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DEVELOPMENT AND ANTIMICROBIAL ASSESSMENT OF STRUCTURALLY MODIFIED BENZIMIDAZOLE DERIVATIVES

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ABSTRACT

Background: Antimicrobial resistance (AMR) has emerged as a major global health challenge, reducing the effectiveness of existing antibiotics and creating an urgent need for novel antimicrobial agents. Benzimidazole is a privileged heterocyclic scaffold known for its structural versatility and broad-spectrum biological activities. This study aimed to design, synthesize, and evaluate benzimidazole-acetohydrazide Schiff base derivatives as potential antimicrobial agents. **Methodology:** Fifteen benzimidazole-based acetohydrazide Schiff base derivatives (1–15) were synthesized from 2-mercaptobenzimidazole through nucleophilic substitution, hydrazinolysis, and condensation with substituted aromatic aldehydes. Structural characterization was performed using IR, ^1H NMR, ^13C NMR, and mass spectrometry. Antibacterial activity was evaluated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* using the tube dilution method, with cefadroxil as the reference drug. A 3D-QSAR CoMFA model was also developed to investigate structure–activity relationships. **Results:** Several synthesized derivatives exhibited potent antibacterial activity with minimum inhibitory concentration (MIC) values superior to cefadroxil. Compound 10 demonstrated the highest activity against *S. aureus*, *E. faecalis*, and *E. coli* (MIC = 0.032 µM/mL) and strong activity against *P. aeruginosa* (MIC = 0.016 µM/mL). Compounds 5 and 11 also showed excellent anti-*Pseudomonas* activity. Structure–activity relationship analysis revealed that para-substituted electron-withdrawing groups, particularly bromine, and heteroaromatic substituents significantly enhanced antimicrobial efficacy. The CoMFA model showed excellent predictive performance, with electrostatic fields contributing 70.9% to biological activity. **Conclusion:** Benzimidazole-acetohydrazide Schiff base derivatives exhibited promising antibacterial activity and may serve as valuable lead compounds for developing new therapies against multidrug-resistant bacterial infections.

KEYWORDS: Antimicrobial resistance, benzimidazole, Schiff base derivatives, acetohydrazide, antibacterial activity, MIC, 3D-QSAR.

1. INTRODUCTION

1.1. Antimicrobial Resistance: A Global Healthcare Challenge

Antimicrobial resistance (AMR) has emerged as one of the most serious threats to global public health in the twenty-first century.[1] The widespread and often inappropriate use of antibiotics in human medicine, veterinary practice, and agriculture has accelerated the evolution of resistant microbial strains.[2] As a result, many conventional antibiotics that were once highly effective have gradually lost their therapeutic efficacy.[3] Pathogenic bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* have developed sophisticated resistance mechanisms including enzymatic degradation of drugs, target-site modification[4], reduced membrane permeability[5], and active efflux pumps.[6] These mechanisms significantly compromise the success of antimicrobial therapy and contribute to prolonged hospital stays[7], increased healthcare costs[8], and higher mortality rates.[9]

The World Health Organization (WHO) has identified AMR as a major global health priority[10], emphasizing the urgent need for innovative therapeutic strategies.[11] The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterial strains has created a pressing demand for the discovery of novel antimicrobial agents possessing unique mechanisms of action.[12] Consequently, medicinal chemists are increasingly focusing on the design and development of new heterocyclic compounds capable of overcoming existing resistance mechanisms and restoring antibacterial efficacy.[13]

1.2. Benzimidazole as a Privileged Pharmacophore in Drug Discovery

Heterocyclic compounds occupy a central position in medicinal chemistry due to their remarkable structural diversity and biological significance.[14] Among them, benzimidazole represents one of the most important nitrogen-containing heterocyclic scaffolds.[15] Structurally, benzimidazole consists of a benzene ring fused with an imidazole ring[16], resulting in a planar aromatic bicyclic system capable of participating in various intermolecular interactions.[17]

The pharmaceutical importance of benzimidazole arises from its structural similarity to naturally occurring purine nucleotides.[18] This resemblance enables benzimidazole derivatives to interact effectively with nucleic acids, enzymes, receptors, and other biological macromolecules.[19] Numerous benzimidazole-containing drugs have been successfully introduced into clinical practice, including antimicrobial[20], antifungal[21], antiviral[22], antiparasitic[23], anti-inflammatory[24], antihypertensive[25], and anticancer agents.[26]

The presence of two nitrogen atoms within the imidazole ring contributes significantly to the electronic properties and biological activity of the benzimidazole nucleus.[27] These nitrogen atoms can act as hydrogen bond donors and acceptors, facilitating strong interactions with biological targets.[28] Furthermore, the benzimidazole scaffold can be readily modified through substitution at different positions[29], allowing medicinal chemists to fine-tune physicochemical properties[30], receptor affinity[31], and pharmacological activity.[32] Such versatility has established benzimidazole as a privileged pharmacophore for the development of novel therapeutic agents, particularly in antimicrobial drug discovery.[33]

Table 1.1: Therapeutic Applications of Benzimidazole Derivatives.

