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Research Article

In-Vitro and In-Vivo Analysis of Novel Substituted Benzimidazole Derivatives as Antimicrobial Agents

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Abstract

In present context, development of novel Antimicrobials is become an urgent need to addressing AMR. In the present study, some novel substituted Bezimidazole derivatives have been designed and subjected to molecular- docking against *E. coli* and *S. aureus* by using Auto Dock Vina software. Among all 10 designed derivatives BI-B show highest binding affinity and molecular interaction against selected target. ADMET prediction by using Molsoft L.L.C. Software that help to predicts both physicochemical significant descriptors and pharmacokinetic significant properties. Then 4-chloro-phenyl benzimidazole and 4-nitro-phenyl benzimidazole derivatives were synthesized and structurally characterized by ^{1}H NMR spectroscopy that confirm the structure of synthesized derivatives. *In vitro* antimicrobial analysis of all the synthesized compounds were performed by agar plate technique. The results indicate that the synthesized compounds show considerable antimicrobial action against the selected microorganism at 31.25 µm concentrations as compared to ampicillin as standard.

Keywords: Antimicrobial; Benzimidazole; Molecular Docking; ADMET; *(E. coli)*; *(S. aureus)*

Introduction

The discovery of antibiotics is considered one of the greatest breakthroughs in twentieth-century medicine, as these drugs have saved countless lives and made complex medical interventions such as surgical procedures and cancer chemotherapy possible. However, the rapid emergence and global spread of antibacterial resistance affecting both hospital-acquired and community-acquired infections has severely threatened the effectiveness of these life-saving agents [1].

Antimicrobial resistance not only compromises the treatment of bacterial infections in humans and animals but also imposes significant social and economic burdens, ultimately hindering progress toward achieving the Sustainable Development Goals. Addressing this crisis requires immediate and coordinated efforts at global, regional, and national levels [2]. Although resistance is a natural and unavoidable evolutionary process, its development is greatly accelerated by the misuse and overuse of antibiotics. Therefore, responsible antibiotic stewardship and the development of novel antibacterial agents are essential to combat emerging resistance [3].

Despite this need, the discovery of new antibiotics faces major challenges, including scientific difficulties in identifying effective

compounds and economic barriers related to investment in research and development. Most newly developed antibiotic classes primarily target Gram-positive bacteria, whereas the most urgent unmet need lies in treating Gram-negative bacterial infections, which have been classified by the World Health Organization as critical priority pathogens for antibiotic research and development [4]. The gram- negative bacteria having more complex structure as compare to gram positive bacteria and due to this, new drugs suffer some problem to penetrate through the bacterial cell envelope. Furthermore, the shortage of high-quality lead compounds suitable for screening against Gram-negative bacteria significantly hampers antibiotic discovery efforts.

In recent years, widespread and inappropriate antibiotic use has further intensified the problem of drug resistance, making the search for alternative and effective antibacterial agents increasingly urgent [5].

In medicinal chemistry, heterocyclic compounds are very much important and Benzimidazole derivatives are among one of them. Having unique fused benzene–imidazole ring system enables benzimidazole derivatives to interact efficiently with various biological targets, resulting in a broad spectrum of pharmacological activities. Numerous studies have demonstrated that benzimidazole-based compounds possess significant antimicrobial, antifungal, antiviral, antipa rasitic, anticancer, and anti-inflammatory properties, making them attractive candidates for drug development [6,7].

The rapid emergence of antimicrobial resistance has become a major global health concern, reducing the effectiveness of existing antibiotics and necessitating the discovery of new antimicrobial agents with novel mechanisms of action. In this context, benzimidazole and its derivatives have gained considerable attention due to their potent activity against a wide range of pathogenic microorganisms, including both Gram-positive and Gram-negative bacteria, as well as fungi. Structural modifications at different positions of the benzimidazole nucleus have been shown to enhance antimicrobial efficacy and overcome resistance, highlighting the scaffold's synthetic flexibility and biological relevance [8,9].

Several benzimidazole derivatives exert their antimicrobial action by targeting essential microbial processes such as DNA synthesis, enzyme inhibition, and disruption of cell membrane integrity. The promising biological profile, ease of chemical modification, and ability to generate diverse analogues make benzimidazole derivatives valuable lead compounds in the development of new antimicrobial agents. Consequently, continuous research efforts are focused on the rational design, synthesis, and biological evaluation of novel benzimidazole derivatives to address the growing challenge of drug-resistant microbial infections [10].

