74; N, 17.29; O, 19.71. M.p.: 170–172°C.

General procedure for the preparation of 1H-benzimidazole-2-carboxylic acid hydrazide III

1.621 g (0.01 mol) of 1*H*-benzimidazole-2-carboxylic acid and thionyl chloride 1.78 g (0.01 mol) were refluxed for about 1 h. Excess thionyl chloride was removed by repeated evaporation *in vacuo*. Finally, we get 1*H*-benzimidazole-2-carboxylic acid chloride as intermediate product. An appropriate amount of 1*H*-benzimidazole-2-carboxylic acid chloride (1.803 g, 0.01 mol) and hydrazine hydrate (1.761 g, 0.01 mol) in 15 mL of dry ethanol was refluxed for 3–4 h. After completion of the reaction, the solution was concentrated and the resulting solid was collected, washed with ether, and recrystallized from DMSO / water.

IR (KBr) ν (cm^{-1}): 3370 (aliphatic NH_2), 3356.5 br (ArNH), 2915 (aliphatic NH), 1652 (C=O), 1579 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.3 (s, 2H, aliphatic NH_2, 3.93 (br, s, 1H, NH-benzimidazole), 7.21 (s, 2H, Ar-H), 7.63 (d, *J* = 8.1 Hz, 2H, Ar-H), 8.24 (s, 1H, aliphatic NH); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm): 114.0 (s, C, Ar-C), 121.6 (s, C, Ar-C), 139.9 (s, C, Ar-C), 142.7 (s, C, C of imidazole), 161.04 (s, C, C of C=O); CIMS *m*/*z*: 176.17: Anal. Calcd. for $C_8H_8N_4O$: C, 54.54; H, 4.58; N, 31.80; O, 9.08. Found: C, 54.55; H, 4.57; N, 31.79; O, 9.09. M.p.: 217–219°C.

2-(1H-Benzimidazole-2-ylcarbonyl)-5-methyl-2,4-dihydro-3H-pyrazol-3-one ***1***

A mixture of carbohydrazide (0.01 mol) and ethyl acetoacetate (0.01 mol) in absolute ethanol (20 mL) was heated at reflux temperature for 3–4 h. The reaction mixture was cooled and the formed precipitate was filtered off and recrystalized to yield compound **1**.

IR (KBr) ν (cm^{-1}): 3310 br (ArNH), 744 (C=O of pyrazolone), 1592 (C=O), 1563 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz,) δ (ppm): 2.50 (s, 3H, CH_3 of pyrazolone), 3.86 (br, s, 1H, NH-benzimidazole), 7.33 (d, *J* = 7.8 Hz, 2H, Ar-H), 7.70 (m, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm): 20.70 (s, C, CH_3 of pyrazolone), 115.67 (s, C, Ar-C), 120.9 (s, C, Ar-C), 168.0 (s, C, C=O), 170.75 (s, C, C=O of pyrazolone); CIMS *m*/*z*: 242.23: Anal. Calcd. for $C_{12}H_{10}N_4O_2$: C, 59.50; H, 4.16; N, 23.13; O, 13.21. Found: C, 59.48; H, 4.15; N, 23.10; O, 13.20.

N-(4-Oxo-2-phenyl-1,3-thiazolidin-3-yl)-1H-benzimidazole-2-carboxamide ***2***

A mixture of carbohydrzide (0.01 mol) and the benzaldehyde (0.01 mol) was refluxed in absolute ethanol (20 mL) for 5–6 h. The reaction mixture was concentrated, cooled, and the formed precipitate was filtered off, dried, and then recrystallized to give the intermediate Schiff base. A mixture of the above-mentioned compound (0.005 mol) and thioglycolic acid (0.006 mol) was refluxed in dry benzene (30 mL) for 6–7 h. The solvent was

evaporated and the reaction mixture was neutralized with cold dilute sodium bicarbonate solution, the formed product was filtered off and recrystallized from ethanol / water.

IR (KBr) ν (cm^{-1}): 3240 br (ArNH), 2915 (CH), 2971 m (AlNH), 1723 (C=O of thiazolidinone), 1599 (C=O, CONH), 1552 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.12 (s, 2H, CH_2 of thiazolidinyl), 3.52 (br, s, 1H, NH-benzimidazole), 5.52 (s, 1H, CH of thiazolidinyl), 6.95 (d, *J* = 5.7 Hz, 2H, Ar-H), 7.07 (s, 1H, Ar-H), 7.16 (s, 2H, Ar-H), 7.39 (m, 1H, CONH), 7.28 (d, *J* = 7.4 Hz, 2H, Ar-H), 7.73 (s, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm): 35.6 (s, C, CH_2 of thiazolidinyl), 115.32 (s, C, Ar-C), 133.49 (s, C, Ar-C), 163.01 (s, C, C=O of thiazolidinyl), 167.32 (s, C, CONH); CIMS *m*/*z*: 338.38: Anal. Calcd. for $C_{13}H_{12}N_4O$: C, 64.99; H, 5.03; N, 23.32; O, 6.66. Found: C, 64.98; H, 5.01; N, 23.30; O, 6.65.