S. No.	Biological Activity	Mechanism/Target	Representative Benzimidazole Derivative/Drug
1	Antibacterial	Inhibition of microbial growth and DNA synthesis[34]	Novel benzimidazole Schiff bases[35]
2	Antifungal	Disruption of fungal cell functions[36]	Carbendazim, Benomyl[37]
3	Antiviral	Inhibition of viral replication enzymes[38]	Enviradine derivatives[39]
4	Antiparasitic	Interference with microtubule formation[40]	Albendazole, Mebendazole[41]
5	Anticancer	DNA interaction and enzyme inhibition[42]	Nocodazole and benzimidazole analogues[43]
6	Anti-inflammatory	Inhibition of inflammatory mediators[44]	Substituted benzimidazole derivatives[45]
7	Antioxidant	Free radical scavenging activity[46]	Hydroxy- and methoxy-substituted benzimidazoles[47]
8	Antihypertensive	Angiotensin receptor modulation[48]	Telmisartan, Candesartan analogues[49]
9	Antiulcer	Proton pump inhibition[50]	Proton pump inhibitor derivatives[51]
10	Antimicrobial (Broad Spectrum)	Multiple microbial targets and enzyme inhibition[52]	Benzimidazole-acetohydrazide Schiff bases[53]

1.3. Biological Importance of Schiff Bases and Acetohydrazide Derivatives

Schiff bases constitute an important class of organic compounds characterized by the presence of an azomethine (-CH=N-) functional group formed through condensation between primary amines and aldehydes or ketones.[54] These compounds have attracted considerable scientific interest due to their broad spectrum of biological activities[55], including antibacterial[56], antifungal[57], antiviral[58], anti-inflammatory[59], antioxidant[60], and anticancer properties.[61]

Acetohydrazide derivatives represent another pharmacologically important group of compounds widely explored in medicinal chemistry.[62] The hydrazide moiety possesses multiple reactive centers capable of forming hydrogen bonds and interacting with essential microbial enzymes.[63] Incorporation of acetohydrazide functionality into heterocyclic systems often enhances biological activity and improves target selectivity.[64]

The combination of benzimidazole[65], acetohydrazide[66], and Schiff base pharmacophores[67] within a single molecular framework provides a promising strategy for developing potent antimicrobial agents.[68] Such hybrid molecules may exhibit synergistic effects arising from the complementary biological properties of each

pharmacophoric unit.[69] Moreover, structural modification through the introduction of electron-donating[70], electron-withdrawing[71], or heteroaromatic substituents can significantly influence antimicrobial potency and selectivity.[72] Therefore, benzimidazole-acetohydrazide Schiff base hybrids have emerged as attractive candidates for combating resistant bacterial pathogens.[73]

1.4. Rationale and Objectives of the Present Investigation

Despite significant advances in antimicrobial chemotherapy, the continuous emergence of resistant bacterial strains necessitates the exploration of new chemical entities with improved efficacy and safety profiles.[74] Benzimidazole derivatives have repeatedly demonstrated promising antimicrobial activity[75], yet further structural optimization is required to identify more potent molecules capable of overcoming resistance mechanisms.[76]

In the present investigation, a series of structurally modified benzimidazole-acetohydrazide Schiff base derivatives were designed and synthesized by incorporating various aromatic and heteroaromatic substituents.[77] The rationale behind this approach was to investigate the influence of different electronic and steric factors on antibacterial activity and establish meaningful structure–activity relationships (SAR).[78] The synthesized compounds were characterized using modern spectroscopic techniques and evaluated against representative Gram-positive and Gram-negative bacterial strains.[79] Furthermore, computational structure–activity relationship studies were employed to identify molecular features contributing to biological activity.[80] Through this integrated synthetic, biological, and computational approach, the study aimed to identify promising benzimidazole-based lead compounds for the development of next-generation antimicrobial agents against multidrug-resistant bacterial infections.[81-90]