Materials and Methods

Docking

Proteins download

The target protein of *Staphylococcus aureus* (PDB ID -1Q1Y) and *Escherichia coli* (PDB ID- 4AMV) were selected for molecular docking. These protein files were downloaded from RCSB in PDB format.

Protein preparation

UCSF Chimera 1.7 software is used to prepare protein by removing water molecule.

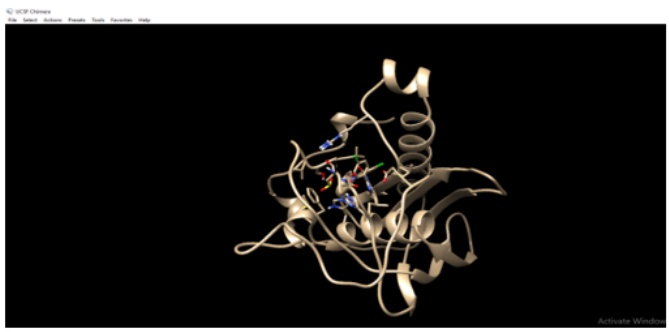


Figure 1: Protein Preparation.

Ligand preparation

Chem Draw Ultra 8.0 was used to draw ligand structure saved in mole. Format.

Defining binding site

Selecting all atom within 10Å, and binding site of protein was determined.

S. No.	PDB ID	X	Y	Z
1.	1Q1Y	-16.554	144.414	46.203
2.	4AMV	5.576	0.117	83.467

Table 1: Binding site of the protein.

Docking in AutoDock

Docking was performed with the help of AutoDock vina software. The .pdb file was first downloaded from major database. Then PDBQT formate file was prepared for both target and ligand and also grid and docking parameter file was prepared as a.gpf and a.dpf by using AutoDock vina software. And then, finally performed docking and docking score was analyzed.

Analysis of docking score

1Q1Y:

The interaction of all designed benzimidazole derivatives bind with 1Q1Y protein was observed. BI_B and BI_E showed highest docking score of -9.13 and -9.42 and thus showed the highest binding affinity and lowest binding energy with 1Q1Y as compared with reference drug Ampicillin.

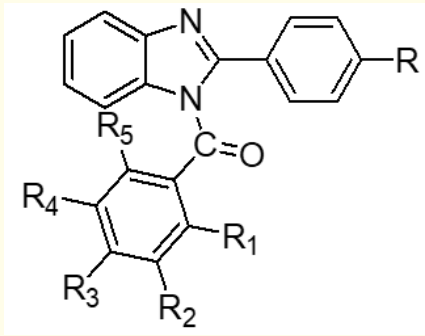


Figure 2: General Structure of Benzimidazole derivative.

Where,

R = Cl, NO2

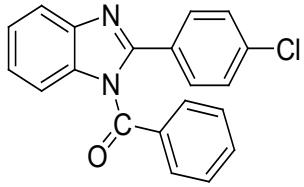
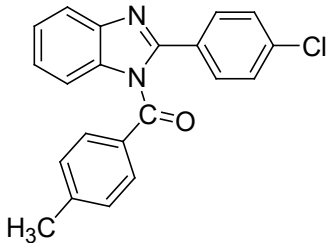
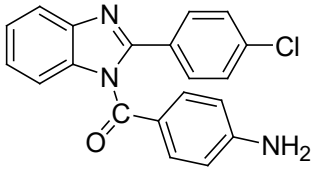
R1 = Cl, H

