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Synthesis, Antimicrobial Evaluation and Chemical Stability Studies of Novel Trisubstitued Benzimidazoles

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Abstract

Background: Development of new potent antimicrobials has remained one of the thrust research areas across the globe due to emergence of resistance among infectious microbe for clinically used antimicrobials. Structural similarity of benzimidazole with purines and its presence in metabolite of vitamin B12 unveils it as a potential nucleus for development of novel antimicrobials.

Objective: With this background, the current study is aim at designing, synthesizing and exploring novel benzimidazole derived compounds as potential antimicrobial agents.

Method: Extensive literature study led to design of two series of 1,2,5-trisubstituted benzimidazole derivatives (4a-4f and 5a-5f). The designed compounds were synthesized from o-phenylenediamine, nicotinic acid, furfuraldehyde and varied benzoyl/benzyl chlorides through a 4-steps synthetic scheme. In-vitro antimicrobial evaluation was carried out by measuring zone of inhibition at different concentrations (1.56-100 µg/mL) of target compounds and streptomycin. Docking analysis was carried out using GlcN-6-P synthase as target enzyme as it is essential for microbial cell wall synthesis. Hydrolytic stability of the most active compound (4d) in GIT was evaluated in non-enzymatic simulated gastric and intestinal fluids through HPLC method.

Results: Compounds 4a, 4d and 4f exhibit good antibacterial activity, whereas 5a-5c have potent antifungal properties. Compound 4d is maximally lethal from the series and equipotent to streptomycin against S. aureus, E. coli and P. aeruginosa with MIC 4.16 ± 1.04, 5.20 ± 1.04 and 10.41 ± 2.08 µg/ml, respectively. Compound 5c (MIC 20.83 ± 4.16 µg/ml) was equipotent to fluconazole against C. albicans. Docking studies show good binding affinity of target compounds toward GlcN-6-P. Compound 4d was found stable in non-enzymatic simulated gastric and intestinal fluids up to 4h.

Conclusion: Compound 4d is identified as a promising lead candidate for development of novel and potent anti-microbial compounds.

Keywords: Benzimidazole, Docking, MIC, Anti-microbial, Antifungal

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Introduction

Infectious microbial diseases remain a pressing problem world-wide due to continuous emergence of resistance among pathogenic microorganisms towards widely used antimicrobial agents [1]. Novel chemotherapeutic agents acting through dissimilar modes of actions are particularly beneficial due to their ability to avoid cross resistance. Benzimidazole (BZ) is an important pharmacophore in novel drug development. Owing to the immense importance and diverse bioactivities exhibited by BZ derived compounds [2], efforts are made from time to time to generate libraries of such compounds and screen them for potential biological activities including antimicrobial [2-5], anticancer [6], antioxidant [7], antiasthmatic, antiallergic [8,9], antiprotozoal [10], anticonvulsant [11], antidiabetic

[12], antifertility [13], anti-inflammatory [14,15], antiviral [16], antitubercular [17], antiparasitic [18] and antiulcer [19]. Biochemical and pharmacological studies has affirmed that benzimidazole derivatives are effective against various strains of microorganisms [2-4]. Based on this affirmation, we surveyed the literature critically to study the substitution patterns around various monosubstituted, disubstituted, trisubstituted, tetrasubstituted BZs, bis-benzimidazoles, fused-benzimidazoles and/or their metal complexes for optimum antimicrobial activity. Critical study of these reports on varied BZ derived antimicrobial compounds led to a general presentation of the most accepted types of substituents at different positions of BZ (Figure 1). N-1, C-2, C-5 and C-6 are the potential sites for substitution on BZ to develop novel compounds. Certain chloro- and nitro-benzimidazoles are found antimetabolites of vitamin B12 [20]. A 5- or 6-membered heteroaryl moiety like pyridine, furan, pyrazole or thiophene at C-2 of BZ proves fruitful for antimicrobial activity [21-26] whereas a benzyl group at N-1 of BZ has been widely explored for antimicrobial activity [27-30].

Based on this generalized SAR, and importance of chloro, nitro, benzyl and heteroaryl groups around BZ nucleus, we aimed at designing, synthesizing and exploring novel BZ derived compounds as potential antimicrobial agents. All designed compounds were found novel by ChemID plus software. The synthesized compounds were also subjected to docking studies using glucosamine-6-phosphate (GlcN-6-P) synthase, which is an elite protein responsible for growth of microbes.

MATERIALS AND METHODS

Chemistry

All chemicals used in the study were purchased from Sigma Aldrich, Merck and S.D. Fine chemicals (India). Commercial grade solvents were used and were distilled before use. Melting points were determined on Digital Auto Melting Point Apparatus (Labtronics, India) and were uncorrected. IR spectra of compounds and intermediate were recorded as KBr pellets on a Bruker Optik FT/IR spectrophotometer with vibrational frequencies expressed as cm^{-1}. Bruker (400 MHz) spectrometer was used to record ^{1}H-NMR and ^{13}C-NMR spectra in deuterated dimethyl sulfoxide (DMSO-d_6) using tetramethylsilane (TMS) as internal standard. Chemical shift values were represented in δ (ppm) scales. Mass spectral analyses were performed on a Thermo Scientific mass spectrometer (Model LTQ, XL). Completion of reactions was ascertained by Thin Layer Chromatography (TLC) on silica gel pre-coated aluminum sheets. The synthetic scheme for target compounds is given in Figure 2. Each target compound was synthesized through a simple two-step reaction. The first step involved generation of intermediates 2 and 3 by coupling of 4-nitro-*o*-phenylenediamine with nicotinic acid and furfuraldehyde, respectively. In second step, intermediates 2 or 3 were coupled with variedly substituted benzyol chloride or benzyl chloride to yield the target compounds 4 and 5.

Synthesis of intermediate 2 (5-Nitro-2-(pyridin-3'-yl)-1H-benzimidazole)

For intermediate 2, 0.38 g (0.0025 mol) of 4-nitro-o-phenylenediamine (1) was refluxed with 0.61 g (0.005 mol) of nicotinic acid in the presence of 0.4 g (1 mg/mol of 1) of polyphosphoric acid (PPA) under nitrogen for 3-3.5 h. The reaction mixture was poured into cold water and basified with aqueous ammonia. The product was filtered at pump, washed with cold water and recrystallized from hot aqueous ethanol. Yield 78%, copper red crystals, melting point 180-181°C. IR (KBr, cm^{-1}): N-H (Ar): 3465; C-H (Ar): 3087, 3054; Ring skeleton bands: 1649, 1604, 1580, 1510; N-O str: 1470, 1390. ^{1}H-NMR (DMSO-d_6): 9.42-9.39 (1H, m, Py); 9.29-9.20 (1H, m, Py); 8.56-8.53 (1H, m, Bz); 8.32-8.29 (1H, m, Py); 8.1 (1H, s, Bz); 7.98-7.91 (1H, m, Bz);

