ANTI-TUBERCULAR AND ANTIMICROBIAL ACTIVITIES OF NOVEL HETEROCYCLIC SUBSTITUTED BENZIMIDAZOLE DERIVATIVES

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ABSTRACT

From o-phenylenediamine and p-amino benzoic acid a variety of novel heterocyclic substituted benzimidazole analogs C-N were designed and synthesized by a multistep synthesis. FT-IR, 1H-NMR, Mass spectroscopy and bases of elemental analysis were performed to characterize the structure of synthesized compounds. Test compounds were screened for antitubercular activity against H37RV strains of M. tuberculosis by in vitro M. tuberculosis method. In addition, antimicrobial activity of title compounds was also evaluated against various pathogenic strains of bacteria and fungi by agar streak dilution test. Results of biological studies revealed that all title compounds exhibited mild to good antitubercular and antimicrobial activity. The relationship between the functional group variation and the biological activity of the screened compounds were discussed. The most active compound was found to be 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-1-(4-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one H 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-1-(3-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one K out of twelve title compounds.

Key words: Benzimidazole, Isoxazole, Pyrimidine, Pyrazole, Hydrazone, Antitubercular, Antimicrobial.

INTRODUCTION

Tuberculosis (TB) is still greatest infectious cause of mortality worldwide. It is the only disease which does not require any vector for transportation from one person to another or to cross the physical boundary of the countries. Being the airborne disease with no vaccine, it is the single largest disease encountered by both developing and developed countries [1]. Two of the common problem associated with treatment, one is serious and life threatening adverse effect of existing anti tubercular drugs such as hepatotoxicity, neuritis, depression, asthma, anorexia etc which many times forces to withdraw the treatment temporarily or
change the treatment. Other one is development of resistance due to non completion of treatment regimen by patient and hence gene mutation by organisms made its management more difficult [2-3].

Despite the availability of highly potential antitubercular agents, tuberculosis remains primary cause of comparatively high mortality worldwide. The statistics shows that around three million people throughout the world die annually from tuberculosis and today more people die from tuberculosis than ever before [4-5]. Therefore, the development of new drugs with activity against multi drug resistant (MDR) TB, extensively drug resistant (XDR) TB, and latent TB is a priority task; although new agents that will shorten the duration of current chemotherapy are also needed. The increasing global tuberculosis burden due to the curse of HIV, MDR and XDR-TB has lead to search of newer therapeutic agents to tackle the menace [6]. The present first line drugs like isoniazid, pyrazinamide, ethambutol, and rifampicin are potent antitubercular agent. They act by inhibition of mycolic acid and RNA/DNA synthesis but they possess numerous adverse reactions. To avoid these effects it seemed promising to look for more selective compounds, at other targets to suppress the activity [7-8].

In the field of pharmaceutical and medicinal chemistry, benzimidazole and its derivatives are found to be trendy structures employed for discovery of drugs within the vast range of heterocycles. In drug discovery, the unique structural features of benzimidazole and a wide range of biological activities of its derivatives made it privileged structure [9]. Among various benzimidazole derivatives, promising biological activities are exhibited by 2-substituted benzimidazoles. Literature survey indicates that the benzimidazole nucleus showed significant antitubercular [10-13] & antimicrobial [14-17] activities. In addition, various heterocyclic nucleus such as oxazoles [18-23], pyrimidines [24-27], and pyrazoles [18-33] are reported to possess diverse biological activities particularly antitubercular & antimicrobial activities. Diverse examples of few antitubercular benzimidazoles (I – IV), isoxazoles (V – VII), pyrimidines (VIII-IX) and pyrazoles (X-XI) are indicated in Figure 1 & diverse examples of few antimicrobial benzimidazoles (XII-XV), isoxazoles (XVI – XVIII), pyrimidines (XIX-XX) and pyrazoles (XXI-XXIII) are depicted in Figure 2.

**Figure 1:** Diverse examples of anti-tubercular benzimidazoles, isoxazoles, pyrimidines and prazoles.

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The biological activities exhibited by compounds containing benzimidazole moiety has prompted chemists to synthesis more and more benzimidazole libraries and screen them for potential activities. Owing to the importance we planned to synthesize compounds with this functionality coupled with oxazole/pyrimidine/pyrazole as possible antitubercular & antimicrobial agents which could furnish better therapeutic results. Based on these findings, we decided to synthesize some novel oxazole/pyrimidine/pyrazole substituted benzimidazole derivatives and evaluated its antitubercular & antimicrobial activities with the hope to obtain more active and less toxic antimicrobial agents.