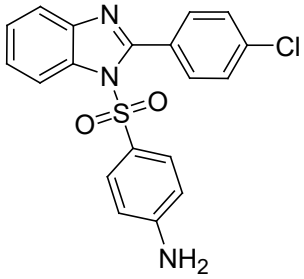
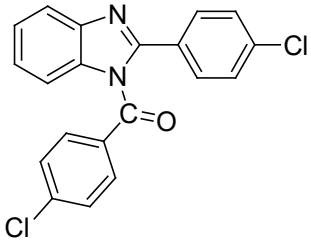
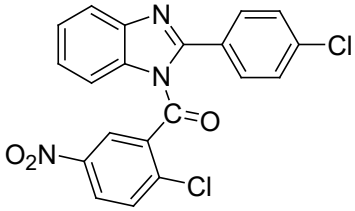
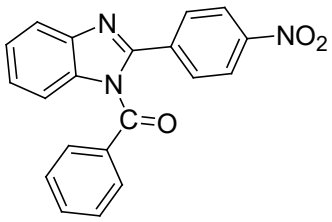
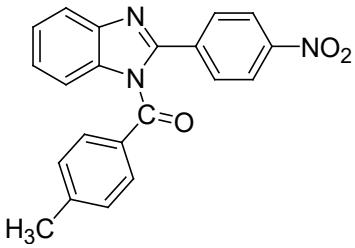
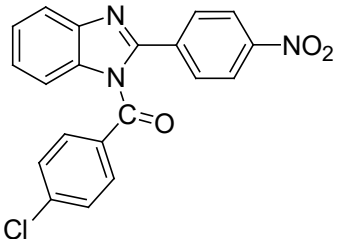
R2 = H

R3 = CH3, NH2, Cl, H

R4 = H, NO2

R5 = H

Compound Code	Compound Structure	IUPAC Name	*S. aureus* (1Q1Y)	*E. coli* (4AMV)
BI_A		[2-(4-chloro-phenyl)-benzoimidazol-1-yl]-phenyl-methanone	-7.79	-6.41
BI_B		[2-(4-cholro-phenyl)-benzoimidazol-1-yl]-p-tolyl-methanone	-9.13	-8.48
BI_C		(4-Amino-phenyl)-[2-(4-chloro-phenyl)-benzo-imidazol-1-yl]-methanone	-8.11	-6.43

BI_D		4-[2-(4-chloro-phenyl)-benzoimidazole-1-sulfonyl]-phenylamine	-8.12	-7.46
BI_E		(4-chloro-phenyl)-[2-(4-chloro-phenyl)-benzo-imidazol-1-yl]-methanone	-9.42	-8.31
BI_F		(2-chloro-5-nitro-phenyl)-[2-(4-chloro-phenyl)-benzoimidazol-1-yl]-methanone	-8.91	-8.10
BI_G		[2-(4-Nitro-phenyl)-ben-zoimidazol-1-yl]-phenyl-methanone	-7.74	-6.58
BI_H		[2-(4-Nitro-phenyl)-ben-zoimidazol-1-yl]-p-tolyl-methanone	-7.73	-6.67
BI_I		(4-chloro-phenyl)-[2-(4-nitro-phenyl)-benzoimid-azol-1-yl]-methanone	-7.40	-6.68

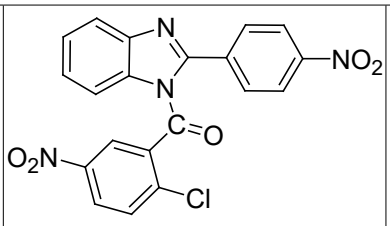
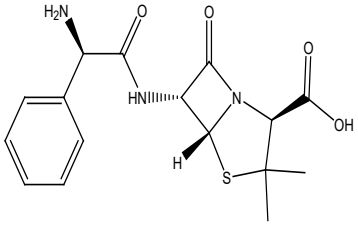
BI_J		(2-chloro-5-nitro-phenyl)-[2-(4-nitro-phenyl)-benzoimidazol-1-yl]-methanone	-7.81	-6.89
BI_Z		Ampicillin	-9.00	-8.18

Table 2: Docking Score of compounds by using AutoDock 4.2 software.

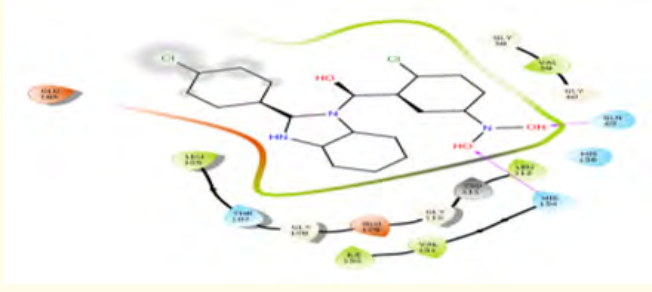


Figure 3: Ligand interaction diagrams of compound BI_B with 1Q1Y protein.