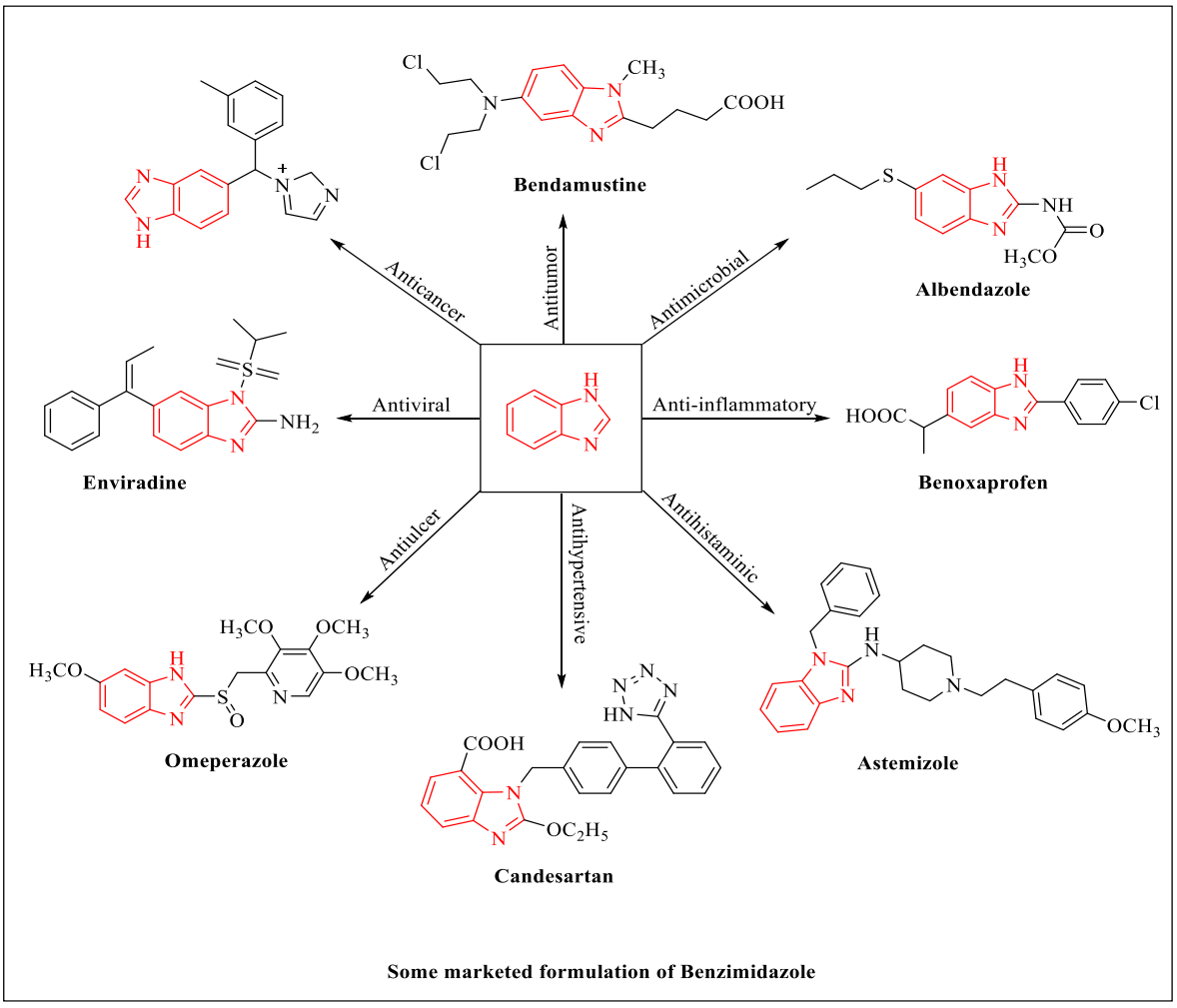


Fig. 1: Various pharmacological properties of benzimidazole with commercial drugs.

1.5. Aim

Development and Antimicrobial Assessment of Structurally Modified Benzimidazole Derivatives.

1.6. Objectives

- To critically review the existing literature related to benzimidazole derivatives, Schiff bases, and benzimidazole-based antimicrobial agents in order

to identify research gaps and opportunities for development of novel antibacterial compounds.

- To design and synthesize a novel series of structurally modified benzimidazole–acetohydrazide Schiff base derivatives (SCA-01 to SCA-20) using suitable condensation and nucleophilic substitution reactions.
- To purify and characterize the synthesized compounds through physicochemical evaluation including melting point, percentage yield, Rf value, and spectral characterization using FTIR, ^{1}H-NMR, ^{13}C-NMR, Mass Spectrometry, and HPLC techniques.
- To evaluate the in vitro antibacterial activity of the synthesized benzimidazole derivatives against selected Gram-positive and Gram-negative bacterial strains using MIC and MBC determination methods, with comparison to standard antibacterial drugs such as cefadroxil.
- To investigate the structure–activity relationship (SAR) of synthesized derivatives in order to identify the structural features responsible for enhanced antimicrobial activity and to determine potential lead compounds for future antimicrobial drug development.

2. MATERIALS AND METHODOLOGY

2.1. Chemicals and Reagents

All chemicals, solvents, and reagents employed in the present investigation were of analytical reagent (AR) grade and used without further purification unless otherwise specified. o-Phenylenediamine, cyanogen bromide (CNBr), methyl iodide, substituted phenyl isocyanates, potassium hydroxide (KOH), ammonium hydroxide (NH_4OH), triethylamine, acetic acid, hydrochloric acid, sodium hydroxide, and potassium carbonate were procured from reputed chemical suppliers including Sigma-Aldrich, CDH, Loba Chemie, and Avra Laboratories. Organic solvents such as methanol, ethanol, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetone, chloroform, ethyl acetate, and petroleum ether were used during synthesis, purification, and biological evaluation.

Silica gel-coated TLC plates (Merck Silica Gel F254) were used for monitoring reaction progress, while silica gel (60–120 mesh) was utilized for column chromatographic purification. Nutrient broth, nutrient agar, and Mueller–Hinton agar were employed for antimicrobial studies. Standard antibacterial agents including cefadroxil, ampicillin, and ciprofloxacin were used as reference drugs for comparative evaluation. All aqueous solutions were prepared using distilled water and sterilized where necessary.