2-[(3,5-Dimethyl-1H-pyrazol-1-yl)carbonyl]-1H-benzimidazole ***3***

A mixture of carbohydrazide (0.01 mol) and acetyl acetone (0.01 mol) in absolute ethanol (20 mL) was heated at reflux temperature for 4 – 5 h. The reaction mixture was cooled and the formed precipitate was filtered off and recrystallized from ethanol / water.

IR (KBr) ν (cm^{-1}): 3445 br (ArNH), 1623 (C=O), 1595 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.50 (d, 6H, CH_3 of pyrazolyl), 4.13 (br, s, 1H, NH-benzimidazole), 7.33 (s, 1H, CH of pyrazolyl), 7.35 (d, *J* = 7.2 Hz, 2H, Ar-H), 7.70 (s, 2H, Ar-H); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm): 13.8 (s, C, CH_3 of pyrazolyl), 122.3 (s, C, Ar-C), 125.3 (s, C, Ar-C), 143.23 (s, C, C=O); CIMS *m*/*z*: 240.26: Anal. Calcd. for $C_{17}H_{14}N_4O_2S$: C, 60.34; H, 4.17; N, 16.56; S, 9.48. Found: C, 60.31; H, 4.16; N, 16.54; S, 9.46.

N-[(2 E or Z)-4-Oxo-2-(phenylimino)-1,3-thiazolidin-3-yl]-1H-benzimidazole-2-carboxamide ***4***

To a solution of carbohydrazide (0.01 mol) in ethanol (5 mL) phenyl isothiocyanate (0.01 mol) and sodium hydroxide (0.01 mol, 2 N) were added. The mixture was stirred for 24 h and filtered. The filtrate was acidified with hydrochloric acid. The precipitate was filtered and recrystallized from ethanol / water, to give the intermediate compound. A mixture of the above-mentioned compound (0.005 mol) and chloroacetyl chloride (0.005 mol) in chloroform (30 mL) was refluxed for 6-7 h. The solvent was distilled off under reduced pressure and the residue was washed with ethanol, filtered, washed again with water, and recrystallized from DMSO / water, to give compound **4**.

IR (KBr) ν (cm^{-1}): 3244 br (ArNH), 3060 m (AlNH), 1753 (C=O of thiazolidinyl), 1583 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.81 (s, 2H, CH_2 of thiazolidinyl), 4.25 (br, s, 1H, NH-benzimidazole), 7.28 (d, *J* = 6.9 Hz, 2H, Ar-H), 7.42 (s, 5H, Ar-H), 7.89 (s, 2H, Ar-H), 10.51 (m, 1H, CONH); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm): 33.20 (s, C, CH_2 of thiazolidinyl), 127.01 (s, C, Ar-C), 130.22 (s, C, Ar-C), 165.33 (s, C, CONH), 173.1 (s, C, C=O of thiazolidinyl); CIMS *m*/*z*: 351.38: Anal. Calcd. for $C_{17}H_{13}N_5O_2S$: C, 58.11; H, 3.73; N, 19.93; S, 9.13; O, 9.11. Found: C, 58.10; H, 3.71; N, 19.91; S, 9.12; O, 9.12.

4-Amino-5-(1H-benzimidazole-2-yl)-4H-[1,2,4] triazole-3-thiol ***5***

To a mixture of carbohydrazide (0.01 mol) in ethanol (20 mL) and a solution of potassium hydroxide (0.015 mol) in ethanol (10 mL) was added followed by carbon di-sulfide (10 mL); the mixture was heated under reflux for 6 – 7 h, then it was concentrated, acidified with dilute HCl, and the resulting solid was collected, washed with water, and recrystallized from a suitable solvent to give the intermediate compound. A mixture of this compound (0.01 mol) and 99% hydrazine hydrate (0.03 mol) in absolute ethanol (20 mL) was refluxed for 6 – 7 h. The solvent and excess hydrazine hydrate were removed under reduced pressure, the residue washed with ether, then recrystallized from DMF / water (1 : 1).