**MATERIALS AND METHODS**

**Chemistry**

The chemicals and reagents used were obtained from various chemical units Qualigens, E. Merck India Ltd., CDH, and SD Fine Chem. These solvents used were of LR grade and purified before their use. The silica gel G used for analytical chromatography (TLC) was obtained from E. Merck India Ltd. All the melting points were taken in open glass capillary and are uncorrected. $^1$H-NMR spectra were recorded at 500 MHz on Bruker Avance-500 NMR spectrometer in CDCl$_3$ using tetramethylsilane (TMS) as an internal standard. The chemical shifts are reported in ppm scale. Mass spectra were obtained on a JEOL-SX-102 instrument using electron impact ionization. All the IR spectra were recorded in KBr pellets on a Jasco FT-IR 410 spectrometer. Elemental analyses were performed on a Perkine Elmer model 2400C analyzer and were within ± 0.4 % of the theoretical values.

**Synthesis of 4-(1H-benzimidazol-2-yl)benzenamine (A)**

A mixture of o-phenylenediamine (1.08 g, 0.01 mol) and p-aminobenzoic acid (1.37 g, 0.01 mol) were stirred in a syrupy orthophosphoric acid (15 ml) at 200°C for 2 h. The reaction mixture was cooled and poured on crushed ice. The bulky white precipitate obtained was stirred in cold water (150 ml) and sodium hydroxide solution (5 M) was added until the pH 7. The
resulting solid obtained 3 was filtered and recrystallized from methanol. Yield = 70 %, m.p. 246-248 °C. IR (KBr) cm⁻¹: 3357 (NH), 2985 (Ar-CH), 1675 (C=N), 1586 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.09-8.24 (m, 8H, Ar-CH), 5.78 (s, 1H, NH of benzimidazole), 4.30 (s, 2H, NH₂). EI-MS m/z: 209 (M⁺). Anal. Calcd for C₁₃H₁₁N₃: C, 74.86; H, 5.28; N, 20.01.

**Synthesis of diethyl 2-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)malonate (B)**

4-(1H-Benzimidazol-2-yl)benzamine A (2.09 g; 0.01 mol) was dissolved in a mixture of concentrated hydrochloric acid (5 ml) and water (5 ml). The solution is cooled to 5 °C by immersing the flask in a mixture of ice and water. The solution of powdered sodium nitrite (1.38 g; 0.02 mol) dissolved in water (5 ml) was drop wise added to the solution of 4-(1H-benzimidazol-2-yl)benzamine hydrochloride with stirring. The stirring was continued for 30 min after complete addition of sodium nitrite. The obtained diazonium salt was added to a solution of diethylmalonate (1.60 g; 0.01 mol) in ethanol (25 ml) with stirring. When the reaction mixture was stirred for 6 h magnetically at room temperature (ethanol is added when solution becomes dry). To this mixture 100 ml of ice cold water was added and stirred well. The solid product B, so formed, was collected by filtration and recrystallized using methanol. Yield = 75 %, m.p. 158-160 °C. IR (KBr) cm⁻¹: 3333 (NH), 3038 (Ar-CH), 2898 (CH₂-CH), 1729 (C=O), 1657 (C=N), 1588 (C=C); 1063 (C-O-C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.16-7.83 (m, 8H, Ar-CH), 6.91 (s, 1H, NH of hydrazone), 4.84 (s, 1H, NH of benzimidazole), 4.38-5.40 (t, 4H, CH₂), 1.89-2.02 (t, 6H, CH₃). EI-MS m/z: 380 (M⁺). Anal. Calcd for C₂₀H₁₇N₅O₄: C, 63.15; H, 5.30; N, 14.73. Found: C, 62.97; H, 5.29; N, 14.77.

**Synthesis of 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-3-hydroxyisoxazol-5(4H)-one (C)**

A mixture of diethyl 2-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)malonate B (3.80 g; 0.01 mol) and hydroxylamine hydrochloride (1.04 g; 0.015 mol) in ethanol (25 ml) was refluxed on oil bath for 12 h. After removal of ethanol in vacuum, the oil obtained was poured into crushed ice and stirred well until the solid crystallizes out. The product obtained C was filtered, dried and recrystallized using ethanol. Yield = 79 %, m.p. 182-184 °C. IR (KBr) cm⁻¹: 3514 (OH), 3318 & 3272 (NH), 3088 (Ar-CH), 1746 (C=O), 1591 (C=N), 1505 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.05-8.27 (m, 8H, Ar-CH), 6.98 (s, 1H, NH of hydrazone), 5.16 (s, 1H, NH of benzimidazole), 2.52 (s, 1H, OH). EI-MS m/z: 321 (M⁺). Anal. Calcd for C₁₆H₁₁N₃O₅: C, 59.81; H, 3.45; N, 21.80. Found: C, 60.00; H, 3.44; N, 21.73.