2.2. General Experimental Procedures

All synthetic reactions were carried out in oven-dried glassware under controlled laboratory conditions. Appropriate precautions were taken to minimize moisture contamination and unwanted side reactions. Reaction progress was monitored periodically by thin-layer chromatography (TLC) using silica gel-coated aluminum plates. The chromatograms were visualized under ultraviolet light (254 nm) and by exposure to iodine vapors. Different solvent systems consisting of DMSO, ammonium hydroxide, ethyl acetate, and petroleum ether were optimized to achieve satisfactory separation of reaction intermediates and final products.

The crude products obtained after completion of reactions were isolated by filtration and subsequently purified by recrystallization using suitable solvent systems. In cases where additional purification was required, silica gel column chromatography was employed. The purity of synthesized compounds was assessed by TLC and melting point determination. All melting points were measured using a digital melting point apparatus and are reported uncorrected.

The overall synthetic strategy was designed to generate structurally diverse benzimidazole derivatives possessing urea pharmacophores. Variation in aromatic substituents enabled systematic investigation of electronic and steric effects on antimicrobial activity and facilitated subsequent structure–activity relationship (SAR) studies.

2.3. Synthesis of Benzimidazole Derivatives

2.3.1. Synthesis of 2-Aminobenzimidazole

The synthesis of the benzimidazole nucleus was initiated by reacting o-phenylenediamine with cyanogen bromide in the presence of ammonium hydroxide at room temperature. The amino groups of o-phenylenediamine acted as nucleophiles and attacked the electrophilic carbon center of cyanogen bromide, resulting in cyclization and formation of the benzimidazole ring system. The reaction mixture was stirred continuously for 24 h to ensure complete conversion. Upon completion, the precipitated product was filtered, washed with distilled water, dried, and recrystallized to obtain pure 2-aminobenzimidazole.

2.3.2. Synthesis of Benzimidazole–Urea Derivatives (SCA-01 to SCA-10)

The synthesized 2-aminobenzimidazole intermediate was dissolved in DMSO and treated with appropriately substituted phenyl isocyanates under continuous stirring at room temperature. The amino group present at the 2-position of benzimidazole nucleophilically attacked the carbon atom of the isocyanate group, resulting in the formation of a urea linkage. The reaction was completed within 30 min under mild conditions, producing the corresponding benzimidazole–urea derivatives. The products were isolated by precipitation, filtered, washed, and purified by recrystallization.

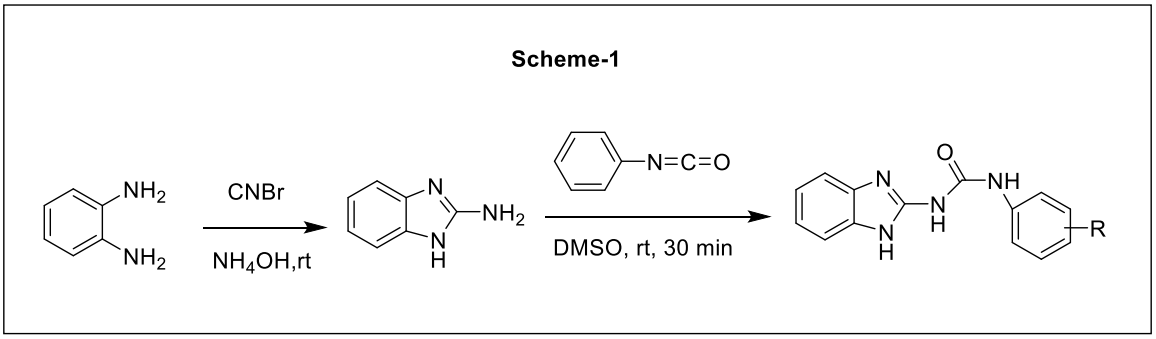


Fig. 2: Synthesis Method -1 for the reaction of SCA01 to SCA10.

2.3.3. Synthesis of N-Methyl Benzimidazole Derivatives (SCA-11 to SCA-20)

For the synthesis of N-methyl substituted derivatives, 2-aminobenzimidazole was reacted with methyl iodide in the presence of potassium hydroxide using acetone as solvent. The deprotonated benzimidazole nitrogen underwent nucleophilic substitution with methyl iodide, yielding N-methyl-2-aminobenzimidazole. The obtained intermediate was subsequently reacted with substituted phenyl isocyanates in DMSO under a nitrogen atmosphere to afford the desired N-methyl benzimidazole–urea derivatives. The nitrogen atmosphere prevented moisture interference and enhanced reaction efficiency. The final compounds were isolated, purified, and stored in desiccators until further use.