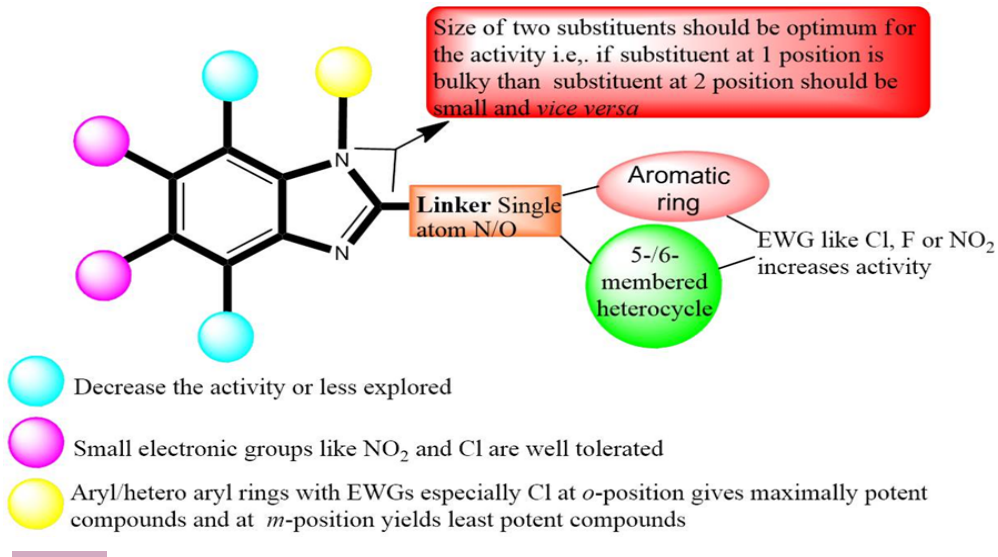

Figure 1 General SAR on the basis of critical analysis of literature of benzimidazole derived compounds.

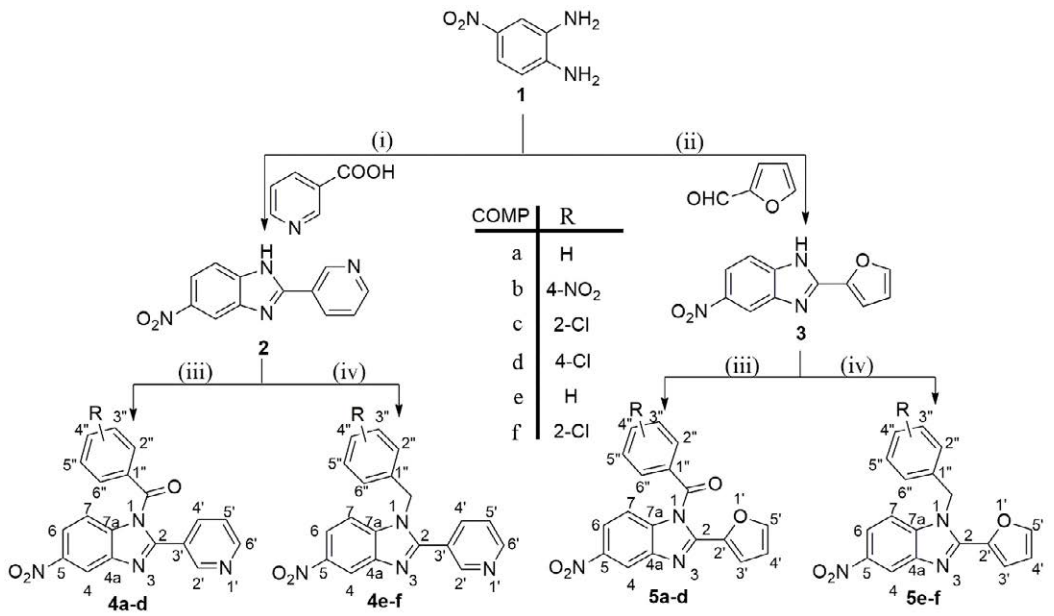

Figure 2 Synthetic scheme of target compounds 4a-f and 5a-f. Reagents and Conditions: (i): PPA, reflux, 3-3.5 h, N2, cold water, NH4OH; (ii): Ethanol, reflux, 4h, r.t, NH4OH; (iii): substituted benzoyl chloride, dry pyridine, reflux, cool, HCl; (iv): substituted benzyl chloride, stirring in acetonitrile, NaOHnzoyl chloride, dry pyridine, reflux, cool, HCl; (iv): substituted benzyl chloride, stirring in acetonitrile, NaOH

7.56-7.49 (1H, m, Py); 5.0 (1H, s, NH). 13C-NMR (DMSO-d6): 155.1 (C2, Py); 152.9 (C2, Bz); 147.9 (C6, Py); 146.8 (C7a, Bz); 144.3 (C5, Bz); 139.8 (C2a, Bz); 135.4 (C4, Py); 132.9 (C3, Py); 124.0 (C5, Py); 188.6 (C6, Bz); 116.1 (C7, Bz); 112.9 (C4, Bz). HRMS (+ESI, *m/z*) calcd. for $C_{12}H_8N_4O_2$ $[M+H]^+$: 241.0721, found: 241.0718.

Synthesis of intermediate 3 (2-(Furan-2'-yl)-5-nitro-1H-benzimidazole)

1.52 g (0.005 mol) of 4-nitro-o-phenylenediamine was refluxed with 0.19 g (0.001 mol) of furfuraldehyde in 20 ml ethanol for 4 h. The reaction mixture was cooled to room temperature and aqueous ammonia was added to it dropwise (2-3 ml) to yield precipitates of 3. These were washed with distilled water and recrystallized from hot aqueous ethanol to obtain 3 as brown powder, Yield 63%, melting point 189-190°C. IR (KBr, cm^{-1}): N-H (Ar): 3470; C-H (Ar): 3079, 3048; C-O: 1300, 1289, 1260; C=C (Ar):1580; N-O: 1470, 1390. ^{1}H-NMR (DMSO-d_6): 8.44 (1H, s, Bz); 8.01-7.97 (1H, m, Bz); 7.65 (1H, d, J=1.6 Hz, Bz); 7.05 (1H, d, J=3.8 Hz, Fr); 6.70 (1H, d, J=8.8 Hz, Fr); 5.03 (1H, s, NH-Bz). ^{13}C-NMR (DMSO-d_6): 154.0 (C2, Fr); 147.8 (C7a, Bz); 144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 139.8 (C3a, Bz); 118.6 (C6, Bz); 116.1 (C7, Bz); 112.9 (C4, Bz); 111.1 (C4, Fr); 107.1 (C3, Fr). HRMS (+ESI, *m/z*) calcd. for $C_{11}H_7N_3O_3$ $[M+H]^+$: 230.0561, found: 230.0556.

General procedure for synthesis of target compounds 4a-d and 5a-d

For the synthesis of compounds 4a-4d and 5a-5d, 0.01 mol of benzoyl chloride of choice and 0.005 mol of 2 or 3, respectively were dissolved in 40 ml of dry pyridine. The reaction mixture was refluxed until both the reactants disappeared on TLC. The reaction mixture was cooled, poured over ice and treated with hydrochloric acid to obtain crude benzoylated products (4a-4d and 5a-5d), which were subsequently purified on silica column with with CHCl3: MeOH solvent system (9:1 v/v) as mobile phase.