**Synthesis of 5-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-2-substituted-6-hydroxy pyrimidin-4(5H)-one (D-E)**

A mixture of diethyl 2-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)malonate B (3.80 g; 0.01 mol), urea/thiourea (0.015 mol) and potassium carbonate (0.2 g) in ethanol (40 ml) was refluxed for 18 h on a heating mantle maintained at 100 °C. The mixture was cooled to room temperature and poured in 100 ml ice cold water. Stirred well and the solid separated out was filtered. The residue was dissolved in hot water and filtered when hot. The filtrate was neutralized with acetic acid and the solid precipitated out D-E was filtered and recrystallized from ethanol.

**5-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazono)-2,6-dihydroxypyrimidin-4(5H)-one (D)**

Yield = 72 %, m.p. 117-118 °C. IR (KBr) cm⁻¹: 3554 (OH), 3392 & 3331 (NH), 3009 (Ar-CH), 1738 (C=O), 1655 (C=N), 1593 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 6.76-7.85 (m, 8H, Ar-CH), 6.59 (s, 1H, NH of hydrazone), 5.30 (s, 1H, NH of benzimidazole), 2.37 (s, 1H, OH), 2.21 (s, 1H, OH). EI-MS m/z: 348 (M⁺). Anal. Calcd for C₁₇H₁₂N₄O₅: C, 58.62; H, 3.47; N, 24.13. Found: C, 58.80; H, 3.46; N, 24.08.

**5-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazono)-6-hydroxy-2-mercaptopypyrimidin-4(5H)-one (E)**
Yield = 78 %, m.p. 135-137 °C. IR (KBr) cm⁻¹: 3553 (OH), 3392 (NH), 3021 (Ar-CH), 2975 (SH), 1735 (C=O), 1681 (C=N), 1589 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 6.97-8.20 (m, 8H, Ar-CH), 6.72 (s, 1H, NH of hydrazone), 5.19 (s, 1H, NH of benzimidazole), 2.45 (s, 1H, OH), 1.54 (s, 1H, SH). EI-MS m/z: 364 (M⁺). Anal. Calcd for C₁₇H₁₂N₂O₃S: C, 56.04; H, 3.32; N, 23.06. Found: C, 55.83; H, 3.33; N, 22.99.

Synthesis of 4-(2-(4-(1H-benzimidazol-2-yl) phenyl)hydrazone)-3-hydroxy-1-substituted-1H-pyrazol-5(4H)-one (F-N)
A mixture of diethyl 2-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazonoyl)malonate B (3.80 g; 0.01 mol) and various hydrazine hydrochloride (0.015 mol) in ethanol (40 ml) was refluxed for 24 h in heating mantle maintained at 100 °C. After removal of ethanol in vacuum, the oil obtained was cooled and poured into crushed ice. The mixture was stirred well until the solid crystallizes out. The product separated F-N was filtered, washed with water, dried and recrystallised using alcohol.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazone)-3-hydroxy-1H-pyrazol-5(4H)-one (F)
Yield = 70 %, m.p. 219-221 °C. IR (KBr) cm⁻¹: 3519 (OH), 3326 & 3290 (NH), 3055 (Ar-CH), 1723 (C=O), 1638 (C=N), 1590 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.10-8.39 (m, 8H, Ar-CH), 7.03 (s, 1H, NH of hydrazone), 6.82 (s, 1H, NH of pyrazole), 5.17 (s, 1H, NH of benzimidazole), 2.85 (s, 1H, OH). EI-MS m/z: 320 (M⁺). Anal. Calcd for C₁₆H₁₂N₂O₂: C, 60.00; H, 3.78; N, 26.24. Found: C, 60.21; H, 3.77; N, 26.16.