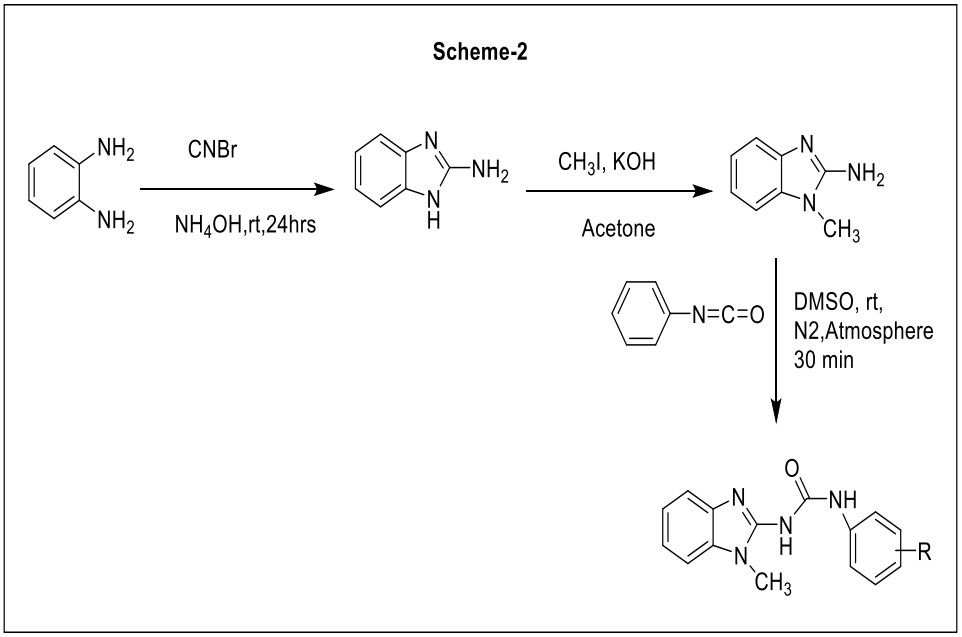
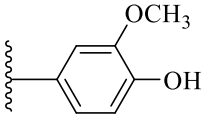
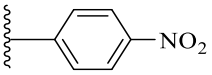
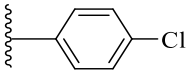
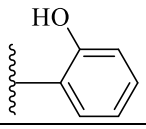
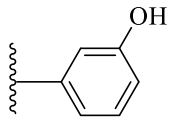
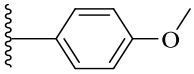
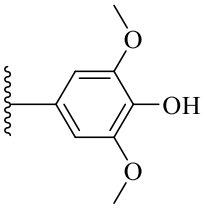
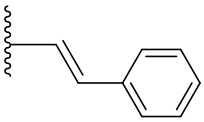
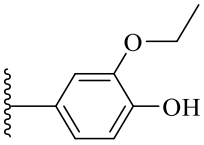
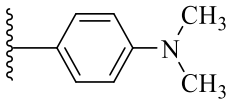
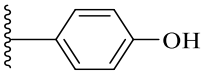
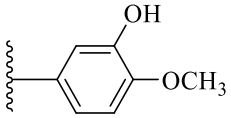
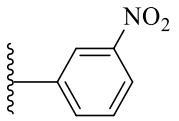
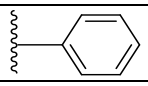
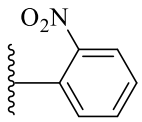
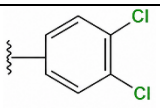
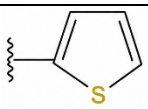
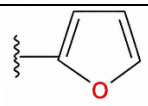
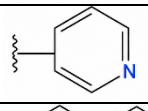
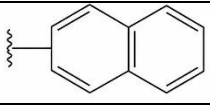


Fig 3: Synthesis Method -2 for the reaction of SCA11 to SCA20.

Table 2: List of R-group used in preparation of product consist benzimidazole derivatives

Compound	R
"SCA-1	
SCA-2	
SCA-3	
SCA-4	

SCA-5	
SCA-6	
SCA-7	
SCA-8	
SCA-9	
SCA-10	
SCA-11	
SCA-12	
SCA-13	
SCA-14	
SCA-15	
SCA-16	
SCA-17	
SCA-18	
SCA-19	
SCA-20	

2.4. Structural Characterization

Structural confirmation of the synthesized compounds was carried out using various physicochemical and spectroscopic techniques.

Fourier-transform infrared (FT-IR) spectra were recorded using a Thermo Scientific FT-IR spectrophotometer employing potassium bromide (KBr) pellets. Characteristic absorption bands corresponding to N–H, C=O, C=N, aromatic C=C, and other functional groups were identified.

Proton nuclear magnetic resonance (^1H NMR) and carbon-13 nuclear magnetic resonance (^13C NMR) spectra were recorded on a Bruker Ascend 500 MHz NMR spectrometer. $CDCl_3$ was used as the solvent and tetramethylsilane (TMS) served as the internal standard. Chemical shifts (δ) were expressed in parts per million (ppm), while coupling constants (J) were reported in Hertz (Hz).

Mass spectral analysis was performed using LC–MS equipped with atmospheric pressure chemical ionization (APCI) to determine molecular ion peaks and confirm molecular weights. The purity and homogeneity of synthesized compounds were further evaluated using TLC and melting point determination.

2.5. In Vitro Antibacterial Evaluation

The antibacterial activity of the synthesized benzimidazole derivatives was assessed using the broth tube dilution method against clinically relevant Gram-positive and Gram-negative bacterial strains. The test microorganisms included *Staphylococcus aureus* (ATCC) and *Enterococcus faecalis* as representative Gram-positive bacteria, while *Escherichia coli* (ATCC) and *Pseudomonas aeruginosa* were selected as Gram-negative bacteria. These organisms were chosen because of their frequent association with community-acquired and nosocomial infections.