5-Nitro-1-benzoyl-2-(pyridin-3'-yl)benzimidazole (4a)

The compound was synthesized from 1.2 g (0.005 mol) of 2 and 1.2 ml (0.01 mol) of benzoyl chloride. Yield: 78%; Melting point: 184-186ºC; IR (KBr, cm^{-1}): C-H (Ar): 3085, 3049; C=O: 1688; Ring skeleton bands: 1607, 1601, 1578, 1548; N-O: 1464, 1380. ^{1}H-NMR (DMSO-d_6): 9.41-9.38 (1H, m, Py); 8.99-8.92 (1H, m, Py); 8.52-8.48 (1H, m, Bz); 8.30-8.28 (1H, m, Py); 8.19 (1H, s, Bz); 7.96-7.93 (2H, m, Ph); 7.86-7.80 (1H, m, Bz); 7.66-7.60 (1H, m, Ph); 7.45-7.39 (2H, m, Ph); 7.19-7.12 (m, 1H, Py). ^{13}C-NMR (DMSO-d_6): 167.7 (C=O); 155.1(C2, Py); 147.9(C6, Py); 144.3 (C5, Bz); 141.5 (C2, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 135.4 (C4, Py); 134.5 (C4, Ph); 133.0 (C1, Ph); 132.9 (C3, Py); 131.1 (C2, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 129.2 (C5, Ph); 124.0 (C5, Py); 118.6 (C6, Bz); 116.1 (C7, Bz);112.9 (C4, Bz). HRMS (+ESI, *m/z*) calcd. for $C_{19}H_{12}N_4O_3$ $[M+H]^+$: 345.0983, found: 345.0974.

1-(4"-Nitrobenzoyl)-5-nitro-2-(pyridin-3'-yl)benzimidazole (4b)

The compound was synthesized from 1.2 g (0.005 mol) of 2 and 1.8 ml (0.01 mol) of 4-nitrobenzoyl chloride. Yield: 75%; Melting point: 205-207ºC; IR (KBr, cm^{-1}): C-H (Ar): 3084, 3050; C=O: 1675; Ring skeleton bands: 1606, 1604, 1575, 1560; N-O: 1460, 1382. ^{1}H-NMR (DMSO-d_6): 9.36-9.31 (1H, m, Py); 9.01-8.98 (1H, m, Py); 8.71-8.69 (1H, m, Bz); 8.26-8.20 (1H, m, Py); 7.93 (1H, s, Bz); 8.09-8.03 (2H, m, Ph); 7.88-7.83 (1H, m, Bz); 7.49-7.43 (2H, m, Ph); 7.18-7.16 (m, 1H, Py). 13C-NMR (DMSO-d6): 167.7 (C=O); 155.1(C2, Py); 153.7 (C4, Ph); 147.9(C6, Py); 144.3 (C5, Bz); 141.5 (C2, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 135.4 (C4, Py); 133.0

(C1, Ph); 132.9 (C3, Py); 131.1 (C2, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 129.2 (C5, Ph); 124.0 (C5, Py); 118.6 (C6, Bz); 116.1 (C7, Bz);112.9 (C4, Bz). HRMS (+ESI, *m/z*) calcd. for $C_{19}H_{11}N_5O_5$ $[M+H]^+$: 390.0833, found: 390.0824.

1-(2"-Chlorobenzoyl)-5-nitro-2-(pyridin-3'-yl)benzimidazole (4c)

It was synthesized from 1.2 g (0.005 mol) of 2 and 1.7 ml (0.01 mol) of 2-chlorobenzoyl chloride. Yield: 78%; Melting point: 191-193ºC: IR (KBr, cm^{-1}): C-H (Ar): 3082, 3052; C=O: 1664; Ring skeleton bands: 1600, 1584, 1570; N-O: 1463, 1389. ^{1}H-NMR (DMSO-d_6): 9.32-9.29 (1H, m, Py); 8.88-8.82 (1H, m, Py); 8.61-8.55 (1H, m, Bz); 8.32-8.29 (1H, m, Py); 7.89 (1H, s, Bz); 7.79-7.73 (1H, m, Bz); 7.69-7.64 (1H, m, Ph); 7.59-7.42 (2H, m, Ph); 7.39-7.36 (1H, m, Ph); 7.01-6.93 (1H, m, Py). 13C-NMR (DMSO-d6): 167.7 (C=O); 155.1(C2, Py); 147.9(C6, Py); 144.3 (C5, Bz); 141.5 (C2, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 136.4 (C4, Py); 135.5 (C4, Ph); 134.1 (C2, Ph);133.0 (C1, Ph); 132.9 (C3, Py); 131.1 (C6, Ph); 129.2 (C3, Ph); 129.2 (C5, Ph); 124.0 (C5, Py); 118.6 (C6, Bz); 116.1 (C7, Bz);112.9 (C4, Bz). HRMS (+ESI, *m/z*) calcd. for $C_{19}H_{11}N_4O_3$ $[M+H]^+$: 379.0593, found: 379.0584.

1-(4"-Chlorobenzoyl)-5-nitro-2-(pyridin-3'-yl)benzimidazole (4d)

It was synthesized from 1.2 g (0.005 mol) of intermediate 2 and 1.7 ml (0.01 mol) of 4-chlorobenzoyl chloride. Yield: 72%; Melting point: 209-210ºC; IR (KBr, cm^{-1}): C-H (Ar): 3079, 3051; C=O: 1671; Ring skeleton bands: 1609, 1601, 1580; N-O: 1466, 1390. ^{1}H-NMR (DMSO-d_6): 9.32-9.28 (1H, m, Py); 9.09-9.01 (1H, m, Py); 8.93-8.89 (1H, m, Bz); 8.85-8.79 (2H, m, Ph); 8.66-8.60 (1H, m, Py); 8.54-8.48 (2H, m, Ph); 8.30 (1H, s, Bz); 7.98-7.93 (1H, m, Bz); 7.63-7.58 (1H, m, Py). 13C-NMR (DMSO-d6): 167.7 (C=O); 155.1 (C2, Py); 153.7 (C4, Ph); 147.9 (C6, Py); 141.5 (C2, Bz); 140.3 (C5, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 135.4 (C4, Py); 133.0 (C1, Ph); 132.9 (C3, Py); 131.1 (C2, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 129.2 (C5, Ph); 124.0 (C5, Py); 118.6 (C6, Bz); 116.1 (C7, Bz);112.9 (C4, Bz). HRMS (+ESI, *m/z*) calcd. for $C_{19}H_{11}ClN_4O_3$ $[M+H]^+$: 379.0593, found: 379.0584.

1-Benzoyl-5-nitro-2-(furan-2'-yl)benzimidazole (5a)

The compound was synthesized from 1.1 g (0.005 mol) of intermediate 3 and 1.2 ml (0.01 mol) of benzoyl chloride. Yield: 74%; Melting point: 174-175ºC; IR (KBr, cm^{-1}): C-H (Ar): 3083, 3050; C=O: 1690; Ring skeleton bands: 1579; N-O: 1465, 1375; C-O: 1298, 1285, 1208. ^{1}H-NMR (DMSO-d_6): 8.59-8.55 (1H, m, Bz); 8.26-8.22 (1H, m, Bz); 8.19-8.15 (1H, m, Fr); 7.93-7.88 (1H, m, Bz); 7.79-7.76 (2H, m, Ph); 7.56-7.50 (1H, m, Ph); 7.33-7.29 (2H, m, Ph); 7.09-6.99 (1H, m, Fr); 6.89-6.81 (1H, m, Fr). 13C-NMR (DMSO-d6): 167.7 (C=O); 154.0 (C2, Fr); 144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 134.5 (C4, Ph); 133.0 (C1, Ph); 131.1 (C2, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 128.2 (C5, Ph); 118.6 (C6, Bz); 116.1 (C7, Bz); 112.9 (C4, Bz); 112.0 (C4, Fr); 107.1 (C3, Fr). HRMS (+ESI, *m/z*) calcd. for $C_{18}H_{11}N_3O_4$ $[M+H]^+$: 334.0823, found: 334.0819.