4AMV

The interaction of all designed benzimidazole derivatives with 4AMV protein was observed. BI_B and BI_E showed highest docking score of -8.48 and -8.31 and thus showed the highest binding affinity and lowest binding energy with 4AMV as compared with reference drug Ampicillin.

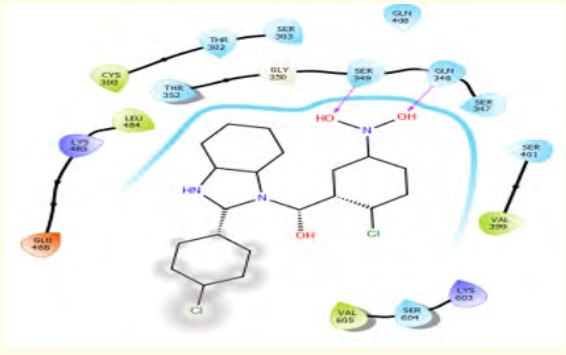


Figure 4: Ligand interaction diagrams of compound BI_E with 4AMV protein.

ADME prediction

ADME properties were calculated by using Molsoft L.L.C. Software. It determines the physicochemical properties. This software help to evaluate suitability of drug and help to select best derivatives that show good Lipinski's rule of five.

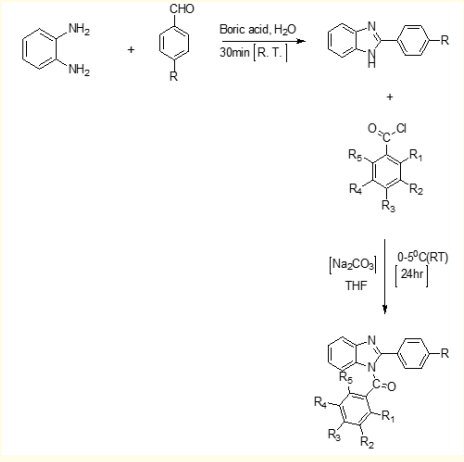


Figure 5: Scheme of reaction.

R= Cl, NO2

R1= Cl, H

R2= H

R3= CH3, NH2, Cl,

R4= NO2, H

R5= H

Synthesis

On the basis of molecular docking results and ADMET prediction results, BI_B and BI_E showed highest docking score and ADMET results against both selected target protein 4AMV and 1Q1Y as compared with reference drug Ampicillin and thus both benzimidazole derivatives (BI_B and BI_E) were selected for synthesis and further antimicrobial evaluation. Following are synthesis method for the synthesis of selected derivatives.

Synthesi of Compound BI_B ([2-(4-cholro-phenyl)-benzoimidazol-1-yl]-p-tolyl-methanone)

2 m mol of 4-chloro-benzaldehyde, 2 m mol of o-phenylenediamine, 0.1 gm boric acid and 1 ml water was taken and mixture was prepared by stirring at room temperature for 30min. TLC method was used to determine the reaction progress by taking ethyl acetate/petroleum ether in 2:8 ration as mobile phase. When the reaction was completed so 5 ml water was added and then stirred for 10 min. After some time, the ppt formed and then filtered the solution and ppt was collected. The collected ppt was purified by recrystallization via ethanol. 4g (19.10 mmol) of product was dissolved in 20 ml of tetrahydrofuran in a RBF at room temperature. The medium was basified by adding Na_2CO_3 (4g, 38.30 mmol) and cooled (0-5 ºC) in ice bath. Then 19.10 m mol *p*-methyl-benzoyl chloride (electrophilic releasing substrate) was added and for 15 min, this reaction mixture was kept on ice bath and then removed it from ice bath and remain it for room temperature for some time so it's temperature was increased and came up to room temperature, then this reaction mixture was stirred for 24 hours. The progress of the reaction mixture was check by using TLC method taking $CHCl_3/CH_3OH$ as mobile phase in 9:1 ratio and spot was observed in UV chamber. After that, we evaporated the solvent from the mixture and then cooled it by adding cold water and filtration was performed by suction filtration method and dried the product. The crude product was purified by recrystallization via DMF.

Yield: 65%.