IR (KBr) ν (cm^{-1}): 3300 (NH_2), 3145 br (ArNH), 2654 (SH), 1595 (C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.62 (br, s, 1H, NH-benzimidazole), 7.27 (s, 2H, Ar-H), 7.57 (d, *J* = 7.9 Hz, 2H, Ar-H), 7.84 (s, 1H, SH), 10.67 (s, 2H, NH_2); ^{13}C-NMR (DMSO-d_6, 75 MHz) δ (ppm): 110.02 (s, C, Ar-C), 127.9 (s, C, Ar-C), 143.33 (s, C, Ar-C), 151.18 (s, C, Ar-C); CIMS *m*/*z*: 232.25: Anal. Calcd. for $C_9H_8N_6S$: C, 46.54; H, 3.47; N, 36.18; S, 13.81. Found: C, 46.52; H, 3.46; N, 36.15; S, 13.80.

3-(1H-Benzimidazole-2-yl)-6-chloro-7H-[1,2,4]triazole [3,4-b] [1,3,4] thiadiazine ***6***

To a suspension of the corresponding compound **5** (0.01 mol) in absolute ethanol (15 mL), chloro acetyl chloride (0.01 mol) was added and the reaction mixture was refluxed for 2 – 3 h, cooled to room temperature and then neutralized with ammonia. The precipitate was filtered off and washed with water. The solid obtained was recrystallized from DMSO / water (1 : 1).

IR (KBr) ν (cm^{-1}): 3430 br (ArNH), 1595, 1551, 1501 & 1462 (4 C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.20 (s, 2H, CH_2 of thiadiazine), 4.76 (br, s, 1H, NH-benzimidazole), 7.28 (s, 2H, Ar-H), 7.90 (d, *J* = 8.5 Hz, 2H, Ar-H) CIMS *m*/*z*: 290.73. Anal. Calcd. for $C_{11}H_7ClN_6S$: C, 45.44; H, 2.43; N, 28.91; S, 11.03; Cl, 12.19. Found: C, 45.42; H, 2.41; N, 28.90; S, 11.01; Cl, 12.18.

2-[1,2,4] Triazolo [3,4-b] [1,3,4] thiadizol-3-yl-1H-benzimidazole ***7***

A mixture of the corresponding compound **5** (0.01 mol) and formic acid (1 mL) in dry benzene (20 mL) was refluxed for 1 h. After evaporating the reaction mixture under reduced pressure, an oily product was obtained. The crude product was crystallized from a suitable solvent. The obtained product (0.001 mol) was treated with cold concentrated sulfuric acid (15 mL) and the formed oily mass was poured into water. The precipitate was recrystallized from DMSO / water (1 : 1).

IR (KBr) ν (cm^{-1}): 3446 br (ArNH), 1595, 1552, 1500 & 1462 (4 C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.45 (br, s, 1H, NH-benzimidazole), 7.27 (d, *J* = 7.0 Hz, 2H, Ar-H), 7.43 (s, 2H, Ar-H), 8.21 (s, 1H, CH of thiadiazole); CIMS *m*/*z*: 242.26: Anal. Calcd. for $C_{10}H_6N_6S$: C, 49.58; H, 2.50; N, 34.69; S, 13.24. Found: C, 49.55; H, 2.47; N, 34.67; S, 13.22.

3-(1H-Benzimidazole-2-yl)-[1,2,4] triazolo [3,4-b] [1,3,4] thiadiazole-6-thiol ***8***

To a solution of the corresponding compound **5** (0.01 mol) in ethanol, KOH (1 g) and carbon disulphide (2 mL) were added and the reaction content was allowed to reflux for 2 – 3 h. The solvent was removed under reduced pressure; then, ice water added onto the reaction content while stirring. The obtained solid was washed with water and recrystallized from ethanol / water.

IR (KBr) ν (cm^{-1}): 3401 br (ArNH), 2655 (SH), 1595, 1552, 1500 & 1462 (4 C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.68 (br, s,

1H, NH-benzimidazole), 7.27 (d, *J* = 7.3 Hz, 2H, Ar-H), 7.57 (s, 1H, SH), 7.89 (d, *J* = 8.1 Hz, 2H, Ar-H); CIMS *m/z*: 274.33. Anal. Calcd. for $C_{10}H_6N_6S_2$: C, 43.78; H, 2.20; N, 30.64; S, 23.38. Found: C, 43.76; H, 2.20; N, 30.63; S, 23.37.