1-(4"-Nitrobenzoyl)-5-nitro-2-(furan-2'-yl)benzimidazole (5b)

The compound was synthesized from 1.1 g (0.005 mol) of intermediate 3 and 1.8 ml (0.01 mol) of 4-nitrobenzoyl chloride. Yield: 78%; Melting pointt: 193-195ºC; IR (KBr, cm-1): C-H (Ar): 3085, 3051; C=O: 1667; Ring skeleton bands: 1580; N-O: 1460, 1385; C-O: 1280, 1269, 1210. ^{1}H-NMR (DMSO-d_6): 8.61-8.54 (1H, m, Bz); 8.49-8.41 (1H, m, Bz); 8.26-8.19 (1H, m, Fr); 8.16-8.11 (2H, m, Ph); 8.00-7.93 (2H, m, Ph); 7.92-7.88 (1H, m, Bz); 7.18-7.11 (1H, m, Fr); 6.77-6.72 (1H, m, Fr). 13C-NMR (DMSO-d6): 167.7 (C=O); 154.0 (C2, Fr); 153.7 (C5, Ph);144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 134.5 (C4, Ph); 133.0 (C1, Ph); 131.1 (C2, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 118.6 (C6, Bz); 116.1 (C7, Bz); 112.9 (C4, Bz); 112.0 (C4, Fr); 107.1 (C3, Fr). HRMS (+ESI, *m/z*) calcd. for $C_{18}H_{10}N_4O_6$ $[M+H]^+$: 379.0674, found: 379.0666.

1-(2"-Chlorobenzoyl)-5-nitro-2-(furan-2'-yl)benzimidazole (5c)

It was synthesized from 1.1 g (0.005 mol) of intermediate 3 and 1.7 ml (0.01 mol) of 2-chlorobenzoyl chloride. Yield: 72%; Melting point: 186-188ºC; IR (KBr, cm-1): C-H (Ar): 3083, 3052; C=O: 1680; Ring skeleton bands: 1579; N-O: 1458, 1390; C-O: 1302, 1289, 1210. ^{1}H-NMR (DMSO-d_6): 8.49-8.44 (1H, s, Bz); 8.22-8.19 (1H, m, Bz); 8.01-7.96 (1H, m, Fr); 7.86-7.70 (1H, m, Bz); 7.69-7.61 (2H, m, Ph); 7.56-7.52 (1H, m, Ph); 7.42-7.39 (1H, m, Ph); 7.01-6.98 (1H, m, Fr); 6.81-6.77 (1H, m, Fr). 13C-NMR (DMSO-d6): 167.7 (C=O); 154.0 (C2, Fr); 144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 139.8 (C3a, Bz); 136.8 (C7a, Bz); 135.5 (C4, Ph); 134.1 (C2, Ph); 133.0 (C1, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 128.2 (C5, Ph); 118.6 (C6, Bz); 116.1 (C7, Bz); 112.9 (C4, Bz); 112.0 (C4, Fr); 107.1 (C3, Fr). HRMS (+ESI, *m/z*) calcd. for $C_{18}H_{10}ClN_3O_4$ $[M+H]^+$: 368.0433, found: 368.0425.

1-(4"-Chlorobenzoyl)-5-nitro-2-(furan-2'-yl)benzimidazole (5d)

The compound was synthesized from 1.1 g (0.005 mol) of intermediate 3 and 1.7 ml (0.01 mol) of 4-chlorobenzoyl chloride. Yield: 78%; Melting point: 185-186ºC; IR (KBr, cm-1): C-H (Ar): 3084, 3053; C=O: 1665; Ring skeleton bands: 1578; N-O: 1461, 1386; C-O: 1300, 1276, 1225. ^{1}H-NMR (DMSO-d_6): 8.33-8.29 (1H, m, Bz); 8.19-8.11 (1H, m, Bz); 8.01-7.98 (2H, m, Fr); 7.86-7.82 (2H, m, Ph); 7.42-7.38 (2H, m, Ph); 7.16-7.10 (1H, m, Fr); 6.78-6.72 (1H, m, Fr). 13C-NMR (DMSO-d6): 167.7 (C=O); 154.0 (C2, Fr); 144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 140.2 (C5, Ph);139.8 (C3a, Bz); 136.8 (C7a, Bz); 134.5 (C4, Ph); 133.0 (C1, Ph); 131.1 (C2, Ph); 131.1 (C6, Ph); 129.2 (C3, Ph); 118.6 (C6, Bz); 116.1 (C7, Bz); 112.9 (C4, Bz); 112.0 (C4, Fr); 107.1 (C3, Fr). HRMS (+ESI, *m/z*) calcd. for $C_{18}H_{10}ClN_3O_4$ $[M+H]^+$: 368.0433, found: 368.0428.

General procedure for synthesis of compounds 4e-f and 5e-f

A mixture of a solution of 2 or 3 (0.002 mol) in 20 ml of acetonitrile and 5 ml of 10% aqueous NaOH solution was stirred for 15 min at room temperature. Subsequently, benzyl chloride or chlorobenzyl chloride (0.0024 mol) was added slowly with stirring at 0 ºC. After addition was complete, temperature was allowed

to rise upto room temperature and stirring was continued for 24 h. Acetonitrile was recovered under reduced pressure, and the residue was extracted with ethyl acetate. Ethyl acetate layer was separated and washed with two 50 ml portions of water followed by two 20 ml portions of brine. After drying the organic layer with anhydrous sodium sulfate, the solvent was evaporated, and residue was dried. The crude product was recrystallized from ethyl acetate to yield pure compound (4e-4f and 5e-5f).

1-Benzyl-5nitro-2-(pyridin-3'-yl)-1H-benzimidazole (4e)

The compound was synthesized from 0.48 g (0.002 mol) of intermediate 2 and 0.33 ml (0.0024 mol) of benzyl chloride. Yield: 77%; Melting point: 176-178 ºC; IR (KBr, cm-1): C-H (Ar): 3089, 3056; Ring skeleton bands: 1609, 1590, 1580; 1560; N-O: 1460, 1385. ^{1}H-NMR (DMSO-d_6): 9.42-9.35 (1H, m, Py); 8.96-8.93 (1H, m, Py); 8.5 (1H, m, Bz); 8.32-8.28 (1H, m, Py); 8.20 (1H, s, Bz); 7.96-7.91 (1H, m, Bz); 7.44-7.38 (2H, m, Ph); 7.26-7.19 (2H, m, Ph); 7.16-7.11 (1H, m, Ph); 7.00-6.98 (1H, m, Py); 5.8 (2H, s, CH2). 13C-NMR (DMSO-d6): 155.1 (C2, Py); 153.3 (C2, Bz); 147.9 (C6, Py); 144.3 (C5, Bz); 139.8 (C3a, Bz); 137.3 (C1, Ph); 136.8 (C7A, Bz); 135.4 (C4, Py); 132.9 (C3, Py); 128.6 (C3, Ph); 128.6 (C5, Ph); 127.6 (C2, Ph); 127.6 (C6, Ph); 125.7(C4, Ph); 124.0 (C5, Py); 118.6 (C6, Bz); 112.9 (C4, Bz); 110.4 (C7, Bz); 52.2 (CH2). HRMS (+ESI, *m/z*) calcd. for $C_{19}H_{14}N_4O_2$ $[M+H]^+$: 331.1190, found: 331.1186.