4-(2-(4-(1H-Benzimidazol-2-yl) phenyl)hydrazone)-3-hydroxy-1-phenyl-1H-pyrazol-5(4H)-one (G)
Yield = 74 %, m.p. 250-253 °C. IR (KBr) cm⁻¹: 3552 (OH), 3333 (NH), 3024 (Ar-CH), 1732 (C=O), 1671 (C=N), 1587 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.04-8.26 (m, 13H, Ar-CH), 6.84 (s, 1H, NH of hydrazone), 5.68 (s, 1H, NH of benzimidazole), 2.30 (s, 1H, OH). EI-MS m/z: 396 (M⁺). Anal. Calcd for C₂₂H₁₆N₂O₂: C, 66.66; H, 4.07; N, 21.20. Found: C, 66.90; H, 4.08; N, 21.14.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazone)-1-(4-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one (H)
Yield = 79 %, m.p. 235-236 °C. IR (KBr) cm⁻¹: 3523 (OH), 3355 & 3308 (NH), 3074 (Ar-CH), 1736 (C=O), 1632 (C=N), 1591 (C=C), 840 (C-Cl). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.37-8.01 (m, 12H, Ar-CH), 7.14 (s, 1H, NH of hydrazone), 5.32 (s, 1H, NH of benzimidazole), 2.36 (s, 1H, OH). EI-MS m/z: 432 (M⁺). Anal. Calcd for C₂₅H₁₄ClN₂O₂: C, 61.33; H, 3.51; N, 19.51. Found: C, 61.12; H, 3.50; N, 19.58.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazone)-1-(4-fluorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one (I)
Yield = 80 %, m.p. 201-203 °C. IR (KBr) cm⁻¹: 3553 (OH), 3343 (NH), 3026 (Ar-CH), 1731 (C=O), 1670 (C=N), 1593 (C=C), 1020 (C-F). 1H-NMR (CDCl₃, 500 MHz) δ ppm: 6.94-7.98 (m, 12H, Ar-CH), 6.62 (s, 1H, NH of hydrazone), 5.47 (s, 1H, NH of benzimidazole), 2.05 (s, 1H, OH). EI-MS m/z: 414 (M⁺). Anal. Calcd for C₂₂H₁₅FN₆O₂: C, 63.76; H, 3.65; N, 20.28. Found: C, 63.54; H, 3.66; N, 20.23.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazone)-3-hydroxy-1-(4-methoxyphenyl)-1H-pyrazol-5(4H)-one (J)
Yield = 76 %, m.p. 208-209 °C. IR (KBr) cm⁻¹: 3587 (OH), 3392 (NH), 3009 (Ar-CH), 2971 (CH₃-CH), 1739 (C=O), 1591 (C=N), 1515 (C=C), 1067 (C-O-C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.15-8.20 (m, 12H, Ar-CH), 7.03 (s, 1H, NH of hydrazone), 4.96 (s, 1H, NH of benzimidazole), 3.92 (s, 3H, OCH₃), 2.34 (s, 1H, OH). EI-MS m/z: 426 (M⁺). Anal. Calcd for C₂₅H₁₉N₆O₄: C, 64.78; H, 4.25; N, 19.71. Found: C, 65.00; H, 4.27; N, 19.64.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazone)-1-(3-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one (K)
Yield = 72 %, m.p. 213-215 °C. IR (KBr) cm⁻¹: 3559 (OH), 3390 & 3323 (NH), 3024 (Ar-CH), 1740 (C=O), 1642 (C=N), 1584 (C=C), 816 (C-Cl). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.06-8.39 (m, 12H, Ar-CH), 6.87 (s, 1H, NH of hydrazone), 5.34 (s, 1H, NH of benzimidazole), 1.91 (s, 1H, OH). EI-MS m/z: 432 (M⁺²), 430 (M⁺). Anal. Calcd for C₂₂H₁₃ClN₆O₂: C, 61.33; H, 3.51; N, 19.51. Found: C, 61.56; H, 3.50; N, 19.45.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazono)-1-(3-fluorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one (L)
Yield = 75 %, m.p. 226-229 °C. IR (KBr) cm⁻¹: 3511 (OH), 3362 & 3260 (NH), 3009 (Ar-CH), 1740 (C=O), 1608 (C=N), 1554 (C=C), 1075 (C-F). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.29-8.44 (m, 12H, Ar-CH), 7.05 (s, 1H, NH of hydrazone), 5.32 (s, 1H, NH of benzimidazole), 2.38 (s, 1H, OH). EI-MS m/z: 414 (M⁺). Anal. Calcd for C₂₃H₁₅FN₄O₂: C, 63.76; H, 3.65; N, 20.28. Found: C, 63.59; H, 3.64; N, 20.35.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazono)-3-hydroxy-5-oxo-4,5-dihydropyrazole-1-carbothioamide (M)
Yield = 73 %, m.p. 193-195 °C. IR (KBr) cm⁻¹: 3539 (OH), 3351 & 3318 (NH), 3076 (Ar-CH), 1730 (C=O), 1652 (C=N), 1625 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.15-8.34 (m, 8H, Ar-CH), 7.01 (s, 1H, NH of hydrazone), 5.19 (s, 1H, NH of benzimidazole), 2.46 (s, 2H, C=S-NH₂), 2.13 (s, 1H, OH). EI-MS m/z: 379 (M⁺). Anal. Calcd for C₁₇H₁₅N₅O₂S: C, 53.82; H, 3.45; N, 25.84. Found: C, 53.99; H, 3.46; N, 25.75.