2-(6-Methyl-[1,2,4] triazolo [3,4-b] [1,3,4] thiadiazol-3-yl-)-1H-benzimidazole ***9***

To a mixture of the corresponding compound **5** (0.01 mol), phosphorus oxychloride (10 mL) was added and the reaction contents were refluxed for 2–3 h on a water bath. After removing the excess of phosphorus oxychloride under reduced pressure, ice water was added to the residue with vigorous stirring. The precipitate was filtered off and washed with 20% sodium bicarbonate solution and water. This white solid was recrystalized from DMSO / water (1 : 1).

IR (KBr) ν (cm^{-1}): 3430 br (ArNH), 1621, 1551, 1500 & 1460 (4 C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 1.62 (s, 3H, CH_3), 3.72 (br, s, 1H, NH-benzimidazole), 7.18 (d, *J* = 7.4 Hz, 2H, Ar-H), 7.61 (s, 2H, Ar-H); CIMS *m/z*: 256.29. Anal. Calcd. for $C_{11}H_8N_6S$: C, 51.55; H, 3.15; N, 32.79; S, 12.51. Found: C, 51.53; H, 3.14; N, 32.77; S, 12.49.

3-(1H-Benzimidazol-2-yl)-5H-[1,2,4] triazolo [3,4–b][1,3,4] thiadiazin-6(7H)-one ***10***

α-Chloroacetic acid (0.012 mol) and (0.01 mol) compound **5** were suspended in absolute ethanol (30 mL) along with anhydrous fused sodium acetate (1 g) and refluxed for 4–6 h on a water bath. The resulting clear solution was then concentrated, cooled, and poured into ice water. The separated solid was filtered and recrystalized from DMSO / water to give compound **10**.

IR (KBr) ν (cm^{-1}): 3410 br (ArNH), 1718 (C=O), 1595, 1551 & 1500 (3 C=N); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 3.80 (br, s, 1H, NH-benzimidazole), 7.27 (s, 2H, CH_2 of thiadiazine), 7.56 (d, *J* = 7.6 Hz, 2H, Ar-H), 7.91 (d, *J* = 8.3 Hz, 2H, Ar-H), 10.67 (br, s, 1H, NH of thidiazine); CIMS *m/z*: 272.29. Anal. Calcd. for $C_{11}H_8N_6OS$: C, 48.52; H, 2.96; N, 30.86; S, 11.78; O, 5.88. Found: C, 48.51; H, 2.95; N, 30.83; S, 11.76; O, 5.89.

7-Methyl-4-{[2-(trifluoromethyl)-1H-benzimidazol-1-yl]methyl}-2H-chromen-2-one ***11***

2-(Trifluoromethyl)-1*H*-benzimidazole (0.01 mol) and anhydrous potassium carbonate (0.01 mol) was stirred in dry acetone (25 mL) for 30 min. 4-Bromomethyl coumarin (0.01 mol) was added and stirring was continued for 24 h. The reaction mixture was concentrated to one fourth of the original volume and poured into ice-cold water. The solid separated was filtered and washed with 50% HCl to neutralize excess potassium carbonate. Then, the solid was washed with 100 mL of cold water and with dilute ethanol. The crude product was dried and recrystallized from DMF.

IR (KBr) ν (cm^{-1}): 1721 (C=O), 1621 (C=N), 1196 (C-O), 1139 (CF_3); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.46 (s, 3H, CH_3), 4.49 (s, 2H, CH_2), 6.47 (s, 1H, Ar-H), 7.19 (s, 1H, Ar-H), 7.26 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.28 (s, 1H, Ar-H), 7.57 (s, 1H, Ar-H), 7.60 (d, *J* = 7.9 Hz, 2H, Ar-H); CIMS *m/z*: 358.31. Anal. Calcd. for $C_{19}H_{13}F_3N_2O_2$: C, 63.69; H, 3.66; N, 7.82; O, 8.93; F, 15.91. Found: C, 63.70; H, 3.68; N, 7.84; O, 8.92; F, 15.90.

*6-Chloro-4-{[2-(trifluoromethyl)-1H-benzimidazol-1-yl]methyl}-2H-*chromen-2-one ***12***

Compound **12** was prepared according to the procedure described above for compound 11.

IR (KBr) ν (cm^{-1}): 1715 (C=O), 1601 (C=N), 1147 (C-O), 1117 (CF_3), 742 (C-Cl); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.98 (s, 2H, CH_2), 5.61 (s, 1H, Ar-H), 5.66 (s, 1H, Ar-H), 7.28 (s, 1H, Ar-H), 7.38 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.61 (s, 1H, Ar-H), 7.64 (d, *J* = 8.0 Hz, 2H, Ar-H); CIMS *m/z*: 378.73. Anal. Calcd. for $C_{18}H_{10}ClF_3N_2O_2$: C, 57.08; H, 2.66; N, 7.40; O, 8.45; Cl, 9.36; F, 15.05. Found: C, 57.09; H, 2.68; N, 7.43; O, 8.44; Cl, 9.37; F, 15.04.