1-(2"-Chlorobenzyl)-5-nitro-2-(pyridin-3'-yl)-1H-benzimidazole (4f)

The compound was synthesized from 0.48 g (0.002 mol) of intermediate 2 and 0.47 ml (0.0024 mol) of 2-chlorobenzyl chloride. Yield: 71%; Melting point: 189-190ºC; IR (KBr, cm^{-1}): C-H (Ar): 3090, 3057; Ring skeleton bands: 1610, 1604,1582; 1570; N-O: 1470, 1380. ^{1}H-NMR (DMSO-d_6): 9.32-9.29 (1H, m, Py); 8.88-8.85 (1H, m, Py); 8.52-8.48 (1H, m, Bz); 8.33-8.28 (1H, m, Py); 8.19 (1H, s, Bz); 8.00-7.96 (1H, m, Bz); 7.76-7.70 (m, 1H, Py); 7.64-7.60 (1H, m, Ph); 7.22-7.19 (2H, m, Ph); 7.00-6.98 (1H, m, Py); 5.5 (2H, s, CH2). 13C-NMR (DMSO-d6): 155.1 (C2, Py); 153.3 (C2, Bz); 147.9(C6, Py); 144.3 (C5, Bz); 139.8 (C3a, Bz); 137.3 (C1, Ph); 136.8 (C7A, Bz); 135.4 (C4, Py); 134.3 (C2, Ph);132.9 (C3, Py); 128.6 (C3, Ph); 128.6 (C5, Ph); 127.6 (C6, Ph); 126.7(C4, Ph); 124.0 (C5, Py); 118.6 (C6, Bz); 112.9 (C4, Bz); 110.4 (C7, Bz); 47.1 (CH2). HRMS (+ESI, *m/z*) calcd. for $C_{19}H_{13}ClN_4O_2$ $[M+H]^+$: 365.0800, found: 365.0800.

1-Benzyl-2-(furan-2'-yl)-5-nitro-1H-benzimidazole (5e)

This compound was synthesized from 0.40 g (0.002 mol) of intermediate 3 and 0.33 ml (0.0024 mol) of benzyl chloride. Yield: 69%; Melting point: 166-167ºC; IR (KBr, cm^{-1}): C-H (Ar): 3088, 3055; Ring skeleton bands: 1579; N-O: 1460, 1385; C-O: 1300, 1289, 1250. ^{1}H-NMR (DMSO-d_6): 8.5 (1H, s, Bz); 8.29-8.26 (1H, m, Bz); 8.00-8.06 (1H, m, Fr); 7.96-7.91 (1H, m, Bz); 7.43-7.38 (2H, m, Ph); 7.33-7.29 (2H, m, Ph); 7.26-7.23 (1H, m, Ph); 7.18-7.13 (1H, m, Fr); 6.83-6.79 (1H, m, Fr); 5.9 (2H, s, CH2). 13C-NMR (DMSO-d6): 154.0 (C2, Fr); 148.5 (C7a, Bz); 144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 139.8 (C3a, Bz); 137.3 (C1, Ph); 128.6 (C3, Ph); 128.6 (C5, Ph); 127.6 (C2, Ph); 127.6 (C6, Ph); 125.7 (C4, Ph); 118.6 (C6, Bz); 112.9 (C4, Bz); 112.0 (C4, Fr); 110.4 (C7, Bz); 107.1 (C3, Fr); 49.8 (CH2). HRMS (+ESI, *m/z*) calcd. for $C_{18}H_{13}N_3O_3$ $[M+H]^+$: 320.1030, found: 320.1025.

1-(2''-Chlorobenzyl)-2-(furan-2'-yl)-5-nitro-1H-benzimidazole (5f)

The compound was synthesized from 0.40 g (0.002 mol) of intermediate 3 and 0.47 ml (0.0024 mol) of 2-chlorobenzyl chloride. Yield: 68%; Melting point: 177-178ºC; IR (KBr, cm^{-1}): C-H (Ar): 3087, 3056; Ring skeleton bands: 1580; N-O: 1466, 1382; C-O: 1305, 1283, 1270. ^{1}H-NMR (DMSO-d_6): 8.4 (1H, s, Bz); 8.26-8.19 (1H, m, Bz); 8.03-7.99 (1H, m, Fr); 7.89-7.85 (1H, m, Bz); 7.69-7.63 (1H, m, Ph); 7.46-7.41 (1H, m, Ph); 7.35-7.31 (1H, t, Fr); 7.23-7.19 (1H, m, Ph); 7.09-7.01 (1H, m, Ph); 6.83-6.81 (1H, m, Fr); 5.5 (2H, s, CH2). 13C-NMR (DMSO-d6): 154.0 (C2, Fr); 148.5 (C7a, Bz); 144.3 (C5, Bz); 142.9 (C5, Fr); 141.5 (C2, Bz); 139.8 (C3a, Bz); 137.3 (C1, Ph); 134.3 (C2, Ph); 128.6 (C3, Ph); 128.6 (C5, Ph); 127.6 (C6, Ph); 125.7 (C4, Ph); 118.6 (C6, Bz); 112.9 (C4, Bz); 112.0 (C4, Fr); 110.4 (C7, Bz); 107.1 (C3, Fr); 44.7 (CH2). HRMS (+ESI, *m/z*) calcd. for $C_{18}H_{12}ClN_3O_3$ $[M+H]^+$: 354.0640, found: 354.0635.

Anti-microbial activity

Disk diffusion method [31] with some modifications was used for evaluation of antimicrobial activity of target test compounds using streptomycin as reference drug. The compounds were tested against one strain of gram +ve bacteria (*Staphylococcus aureus*), two strains of gram –ve bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and one fungal strain (*Candida albicans*). Whatman No. 1 filter paper disk of 9 mm diameter were sterilized in an autoclave for 15 min at 121°C. The sterile disks were impregnated with the compounds of varying concentrations (1.56, 3.12, 6.25, 12.50, 25, 50 µg/ml). Agar plates were surface inoculated uniformly from broth culture of the test microorganisms (sabouraud broth and agar were used for *C. albicans*). The impregnated disks were placed with suitably apart on the cultured medium, the plates were incubated at 5°C for 1 h to permit good diffusion and then placed in an incubator at 37°C for 24 h for bacteria, and at 28°C for 48 h for fungi. The inhibition zones created by the test compounds as well as streptomycin were examined after the incubation periods.