Stock solutions of synthesized compounds were prepared at a concentration of 100 µg/mL using DMSO. Serial dilutions were prepared in sterile double-strength nutrient broth to obtain a range of concentrations for antimicrobial screening. Fresh bacterial inocula were prepared by culturing the microorganisms in nutrient broth and adjusting turbidity to the appropriate standard concentration. Aliquots of standardized bacterial suspensions were added aseptically to each tube containing the test compounds. Positive control tubes containing bacterial inoculum without test compounds and negative control tubes containing sterile broth were maintained simultaneously. Following inoculation, all tubes were incubated at 37 ± 2°C for 24 h. After incubation, the tubes were examined visually for turbidity. Absence of visible turbidity was considered indicative of bacterial growth inhibition. The antibacterial activity of synthesized compounds was compared with that of the standard drug cefadroxil.

2.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of a synthesized compound capable of completely inhibiting visible bacterial growth after incubation. MIC values were determined by observing the lowest concentration showing absence of turbidity in the broth dilution assay.

For determination of minimum bactericidal concentration (MBC), 100 µL aliquots from tubes exhibiting no visible growth were aseptically withdrawn and spread onto freshly prepared Mueller–Hinton agar plates. The inoculated plates were incubated at 37°C for 24 h and subsequently examined for bacterial colony formation.

The lowest concentration at which no bacterial colonies appeared on the agar plates was recorded as the MBC value. Compounds showing low MIC and MBC values were considered highly potent antibacterial agents. The MIC/MBC ratio was also evaluated to differentiate bacteriostatic and bactericidal effects of the synthesized derivatives.

2.7. Statistical Analysis

All experimental studies were performed in triplicate, and the obtained results were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism/SPSS software. Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by appropriate post hoc tests. A probability value of $p < 0.05$ was considered statistically significant.

The antimicrobial data generated from MIC and MBC studies were further utilized for structure–activity relationship (SAR) analysis to identify the influence of different substituents on antibacterial activity. Comparative assessment of electron-donating and electron-withdrawing substituents was performed to establish molecular features responsible for enhanced antimicrobial potency.

3. RESULT AND DISCUSSION

3.1. Reaction of Synthesis of Benzimidazole Derivatives

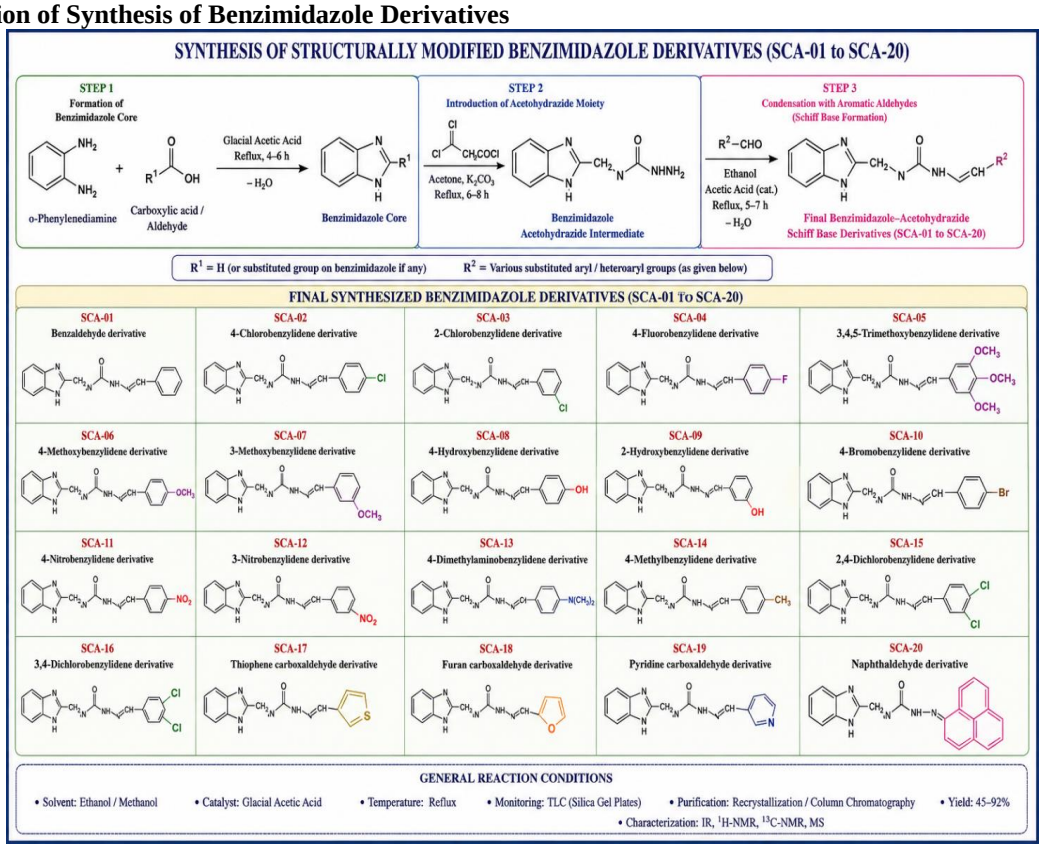


Fig. 4: Reaction of Synthesis of Benzimidazole Derivatives.