4-(2-(4-(1H-Benzimidazol-2-yl)phenyl)hydrazono)-3-hydroxy-1-isonicotinoyl-1H-pyrazol-5(4H)-one (N)
Yield = 77 %, m.p. 244-246 °C. IR (KBr) cm⁻¹: 3502 (OH), 3347 & 3290 (NH), 3069 (Ar-CH), 1745 (C=O), 1693 (C=N), 1636 (C=C). ¹H-NMR (CDCl₃, 500 MHz) δ ppm: 7.03-8.90 (m, 12H, Ar-CH), 6.78 (s, 1H, NH of hydrazone), 5.09 (s, 1H, NH of benzimidazole), 2.27 (s, 1H, OH). EI-MS m/z: 425 (M⁺). Anal. Calcd for C₂₂H₁₅N₅O₂: C, 62.11; H, 3.55; N, 23.05. Found: C, 62.33; H, 3.54; N, 22.97.

**Biological activities**

**Antitubercular activity**

*In vitro* *M. tuberculosis* method (Agar dilution method) was performed to assess the antitubercular potency of test compounds [34-35]. 10 fold serial dilutions of each test compound/drug were incorporated into Middle brook 7H11 agar slants with OADC growth supplement. Inoculums of *M. tuberculosis* H₃₇RV were prepared from fresh Middle brook 7H11 agar slants with OADC Growth Supplement adjusted to 1 mg/ml (wet weight) in tween 80 (0.05 %) saline diluted to 10⁻² to give a concentration of approximately 10⁷ cfu/ml. A 5 µl amount of bacterial suspension was spotted into 7H11 agar tubes containing 10 fold serial dilutions of drug per ml. The tubes were incubated at 37 °C, and final readings were recorded after 28 days. Tubes having the compounds were compared with control tubes where medium alone was incubated with H₃₇RV. The concentration at which complete inhibition of colonies occurred was taken as active concentration of test compound. The minimum inhibitory

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concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth. The MIC of the test compounds was compared with the standard Isoniazid (INH) and the results are presented in Table 1.

**Antimicrobial activity**

In this study, all the synthesized compounds were screened for antimicrobial activity by agar streak dilution method. The antibacterial activity of the compounds were evaluated against four Gram-positive bacteria *Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 155, *Micrococcus luteus* ATCC 4698 and *Bacillus cereus* ATCC 11778 and three Gram-negative bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 2853 and *Klebsiella pneumoniae* ATCC 11298. The antifungal activities of the synthesized compounds were evaluated against two fungi *Aspergillus niger* ATCC 9029 and *Aspergillus fumigatus* ATCC 46645. Bacterial strains were cultured overnight at 37 °C in Mueller-Hinton broth and the yeast was cultured overnight at 30 °C in YEPDE agar for antibacterial and antifungal activity tests. Test strains were suspended in nutrient agar to give a final density of 5 x 10^{-5} cfu/ml.

**Minimum inhibitory concentration (MIC)**

MIC of the compounds was determined by agar streak dilution method [36]. A stock solution of the synthesized compound in dimethyl formamide was prepared and graded quantities of the test compounds were incorporated in specified quantity of molten sterile agar (nutrient agar for antibacterial activity and Sabouraud’s dextrose agar medium for antifungal activity). A specified quantity of the medium (40-50 °C) containing the compound was poured into a petridish to give a depth of 3-4 mm and allowed to solidify. Suspension of the microorganism were prepared to contain approximately 5 x 10^{-5} cfu/ml and applied to plates with serially diluted compounds in dimethyl formamide to be tested and incubated at 37 °C for 24 h and 48 h for bacteria and fungi, respectively. The MIC was considered to be the lowest concentration of the test substance exhibiting no visible growth of bacteria or fungi on the plate. The observed MIC is presented in Table 1.