Molecular docking studies

The docking analysis was done by Autodock Tools (ADT) version 1.5.6 and Autodock Vina version 1.1.2., which is an open-source program for molecular docking from Scripps Research Institute (http://vina.scripps.edu/). 3D structure of glutaminase domain of GlcN-6-P (PDB entry code 1XFF) was retrieved from Protein Data Bank (PDB) (http://www.pdb.org). The protein obtained from PDB was complexed with glutamate, which is removed from the structure to free the docking site. The structure was then saved in PDBQT file format for input into docking. eFindSite webserver (Department of Biological Sciences, Louisiana State University) was used for ligand binding site prediction. It is a virtual screening algorithm that detects common ligand binding

sites [32]. Best fit binding sites with higher score were chosen for further procedures. 2D structures of ligands were drawn using ChemBio Draw Ultra 12.0 (ChemOffice 2010). PRODRG2 server (GlycoBioChem) [33] was used for energy minimization and to convert 2D structure into 3D. All structures were saved as PDBQT using AutoDock Tool (ADT). A configuration file having grid dimensions of 22×22×20 Å with 1 Å centered on (x, y, z) 4.874, 28.786, 27.087 was created around the binding site on protein. All the ligands were docked into target protein complexes, with a few residues near binding site of protein molecule considered as flexible (CYS1, ARG73, THR76, ASN84, and ILE100) and rest of the body as rigid. The search was carried out with Broyden-Fletcher-Goldfarb-Shanno algorithm [34] with total number of runs kept 50. Evaluation of results was done by sorting different complexes with respect to their predicted binding energy. The outputs were exported to Discovery Studio for visual inspection of the binding modes and interactions of the compounds with amino acid residues in the active sites.

Chemical stability evaluation

Chemical stability of compound 4d was studied in non-enzymatic Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF). For non-enzymatic SGF, sodium chloride (2 g) was dissolved in 7 ml of hydrochloric acid and diluted with a sufficient volume of water to 1000 ml. pH of the resulting solution was approximately 1.2. For non-enzymatic SIF, monobasic potassium phosphate (6.8 g) was dissolved in 250 ml of water. To this, sodium hydroxide solution (0.2 N, 77 ml) and 500 ml of water were added. The solution was adjusted to pH 6.8 ± 0.1 with sodium hydroxide or hydrochloric acid solution, and then diluted with water to 1000 ml. Stock solutions (2×10-4 M) of reactant 4-nitrobenzoic acid and product (4d) were prepared in 10% DMSO. The concentration of these solutions was found to be 76 µg/ml. These solutions were diluted with 10% DMSO to obtain working standard solution of concentration of 3.8 µg/ml for HPLC analysis.

Stability studies

Stock solution of 4d (0.5 ml) was taken in two 10 ml volumetric flasks labeled as A and B. Volume in A and B was adjusted with SGF and SIF, respectively. These flasks were placed in a hot air oven maintained at 37 ± 1ºC. The samples were withdrawn at 0, 1, 2, 3 and 4 h and analyzed by HPLC.

HPLC method

The HPLC system consisted of binary pumps (515), PDA detector 2998 and Rheodyne manual injector (Waters, Milford MA, USA). The data was acquired and processed in Empower 3 software (Waters, MA, USA). The intermediate 2,4-nitrobenzoic acid, and compound 4d were optimally resolved by isocratic elution with mobile phase composed of water and methanol (50:50 v/v, pH 8.4 with 1% NH_4OH) on Kromasil C18 (250×4.6 mm; 5 µ) column. The injection volume was fixed at 20 µl. Well defined sharp peaks were obtained when eluent was detected at 239 nm.

RESULTS AND DISCUSSION

Chemistry

The starting key intermediates, 2-substituted benzimidazole derivatives (2 and 3) were synthesized by slight modifications in the reported methods [35,36]. o-Phenylenediamine was refluxed with nicotinic acid in the presence of PPA under nitrogen flux to yield intermediate 2, and with furfuraldehyde in ethanol for 4 h to yield intermediate 3. Structures of 2 and 3 were ascertained through IR spectra by the appearance of single absorption band in the range of 3350-3500 cm^{-1} for secondary amine (-NH-) in contrast to two absorption bands of primary amine (-NH2) functionality of 5-nitro-o-phenylenediamine. Symmetric and asymmetric N=O stretch due to presence of nitro group was observed at 1470 cm^{-1} and 1390-1380 cm^{-1} respectively. The 1H-NMR spectra showed signals due to aromatic and N-H protons of BZ nucleus in addition to the signals of the corresponding pyridyl/furyl moiety. The target compounds 4 and 5 were obtained in good to moderate yields and characterized by comprehensive spectral analysis. In IR spectra, presence of carbonyl stretch at 1660-1690 cm^{-1} and disappearance of broad band in the range of 3350-3400 cm^{-1} prove the coupling of NH of BZ with benzoyl/benzyl moiety. The signals due to protons of BZ as well as pyridyl/furyl were found similarly as in 1H-NMR spectra of 2 or 3. In addition, signals due to benzoyl/benzyl rings were noted at chemical shift values that corresponds to the values reported in literature [37]. The signals due to –CONH and–NH– protons were ascertained through D2O exchange experiments. Structures of all compounds (2-5) were confirmed by high resolution mass spectral data (+ESI), wherein the compounds were detected as $[M+H]^+$ at *m/z* values corresponding to their theoretical masses.

Anti-microbial activity

Minimum Inhibitory Concentration (MIC) of all target compounds against *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* is summarized in Figure 3. All compounds were found effective in controlling growth of the tested micro-organisms. The results revealed that replacement of 2-pyridnyl (4a, 4d and 4f) with 2-furyl ring (5a-c) rendered the compound less active against bacterial strains but more active against fungal strain. Compounds with electron withdrawing group like chloro or nitro on benzoyl/benzyl (4d, 4f, 5b and 5c) moiety imparted antimicrobial activity better as compared to the unsubstituted ones (4a and 5a). Compound 4d exhibited the most potent antibacterial activity against *S. aureus* (MIC 4.16 ± 1.04 µg/ml), *E. coli* (MIC 5.20 ± 1.04 µg/ml) and *P. aeruginosa* (MIC 10.41 ± 2.08 µg/ml), which was comparable to that of streptomycin (MIC 2.08 ± 0.52, 2.60 ± 0.51 and 10.41 ± 2.08 µg/ml, respectively). Out of all tested compounds, compound 5c was most active antifungal (MIC 20.83 ± 4.16 µg/ml) and equipotent to fluconazole against *C. albicans*.

Molecular docking studies

GlcN-6-P synthase catalyses a complex reaction involving ammonia transfer from L-glutamine to Fru-6-P, followed by isomerisation of fructosamine-6-phosphate to glucosamine-

6-phosphate. This reaction is the first step of the pathway leading to formation of UDP-GlcNAc, which is used to build macromolecules important for the cell wall assembly in fungi and bacteria. In mammals, UDP-GlcNAc is utilised for biosynthesis of glycoproteins and mucopolysaccharides. Thus, even a short time inactivation of this enzyme is lethal for micro-organism, whereas inhibition of enzyme in mammals is not lethal because of rapid expression of mammalian gene that encodes GlcN-6-P synthase [38]. Therefore, GlcN-6-P synthase was taken as target receptor for molecular docking study with the compounds 4 and 5 with an aim of rationalizing the antimicrobial activity of the compounds.