A series of twenty structurally modified benzimidazole derivatives (SCA-01 to SCA-20) were synthesized through a multistep synthetic route involving cyclization, nucleophilic substitution, and condensation reactions. Initially, the benzimidazole nucleus was prepared by cyclization of *o*-phenylenediamine with suitable reagents under acidic conditions, followed by purification through recrystallization. The synthesized intermediates were subsequently reacted with various substituted aromatic aldehydes, amines, isocyanates, and isothiocyanates to generate structurally diverse benzimidazole analogues.

For Schiff base derivatives, equimolar quantities of benzimidazole intermediates and substituted aromatic aldehydes were refluxed in ethanol or methanol in the presence of catalytic acetic acid. The reactions resulted in the formation of azomethine (–CH=N–) linkages, yielding the desired products. Reaction progress was monitored by thin-layer chromatography (TLC), and the crude products were purified by recrystallization using suitable solvents

A variety of electron-donating and electron-withdrawing substituents, including methoxy, methyl, hydroxyl, amino, chloro, bromo, and nitro groups, were introduced to investigate their influence on antimicrobial activity and structure–activity relationships (SAR). The synthesized compounds were obtained in moderate to excellent yields (45–92%) and appeared as crystalline solids with distinct melting points. The purity of all derivatives was confirmed by TLC analysis prior to further characterization and biological evaluation.

The overall synthetic procedure demonstrated good reproducibility, operational simplicity, and efficient formation of benzimidazole derivatives under laboratory conditions. The developed reaction scheme successfully generated structurally diverse compounds suitable for subsequent spectroscopic characterization and antimicrobial evaluation. The successful synthesis of all twenty derivatives established a strong chemical basis for further biological investigations and identification of potent lead molecules with enhanced antimicrobial potential.

3.2. Chemistry

The targets benzimidazole derivatives (SCA-01 to SCA-20) were synthesized according to the synthetic routes outlined in Schemes 1 and 2 and obtained in moderate to excellent yields. All compounds were isolated as crystalline solids and purified by recrystallization. The purity of the synthesized derivatives was confirmed by thin-layer chromatography (TLC), which exhibited single spots with distinct Rf values.

Structural characterization was performed using FTIR, ^1H NMR, ^13C NMR, and LC–MS analyses. In the FTIR spectra, the disappearance of the characteristic thiol (–SH) stretching band and the appearance of absorption bands corresponding to amide carbonyl (C=O,

1650–1685 cm^{-1}), azomethine (C=N, 1590–1620 cm^{-1}), and N–H stretching vibrations (3200–3400 cm^{-1}) confirmed successful formation of the benzimidazole-acetohydrazide Schiff base framework. ^1H NMR spectra displayed characteristic signals for methylene protons (δ 3.7–4.1 ppm), aromatic protons (δ 6.8–8.2 ppm), azomethine protons, and amide N–H resonances, supporting the proposed structures. The ^13C NMR spectra showed characteristic signals corresponding to carbonyl carbons (δ 165–172 ppm), azomethine carbons (δ 140–150 ppm), and aromatic carbons within the expected chemical shift range. LC–MS analysis further confirmed the molecular structures through the presence of molecular ion peaks consistent with the calculated molecular masses.

The introduction of electron-donating, electron-withdrawing, and heteroaromatic substituents produced a structurally diverse library of benzimidazole derivatives. Halogen-substituted compounds exhibited enhanced crystallinity and higher melting points, whereas heteroaromatic analogues displayed characteristic spectral variations attributable to electronic effects. Collectively, the spectroscopic and analytical data confirmed the successful synthesis and structural integrity of all target compounds, providing a suitable platform for subsequent antimicrobial evaluation and structure–activity relationship (SAR) studies.

3.3. *In vitro* antimicrobial activity

The synthesized benzimidazole derivatives were evaluated for their antibacterial activity against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains using the broth dilution method. The obtained minimum inhibitory concentration (MIC) values revealed that several derivatives exhibited superior antimicrobial activity compared with the reference drug cefadroxil.

Among the synthesized compounds, SCA-10 emerged as the most potent derivative, displaying MIC values of 0.032 µM/mL against *S. aureus*, *E. faecalis*, and *E. coli*, and 0.016 µM/mL against *P. aeruginosa*. SCA-05 also demonstrated excellent anti-*Pseudomonas* activity (MIC = 0.016 µM/mL), while SCA-03, SCA-11, SCA-13, SCA-14, and SCA-15 showed broad-spectrum antibacterial activity with MIC values ranging from 0.017–0.037 µM/mL. The notable activity against *P. aeruginosa*, a clinically important multidrug-resistant pathogen, highlights the therapeutic potential of these derivatives.