Synthesis of Compound BI_E (4-Amino-phenyl)-[2-(4-chloro-phenyl)-benzoimidazol-1-yl]-methanone

2 m mol of 4-chloro-benzaldehyde, 2 m mol of o-phenylenediamine, 0.1 gm boric acid and 1 *ml* water was taken and mixture was prepared by stirring at room temperature for 30min. TLC method was used to determine the reaction progress by taking ethyl acetate/petroleum ether in 2:8 ration as mobile phase. When the reaction was completed so 5 ml water was added and then stirred for 10 min. After some time, the ppt formed and then filtered the solution and ppt was collected. The collected ppt was purified by recrystallization via ethanol to give 2-(4-chloro-phenyl)-1*H*-benzoimidazole ppt. 4g (19.10 mmol) of product was dissolved in 20ml of tetrahydrofuran in a RBF at room temperature. The medium was basified by adding Na_2CO_3 (4g, 38.30 mmol) and cooled (0-5 ºC) in ice bath. Then 19.10 m mol *p*-amino-benzoyl chloride (electrophilic releasing substrate) was added and for 15 min, this reaction mixture was kept on ice bath and then removed it from ice bath and remain it for room temperature for some time so it's temperature was increased and came up to room temperature, then this reaction mixture was stirred for 24 hours. The progress of the reaction mixture was check by using TLC method taking $CHCl_3/CH_3OH$ as mobile phase in 9:1 ratio and spot was observed in UV chamber. After that, we evaporated the solvent from the mixture and then cooled it by adding cold water and filtration was performed by suction filtration method and dried the product. The crude product was purified by recrystallization via DMF.

Yield: 61%.

Microbiological assay

Bacterial cells, growth media and chemicals

The solvent DMSO and 96-well microliter plates were used. The bacterial cells used in the present study were procured from the Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacterial cells *Staphylococcus aureus* MTCC 3160 and *Escherichia coli* MTCC-2961 were cultured in Mueller Hinton Broth.

For determining the MIC value of synthesized derivatives we used the following mention nutrient broth.

Minimum Inhibitory Concentration (MIC)

Antibacterial activity of synthesized derivatives was determining by measuring MIC value (Minimum inhibitory concentration activity) against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). The bacterial cells (*S. aureus* and *E. coli*) were grown overnight and diluted in Mueller-Hinton broth (MHB) to a density of 10^5 CFU/mL (Colony Forming Unit (CFU). Further, the bacterial

S. No.	Ingredient	Nutrient broth
1.	Beef Extract	2.0 gm
2.	Acid Hydrolysate of Casein	17.5 gm
3.	Starch	1.5 gm
4.	Agar	17.0 gm
4.	Distilled Water	Up to 1000 ml

Table 4: Composition of nutrient broth.

pH was 7.3 ± 0.1 at room temperature.

Reference standard: Ampicillin was taking as a reference standard.

cells (100 µl) and synthetic compounds concentrations from 250µM to 0.095 µM were added into the 96-well flat-bottom microliter plate and incubated for 24 hrs at 37ºC without shaking. The optical density (O.D) and visual growth of bacterial cells was measured at 600 nm by using microplate reader (Thermos scientific, Model 680). In the above mention step, ampicillin was taken as reference standard [11].

S. No.	Compounds	*Staphylococcus aureus*	*Escherichia coli*
1.	BI_B	31.25	31.25
2.	BI_E	62.50	31.25
3.	Benzimidazole	>250	>250
4.	Ampicillin	250	100

Table 5: Antibacterial activity of Synthesized Compounds (in µM).

The synthetic compounds BI_B and BI_E were shown the potent antibacterial activity against *S. aureus* and *E. coli* respectively at 31.25 µM concentrations as compared to ampicillin.

Result and Discussion

The results of molecular docking, synthesis, characterisation and anti-bacterial evaluation are discussed below.

Molecular docking results

On the basis of literature search ligands were prepared by using 2D chemdraw ultra 8.0 and energy minimized in 3D chemdraw ultra 8.0, these structures were used for docking. 1Q1Y and 4AMV both were selected as target protien and it was downloaded from RCSB (protein data bank).

Docking of ligand was performed by using AutoDock 4.2 software. On the basis of docking score and ADMET prediction results the highly scored compounds BI_B and BI_E were selected for synthesis and antimicrobial evaluation.

Characterization of synthesized compounds

Two compounds were synthesized on the basis of the docking score and structures were confirmed by ^{1}H NMR spectroscopy.