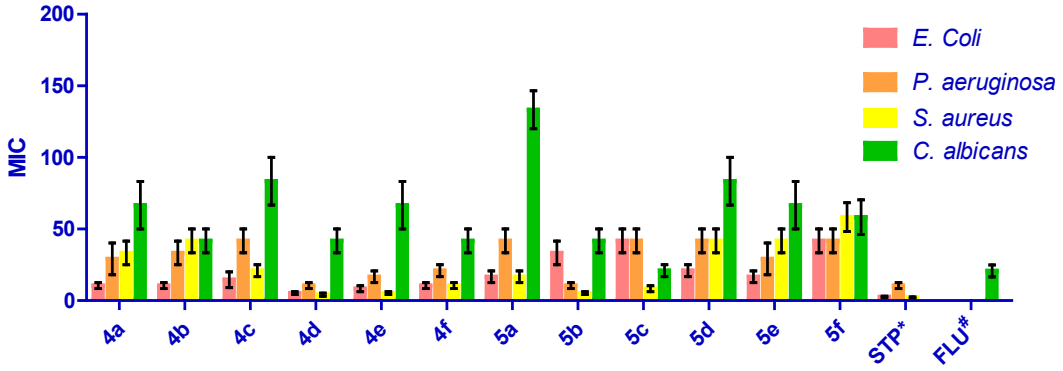

Figure 3 Antimicrobial activity of test compounds. *STP: Streptomycin. #FLU: Fluconazole.

Table 1: Docking results of target compounds 4a-f and 5a-f

Comp.	Binding Energy (Kcal/mol)	No. of H-bonds	H-bonding*	Bond length
4a	-8.6	3	:4a:N3::A:CYS1 :4a:N⋯O::A:HIS86 :4a:N⋯O::A:HIS97	2.73389 2.34322 2.71599
4b	-7.8	3	:4b:N⋯O::A:CYS1 :4b:N⋯O_b::A:ARG26 :4b:N⋯O::A:TRP74	2.27025 2.84155 2.14125
4c	-7.8	2	:4c:N⋯O::A:THR76 :4c:N⋯O::A:HIS86	2.28184 2.78639
4d	-9.1	6	:4d:C=O::A:CYS1 :4d:N⋯O::A:THR76 :4d:N⋯O::A:HIS77 :4d:N⋯O::A:HIS86 :4d:C=O::A:GLY99 :4d:N⋯O::A:THR124	3.06027 2.03864 2.31105 1.95978 2.25986 2.45146
4e	-8.5	4	:4e:N⋯O::A:THR76 :4e:N⋯O::A:HIS77 :4e:N⋯O::A:HIS86 :4e:N⋯O::A:THR124	1.86614 2.30793 1.90807 2.6343
4f	-8.9	4	:4f:N⋯O::A:THR76 :4f:N⋯O::A:HIS77 :4f:N⋯O::A:HIS86 :4f:N⋯O::A:THR124	1.87748 2.25194 1.88572 2.60096
5a	-8.2	1	:5a:N3::A:CYS1	2.76635
5b	-8.2	3	:5b:N⋯O_b::A:THR76 :5b:N3::A:SER176 :5b:N⋯O::A:SER191	2.76342 2.77933 2.90037
5c	-9.1	4	:5c:N⋯O::A:THR76 :5c:N⋯O::A:HIS77 :5c:N⋯O::A:HIS86 :5c:N⋯O::A:THR124	1.79097 2.30302 1.90157 2.71409
5d	-7.7	2	:5d:C=O::A:CYS1 :5d:OFU::A:GLY99	2.69404 2.05137
5e	-7.6	3	:5e:N⋯O::A:HIS86 :5e:N⋯O::A:THR124 :5e:N⋯O::A:GLY99	2.07712 2.47206 2.5937
5f	-8.0	1	:5f:OFU::A:TRP74	2.13726

All compounds exhibited well established interactions with one or more amino acids in active pocket of the receptor. Table 1 shows the binding energies, number of hydrogen bonds and bond length of all the docked compounds. All compounds were completely folded in the active pocket of the enzyme and possessed good binding energy ranging from -9.1 to -7.6 Kcal/mol. Compounds 4d and 5c showed maximum interactions with highest binding energies of -9.1 Kcal/mol. Compound 4d proved to be the best fit as it bind with GlcN-6-P synthase through six H-bonds and one pi-alkyl bond as depicted in Figure 4. This increased interaction is due to nitro group at 5-position of benzimidazole that contributes to four out of six H-bond interactions. In compound 4d, a chloro group at para position on benzoyl oriented the benzoyl moiety for two additional H-bonds with the enzyme. In addition to these interactions, pyridine at 2-position of BZ showed some non-bonding interactions with the enzyme due to which its binding with the enzyme is highly increased. Energy minimized binding pose of compound 5c showed four H-bonding interactions of NO_2 group with THR124, HIS86, HIS77, and THR76 residues. Furyl ring at 2-position interacted *via* π-σ interaction with GLY99 residue. A significant correlation was observed between experimental antimicrobial activity and molecular docking scores wherein active analogs (4a, 4d, 4f and 5c) exhibited high docking score while those with relatively low inhibition were predicted to possess low scores.

Chemical stability

Majority of the synthesized compounds (4a-d and 5a-d) were suspected to degrade to respective 5-nitro benzimidazole intermediate (2 or 3) and the corresponding substituted benzoic acid due to hydrolytic susceptibility of amide (NH-CO) linkage at N-1 of BZ (Figure 5) under the acidic pH in gastric medium and/or in alkaline pH in small intestine [39]. In order to evaluate stability of these compounds in gastric and intestinal pH, chemical stability study on the most potent compound in this series (4d) was performed by exposing the compound to non-enzymatic SGF as well as to non-enzymatic SIF for 4 h each. Tough these studies cannot be correlated to CYP metabolism but will provide a better understanding of stability of compounds in acidic and alkaline pH in gastro-intestinal tract. LC-UV chromatograms of blank, intermediate 2, *p*-chloro benzoic acid (PCBA) and 4d in non-enzymatic SGF and SIF are shown in Figures 6 and 7. Components of SGF and SIF eluted at 2.5-3.0 min and DMSO eluted at 3.4 min. PCBA, intermediate 2 and 4d were detected as distinct peaks at about 1.8, 4.0 and 9.8 min, respectively. Comparison of LC-UV chromatograms of control sample (0 h) with those of stability

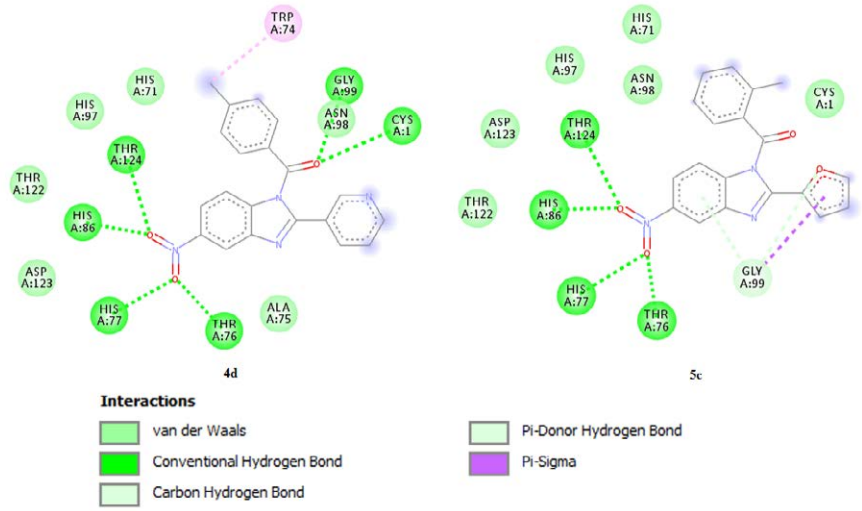

Figure 4 2-D docking images of potent compounds 4d and 5c.