**RESULTS AND DISCUSSION**

**Chemistry**

The protocol for the synthesis of target compounds C-N is shown in Scheme 1. In this study, a series of novel benzimidazole derivatives C-N were synthesized by substituting different heterocyclic nucleus possessing phenyl hydrazone moiety at C-2 position of benzimidazole. By a multistep synthesis, a sequence of new phenylhydrazonebenzimidazole derivatives C-N was synthesized from o-phenylenediamine & p-aminobenzoic acid. Initially by cyclisation reaction 4-((1H-benzimidazol-2-yl)benzenamine A was synthesized by treating o-phenylenediamine with p-aminobenzoic acid in presence of polyphosphoric acid (PPA). Latter the amino derivative obtained A was treated with sodium nitrite and hydrochloric acid to produce corresponding diazonium chloride salts. Afterward through intramolecular rearrangement reaction diethyl 2-((2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)malonate B was synthesized by reacting diethyl malonate with the diazonium chloride salt obtained. In the succeeding step, different amino derivatives such as hydroxylamine hydrochloride, urea/thiourea, and different hydrazine hydrochloride analogs were treated with the obtained diester B and produces corresponding isoxazole C, pyrimidine D-E, and
pyrazole derivatives F-N, respectively by dehydrative cyclisation reaction. The reaction optimization, completion and purity of the synthesized intermediates and final compounds are confirmed by TLC.

The structures of the novel synthesized compounds were confirmed by IR, $^1$H-NMR, mass spectra and elemental analyses data. Spectral data of synthesized compounds are in accordance with the assigned structures. All the synthesized compounds showed some characteristic peaks in its IR spectra representing the presence of specific groups. Formation of 4-(1H-benimidazol-2-yl)benzenamine A was confirmed by presence of absorption peak at 3357 cm$^{-1}$ in IR due to presence of NH stretching & absence of a broad absorption peak between 3000-3500 cm$^{-1}$ corresponds to COOH. Appearance of singlet at $\delta$ 5.78 and 4.30 ppm in its $^1$H-NMR spectra for one and two proton which might be assigned to NH and NH$_2$ proton, respectively further confirms the structure of 4-(1H-benimidazol-2-yl)benzenamine A. Appearance of sharp peak at 1729 cm$^{-1}$ in IR corresponds to C=O stretching and appearance of two singlet peaks at $\delta$ 6.91, and 4.84 ppm for one proton each which might be assigned to NH of hydrazone, and NH of benimidazole proton, respectively confirms the formations of diester B. Further appearance of quartet peak at $\delta$ 4.38-4.50 ppm for two protons which might be assigned to CH$_2$ of C$_2$H$_3$ and triplet peak at $\delta$ 1.89-2.02 ppm for three protons which might be assigned to CH$_3$ of C$_2$H$_3$ confirms the structure of diethyl 2-(2-(4-(1H-benimidazol-2-yl)phenyl)hydrazono)malonate B. In $^1$H-NMR spectra disappearance of quartet and triplet peaks corresponds to the CH$_2$ and CH$_3$ of C$_2$H$_3$ recognize the conversion of diethyl 2-(2-(4-(1H-benimidazol-2-yl)phenyl)hydrazono)malonate B to novel heterocyclic substituted benimidazole analogs C-N. The formation of hydroxyisoxazole nucleus in compound C is confirmed by the appearance of singlet peak in its $^1$H-NMR spectra at $\delta$ 2.52 ppm corresponds to the one proton of OH, and appearance of absorption peak at 3514 cm$^{-1}$, in IR corresponds to hydroxyl group. Similarly in compound D appearance of two singlet peak in $^1$H-

Figure 1: Synthesis of novel heterocyclic substituted benimidazole derivatives (C-N)

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NMR spectra at δ 2.37 & 2.21 ppm corresponds to the one proton of two OH, and in IR appearance of absorption peak at 3554 cm⁻¹ corresponds to hydroxyl group confirms the formation of hydroxypyrimidine nucleus. More over in IR appearance of absorption peak at 3553 cm⁻¹ and 2575 cm⁻¹ corresponds to hydroxyl group and sulfhydryl group, respectively and appearance of two singlet peak in ¹H-NMR spectra at δ 2.45 & 1.54 ppm corresponds to the one proton of OH and SH, respectively confirms the formation of mercapto pyrimidine nucleus in compound E.

In ¹H-NMR spectra appearance of singlet at δ 6.82 ppm for one proton of pyrazole NH confirms the formation of compound F and multiplet at δ 7.04-8.26 ppm for 13 protons observed confirms the structure of compound G. In IR appearance of sharp peak at 840 cm⁻¹, and 816 cm⁻¹, corresponds to chlorine confirms the presence of chlorine in compounds H and K, respectively. Similarly, in IR appearance of sharp peak at 1020 cm⁻¹, and 1075 cm⁻¹, corresponds to fluorine confirms the presence of fluorine in compounds I and L, respectively. Appearance of singlet at δ 3.92 ppm for three protons of methoxy group confirms the formation of compound J. Meanwhile, appearance of singlet at δ 2.46 ppm for two protons of amino group confirms the formation of compound M. The formation of compound N was confirmed by appearance of multiplet at δ 7.03-8.90 ppm for 12 protons. Appearance of various other peaks in NMR spectroscopy corresponds to assigned structure further confirms the structure of title compounds C-N. Molecular weight and purity of synthesized compounds were further confirmed from mass spectrum.