^{1}H NMR spectroscopy

^{1}H NMR spectra of the synthesized compounds were determined in DMSO-d_6 using TMS as a reference by BRUKER ADVANCE Ⅲ (500MHz) instrument from CIF NMR facility IISER Bhopal. Table 6 show results of ^{1}H NMR.

S. No.	Compound Code	^{1}H NMR Shifts in reference to TMS
1.	BI_B	DMSO (2.5), 2.3(H, s, Ph), 7.02(H, d, Ph), 7.25(H, d, Ph), 7.2(H, Q, Ar), 7.33(H, Q, Ar), 7.4(H, d, Ph), 7.6(H, d, Ph), 7.75(H, d, Ph), 7.8(2H, d, Ph), 8.1(2H, d, Ar), 8.25(H, d, Ar).
2.	BI_E	DMSO (2.5), 4.3 (H, Q, Ph), 7.2 (2H, Q, Ar), 7.33 (2H, d, Ph), 7.4 (2H, t, Ph), 7.6 (2H, d, Ph), 7.75 (H, d, Ph), 8.2 (H, d, Ph).

Table 6: ^{1}H NMR Spectroscopy results of synthesized derivatives.

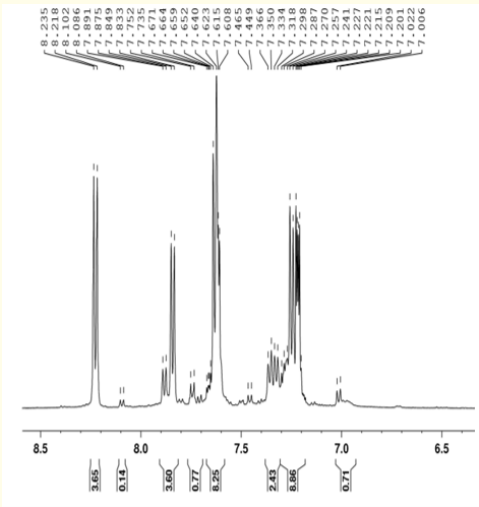


Figure 6: ^{1}H NMR spectra of compound BI_B.

Determination of physicochemical properties of synthesized compounds

Melting point

Open capillary method was used for determination of melting point of synthesized compounds. Melting Point is reported in table 7.

Rf value

TLC method was used to determine Rf value of synthesized derivatives by using silica gel 60 F 254 and taking Chloroform: Methanol in 9:1 ratio as mobile phase. The results were analyzed in UV chamber. Rf value of synthesized derivatives are reported in table 7.

Solubility

Various solvent Ethanol, Acetone, DMF, and DMSO were used for solubility determination and it was found that all synthesized derivatives was soluble qualitatively.

Partition coefficient

Octanol and water in equal ration was taken and Partition Coefficient of synthesized compounds was determined and LogP value of compounds are given below.

S. No	Compound Code	Molecular Weight	R_f Value	Melting Point	Partition Coefficient (Log P)
1.	BI_B	346.81	0.72	242-244	0.170
2.	BI_E	347.80	0.71	261-264	0.314

Table 7: Physicochemical properties of synthesized compounds.

Summary and Conclusion

Benzimidazole derivatives are important and useful antimicrobial drugs. The 4-chloro-phenyl benzimidazole and 4-nitro-phenyl benzimidazole derivatives were synthesized and purified by recrystallization. The structure of all the synthesized compounds was confirmed by ^{1}H NMR and mass spectroscopy. The synthesized derivatives of benzimidazole was evaluated for their anti-bacterial activities against *S. aureus* and *E. coli* and MIC value was determined and considerable anti-bacterial activity was observed as compare to reference standard.

Compounds (BI_B and BI_E) showed potent antibacterial activity at 31.25 µM concentration against *S. aureus* and *E. coli* as compared to parent nucleus benzimidazole and standard drug ampicillin. The compound BI_B have p-tolyl group at position 1 and 4- chloro phenyl group at position 2 of benzimidazole ring and the compound BI_E have p-choro phenyl group at both 1 & 2 position of benzimidazole ring showed best activity among synthesized compounds. The overall results of anti-bacterial evaluation revealed that the synthesized compound show considerable anti-bacterial activity and the parent nucleus was further more explored to obtain derivatives with enhanced potency.

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