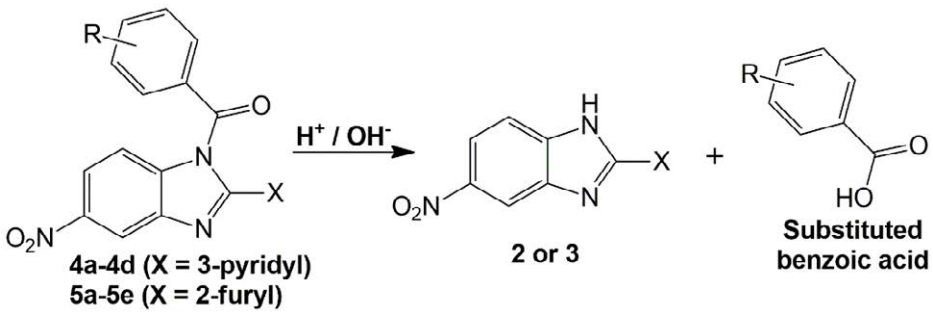

Figure 5 Most probable degradation products of compounds 4a-d and 5a-d.

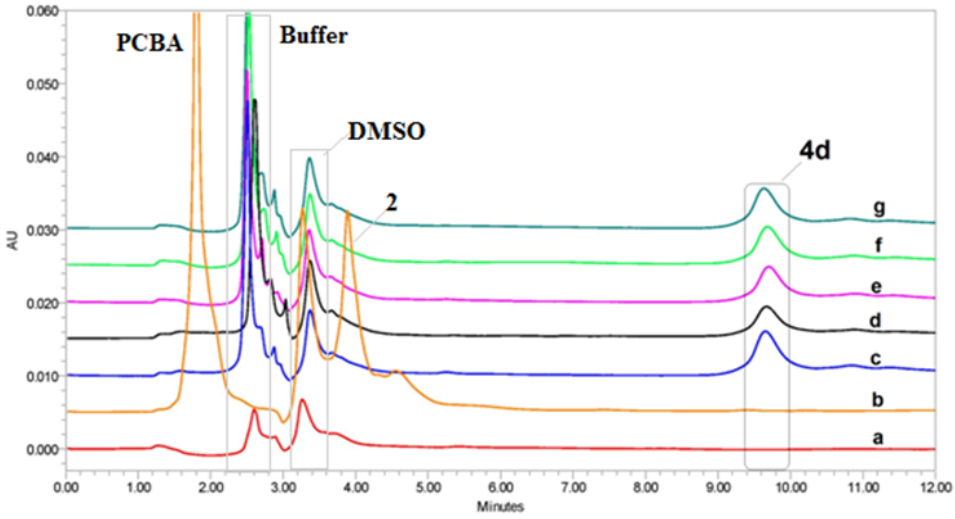

Figure 6 LC-UV chromatograms of blank non-enzymatic SGF containing buffer and DMSO (a), of intermediate 2 and PCBA (b), of compound 4d at 0 h (c), and of 4d after degradation in non-enzymatic SGF for 1 h (d), 2 h (e), 3 h (f), and 4 h (g).

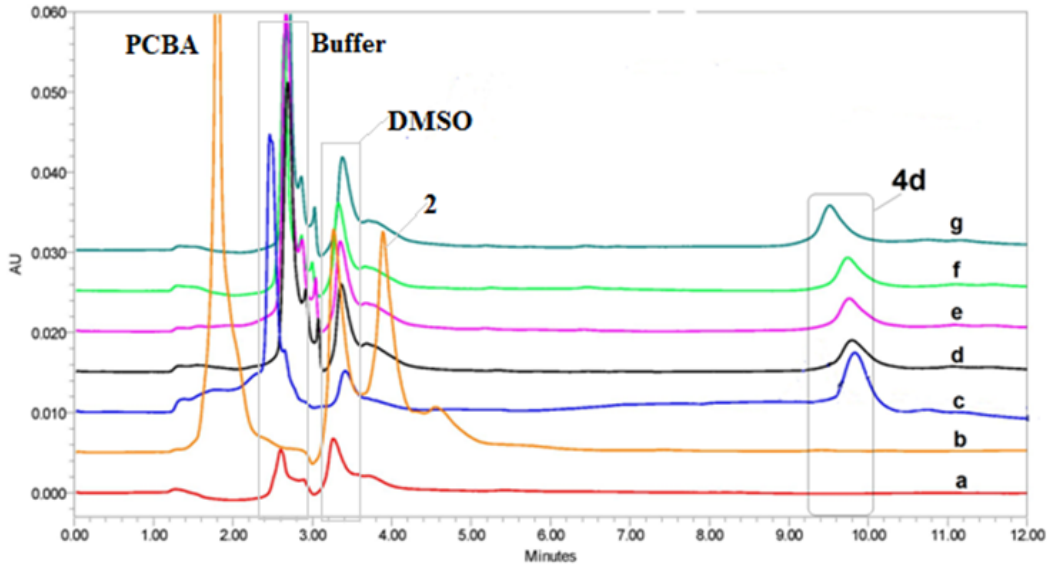

Figure 7 LC-UV chromatograms of blank non-enzymatic SIF containing buffer and DMSO, (a) of intermediate 2 and PCBA (b), of compound 4d at 0 h (c), and of 4d subjected to degradation in non-enzymatic SGF for 1 h (d), 2 h (e), 3 h (f), and 4 h (g).

samples (1, 2, 3 and 4 h) revealed that there was no trace of any peak of PCBA, intermediate 2 or any other additional compound in any stability sample of 4d in both SGF and SIF. It suggested that 4d remain stable in SGF and SIF and hence can be expected to remain intact *in-vivo*.

CONCLUSION

In the present study, twelve 1,2,5-trisubstitued benzimidazole derivatives were designed on the basis of proposed general SAR and synthesized using simple chemical reactions. All intermediates and target compounds were synthesized with good to moderate yields. Structures of the compounds were ascertained through comprehensive spectral analysis. Antimicrobial evaluation study revealed compound 4d as the most potent antibacterial compound from the series and equipotent to streptomycin. Lower MIC values of 2-furyl analogs in comparison to 2-pyridyl analogs suggests pyridine as an important structural component for development of potent antibacterial. Docking studies suggested that the compounds might be eliciting the by binding with GlcN-6-P synthase. Chemical stability study of 4d in non-enzymatic simulated gastric and intestinal fluids through HPLC has revealed that it remains stable in both fluids for 4 h. Hence, compound 4d has emerged as a novel compound that can be further explored or modified to develop potent antibacterial compounds.

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