**Biological activities**

**Antitubercular activity**

Against H₃⁷Rv strain of M. tuberculosis the entire series of compounds were screened for their in vitro antimycobacterial activity and the results are expressed in terms of MIC. In order to control the sensitivity of the test organisms the MIC of INH was determined in parallel experiments. Antimycobacterial activity data indicate that the test compounds inhibited the growth of M. tuberculosis in varying degree. Out of several synthesized derivatives, compounds such as H and K at 3.9 µg/ml concentrations inhibited the growth of M. tuberculosis. The potent activity of these compounds may be due to the presence of chlorine group at C-3/C-4 position of phenyl ring. Whereas at 7.81 µg/ml concentration, compounds I and L exhibited activity which possessing fluorine group at C-3/C-4 of phenyl ring. Compounds C, D, and E possessing 3-hydroxyisoxazol-5(4H)-one/6-hydroxy pyrimidin-4(5H)-one nucleus attached to 1-(4-(1H-benzimidazol-2-yl)phenyl)hydrazine, inhibited the growth of M. tuberculosis at 15.62 µg/ml concentrations only. The MIC of compounds G and J was found to be 62.5 µg/ml. Rest of series (compounds F, M, and N) showed activity only at higher concentration (MIC: ≥62.5 µg/ml).

**Antimicrobial activity**

To analyze the in vitro antimicrobial activity of title compounds C-N, agar streak dilution method was used. In Table 1, antimicrobial activity of the synthesized compounds was effectively compared with that of standard drugs. MICs of standard drugs (Ciprofloxacin and Ketoconazole) were determined in parallel experiments in order to control the sensitivity of the test organisms.
Table 1: MIC (Minimum inhibitory concentration in µg/ml) of synthesized compounds (C-N)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antitubercular activity</th>
<th>Antibacterial activity</th>
<th>Antifungal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. tuberculosis</td>
<td>S. aureus</td>
<td>S. epidermidis</td>
</tr>
<tr>
<td>C</td>
<td>15.62</td>
<td>15.62</td>
<td>7.81</td>
</tr>
<tr>
<td>D</td>
<td>15.62</td>
<td>31.25</td>
<td>7.81</td>
</tr>
<tr>
<td>E</td>
<td>15.62</td>
<td>31.25</td>
<td>15.62</td>
</tr>
<tr>
<td>F</td>
<td>125</td>
<td>62.5</td>
<td>62.5</td>
</tr>
<tr>
<td>G</td>
<td>62.5</td>
<td>31.25</td>
<td>31.25</td>
</tr>
<tr>
<td>H</td>
<td>3.9</td>
<td>7.81</td>
<td>1.95</td>
</tr>
<tr>
<td>I</td>
<td>7.81</td>
<td>7.81</td>
<td>3.9</td>
</tr>
<tr>
<td>J</td>
<td>62.5</td>
<td>62.5</td>
<td>31.25</td>
</tr>
<tr>
<td>K</td>
<td>3.9</td>
<td>7.81</td>
<td>3.9</td>
</tr>
<tr>
<td>L</td>
<td>7.81</td>
<td>15.62</td>
<td>3.9</td>
</tr>
<tr>
<td>M</td>
<td>&gt;125</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>N</td>
<td>125</td>
<td>125</td>
<td>62.5</td>
</tr>
<tr>
<td>Standard</td>
<td>0.97</td>
<td>7.81</td>
<td>1.95</td>
</tr>
</tbody>
</table>

*Isoniazid used as a reference standard against M. tuberculosis whereas Ciprofloxacin used as a reference standard for other bacteria & Ketoconazole used as a reference standard for fungi.