1-{[2-(Trifluoromethyl)-1H-benzimidazol-1-yl]methyl}-3H-benzo [f] chromen-3-one ***13***

Compound **13** was prepared according to the procedure described above for compound 11.

IR (KBr) ν (cm^{-1}): 1726 (C=O), 1624 (C=N), 1169 (C-O), 1123 (CF_3); ^{1}H-NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.46 (s, 3H, CH_3), 4.79 (s, 2H, CH_2), 6.17 (s, 1H, Ar-H), 7.12 (s, 1H, Ar-H), 7.19 (s, 2H, Ar-H), 7.28 (d, *J* = 7.7 Hz, 2H, Ar-H), 7.48 (s, 1H, Ar-H), 7.61 (s, 2H, Ar-H), 7.72 (d, *J* = 8.2 Hz, 2H, Ar-H); CIMS *m/z*: 394.35: Anal. Calcd. for $C_{22}H_{13}F_3N_2O_2$: C, 67.01; H, 3.32; N, 7.10; O, 8.11; F, 14.45. Found: C, 67.00; H, 3.31; N, 7.10; O, 8.10; F, 14.46.

Biological evaluation

Antibacterial activity

The antibacterial activity of the benzimidazole derivatives was tested by the agar disc-diffusion method against Gram-positive and Gram-negative bacteria. Test-compound solutions were prepared in DMSO, were serially diluted and loaded (10 µL) to sterile filter-paper discs (6 mm diameter), which finally contained 25, 50, and 100 µg/mL of the compound per disc, respectively. Impregnated disks were then dried for 1 h and placed on inoculated plates. The seeded plates were incubated at 37°C for 16 h. The radii of inhibition zones (in mm) of triplicate sets were measured and the standard MIC for streptomycin is *Vancomycin-resistant enteroccoccus* (0.88–1.81), *Staphylococcus aureus* (≤0.88), *Micrococcus* (≤0.88), *Bacillus subtilis* (0.88–1.81), *Shigella dysentery* (1.81–3.36), and *Escheria coli* (≤0.88). Every experiment in the antibacterial assay was replicated thrice in order to define the MIC values, as shown in Table 2.

Among all compounds tested, **2**, **4**, and **13** exhibited better antibacterial activity against the Gram-positive *Bacillus subtilis* than the antibiotic streptomycin. On the other hand, compound **7** acted against the Gram-negative *Escheria coli* in a way comparable with the antibiotic streptomycin.

Antifungal activity

The synthesized benzimidazole derivatives were tested for their antifungal activity *in vitro* in comparison with Nystatin (Nystatin Ns 100, HIMEDIA, 100 units/disc) as a reference drug using the standard agar disk diffusion method against three strains of fungi (*Aspergillus niger*, *Penicillium*, and *Candida albicans*). A spore suspension in sterile distilled water was prepared from 3 to 5 days-old culture of the test fungi growing on Potato Dextrose Agar (PDA) media. The final spore concentration was 5×10^{-4} spores/mL. About 15 mL of the growth medium was placed into sterilized Petri dishes of 9 cm diameter and inoculated with 100 µL of the spore suspension. Sterile 6-mm filter paper disk (HiMedia Laboratories Pvt. Ltd, India) was saturated with 10 µL

of the test compound solution. Impregnated disks were then dried for 1 h and placed on inoculated plates. The seeded plates were incubated at 27°C for four days. The radii of inhibition zones (in mm) of triplicate sets were measured and the standard MIC for Nystatin is *Aspergillus niger* (≤3.54), *Penicillium* (3.54), and *Candida albicans* (≤1.82) at 100 units, as showed in Table 3.

It is evident from the screening data (MIC shown in Table 3) that compounds **2** and **4** were more effective against *Aspergillus niger*, compared with the standard Nystatin. The remaining compounds showed moderate antifungal activity against all fungal species with a MIC of 50 μg/mL.

Cytotoxic activity

As can be seen from the data recorded in Table 4, only compounds **2**, **4**, and **13** displayed cytotoxic activity as LD_{50} at concentrations of 1×10^{-3} M against *Artemia salina*, while the remaining compounds were almost inactive in this assay.

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