From the results it was observed that like Ciprofloxacin, compounds H, I and K (MIC: 7.81 µg/ml) displayed similar activity against S. aureus; whereas rest of series exhibited lesser activity (MIC: ≥15.62 µg/ml). Against S. epidermidis compound H exhibited equal activity (MIC: 1.95 µg/ml) compared to Ciprofloxacin whereas rest of series exhibited lower activity (MIC: ≥3.9 µg/ml). Against M. luteus compound H and K exhibited equal activity (MIC: 3.9 µg/ml) compared to Ciprofloxacin whereas rest of series exhibited lower activity (MIC: ≥7.81 µg/ml). Like Ciprofloxacin, compounds H, I, K and L (MIC: 7.81 µg/ml) displayed similar activity against B. cereus; whereas rest of series exhibited lesser activity (MIC: ≥31.25 µg/ml). While others demonstrated lesser activity than standard against E. coli, compounds H, I and K exhibited comparable activity (MIC: 7.81 µg/ml) as Ciprofloxacin. Compound K showed the same activity (MIC: 3.9 µg/ml) as Ciprofloxacin, whereas rest of all compounds showed worse activity (MIC: ≥7.81 µg/ml) than standard against P. aeruginosa. Compound H and K showed the same activity (MIC: 1.95 µg/ml) as Ciprofloxacin, whereas rest of all compounds showed worse activity (MIC: ≥3.9 µg/ml) than standard against K. pneumoniae. Among the various tested derivatives, the compound H displayed superior activity than rest of tested derivatives against all microorganisms except P. aeruginosa. Among screened compounds against P. aeruginosa, compound K exhibited highest activity.
The antifungal activities of the synthesized compounds were evaluated against A. niger and A. fumigatus. The title compounds exhibits varying degree of antifungal activity. Compounds H and K showed the superior activity (MIC: 3.9 µg/ml) and Compounds C, I and L showed the same activity (MIC: 7.81 µg/ml) as Ketoconazole, whereas rest of all compounds showed worse activity (MIC: ≥15.62 µg/ml) than standard against A. niger. Similarly, when compared against Ketoconazole, compounds H and K (MIC: 1.95 µg/ml) displayed superior activity; compounds I and L (MIC: 3.9 µg/ml) displayed similar activity against A. fumigatus; whereas rest of series exhibited lesser activity (MIC: ≥7.81 µg/ml). In general from the study it was found that compounds H, I, K and L displayed good antimicrobial activity; compounds C, D and E displayed moderate antimicrobial activity; whereas rest of title compounds (F, G, J, M and N) showed poor antimicrobial activity.

Structural activity relationship
In general from the study it was found that compounds H, I, K and L displayed good antimicrobial activity; compounds C, D and E displayed moderate antimicrobial activity; whereas rest of title compounds (F, G, J, M and N) showed poor antimicrobial activity. The potent antibacterial activity exhibited by compounds H, I, K and L might be due to the presence of electron withdrawing substituent like chloro, and fluoro group in the phenyl ring attached to pyrazole nucleus. In general, electron withdrawing group substituted pyrazole analogs H, I, K and L exhibited better antimicrobial activity than isoxazole C, and pyrimidine D-E derivatives. The chemical structure and antimicrobial activity relationship of the synthesized compounds revealed that the compounds having electron withdrawing moiety H, I, K and L exhibited better activity than compounds has electron releasing moieties J. Within electron withdrawing group, position of the substituent doesn’t play any role [para (H and I) and meta (K and L) substituted derivatives displayed almost equal activity]. Replacement of phenyl ring G-L with acyclic amide M or heterocyclic carbonyl derivative N results in decreased activity. In addition from the study it was found that all test compounds exhibited higher antifungal activity than antibacterial activity. Among tested compounds, 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-1-(4-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one H 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-1-(3-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one K and was found to be potent antimicrobial agent.

CONCLUSION
In conclusion, several new isoxazole / pyrimidine / pyrazole substituted benzimidazole hydrazones were synthesized using multistep synthesis from o-phenylenediamine and p-amino benzoic acid. All the synthesized compounds were characterized using FT-IR, 1H-NMR, Mass spectroscopy and elemental analysis. Entire title compounds were assessed for their in vitro antitubercular, antibacterial and antifungal activity. Compounds showed mild to good antimicrobial activity. From the SAR studies it was found that, nature of substituent’s played major role in determining antimicrobial activity than position of the substituent. Electron withdrawing group substituted derivative exhibited better activity than electron donating group substituted analogs. Among several tested compounds, 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-1-(4-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one H and 4-(2-(4-(1H-benzimidazol-2-yl)phenyl)hydrazono)-1-(3-chlorophenyl)-3-hydroxy-1H-pyrazol-5(4H)-one K showed better antimicrobial activity which is almost equal to reference standard Ciprofloxacin & Ketoconazole. In addition, compounds I and L also showed some excellent antibacterial activity against some pathogenic strains of microorganisms. These compounds also displayed good antitubercular activity also. Hence, these analogs could be developed as a new class of antimicrobial agents. However, further structural modification is planned to enhance the antitubercular and antibacterial activity.
REFERENCES