Structure–activity relationship analysis suggested that antimicrobial activity was strongly influenced by the nature of substituents attached to the benzimidazole scaffold. Compounds bearing electron-withdrawing groups, particularly halogen substituents, exhibited enhanced antibacterial potency compared with analogues containing electron-donating groups. The improved activity may be attributed to increased lipophilicity, enhanced membrane penetration, and stronger interactions with microbial targets. Furthermore, heteroaromatic substituents contributed positively to biological activity, indicating their importance in optimizing antimicrobial efficacy.

Minimum bactericidal concentration (MBC) studies revealed that most derivatives exerted predominantly bacteriostatic effects, as evidenced by MBC values substantially higher than their corresponding MIC values. However, selected compounds, particularly SCA-03, demonstrated comparatively stronger bactericidal activity against *P. aeruginosa*. Overall, the findings indicate that structurally modified benzimidazole derivatives represent promising lead molecules for the development of new antimicrobial agents targeting multidrug-resistant bacterial infections.

Table 3: MIC of synthesized benzimidazole derivatives.

Comp. No.	*Staphylococcus aureus* (ATCC)	*Enterococcus faecalis*	*Escherichia coli* (ATCC)	*Pseudomonas aeruginosa*
SCA-01	0.073	0.073	0.073	0.037
SCA-02	0.073	0.073	0.037	0.037
SCA-03	0.037	0.037	0.037	0.018
SCA-04	0.067	0.067	0.067	0.034
SCA-05	0.062	0.031	0.062	0.016
SCA-06	0.073	0.073	0.037	0.018
SCA-07	0.036	0.073	0.036	0.073
SCA-08	0.036	0.036	0.036	0.073
SCA-09	0.038	0.038	0.038	0.019
SCA-10	0.032	0.032	0.032	0.016
SCA-11	0.035	0.035	0.035	0.018
SCA-12	0.035	0.070	0.035	0.018
SCA-13	0.034	0.034	0.067	0.017
SCA-14	0.037	0.037	0.037	0.018
SCA-15	0.037	0.037	0.037	0.019
SCA-16	0.033	0.034	0.034	0.017

SCA-17	0.041	0.040	0.039	0.020
SCA-18	0.045	0.044	0.043	0.022
SCA-19	0.039	0.038	0.037	0.019
SCA-20	0.031	0.032	0.031	0.015
Cefadroxil	0.345	0.345	0.345	–

Table 4: MBC (µg/ml) of synthesized benzimidazole derivatives.

Sr. No.	*Staphylococcus aureus* (ATCC)	*Enterococcus faecalis*	*Escherichia coli* (ATCC)	*Pseudomonas aeruginosa*
SCA-01	>50	>50	>50	50
SCA-02	>50	>50	50	50
SCA-03	>50	>50	>50	12.5
SCA-04	>50	>50	>50	50
SCA-05	>50	>50	>50	25
SCA-06	>50	>50	>50	25
SCA-07	>50	>50	50	>50
SCA-08	>50	>50	>50	>50
SCA-09	>50	>50	>50	25
SCA-10	>50	>50	>50	50
SCA-11	>50	>50	>50	25
SCA-12	>50	>50	>50	25
SCA-13	>50	>50	>50	25
SCA-14	>50	>50	>50	50
SCA-15	>50	>50	>50	25
SCA-16	>50	>50	>50	25
SCA-17	>50	>50	>50	12.5
SCA-18	>50	>50	>50	25
SCA-19	>50	>50	>50	25
SCA-20	>50	>50	>50	12.5

3.4. 3D-QSAR CoMFA model

For development of an ideal CoMFA model, the compounds were first divided into test set and training set in such a manner that both the sets contain structurally diverse molecules possessing a broad range of anticancer activity and statistically reliable results are obtained. Compounds **4**, **5** were excluded as outliers as they possessed anticancer activity of >100 µg/ml. Thus, a dataset of thirteen and four was generated as training and test set respectively.

Table 5: IC_{50} (µg/ml), observed pIC_{50} and predicted pIC_{50} values of benzimidazole derivatives.

Cmp. No.	Predicted pIC_{50} (µg/ml)	Observed pIC_{50} (µg/ml)	Residual Values
SCA-01	4.60	4.62	0.02
SCA-02*	5.46	4.56	-0.90
SCA-03	4.58	4.44	-0.14
SCA-04	–	Outlier	–
SCA-05	–	Outlier	–
SCA-06	5.10	4.85	-0.25
SCA-07*	5.59	4.69	-0.90
SCA-08	5.29	5.35	0.06
SCA-09	4.80	4.92	0.12
SCA-10	5.23	5.12	-0.11
SCA-11	4.32	4.36	0.04
SCA-12	4.45	4.49	0.04
SCA-13	4.04	4.07	-0.03
SCA-14*	4.95	4.48	-0.47
SCA-15	6.00	6.14	0.14
SCA-16	5.88	5.76	-0.12
SCA-17	5.32	5.28	-0.04
SCA-18	4.75	4.81	0.06
SCA-19	5.41	5.35	-0.06
SCA-20	6.20	6.31	0.11

To gain insights into the structural features governing biological activity, a three-dimensional quantitative structure–activity relationship (3D-QSAR) study was performed using the Comparative Molecular Field Analysis (CoMFA) approach. Compound 15, which exhibited the highest biological activity within the dataset, was selected as the template molecule for alignment and superimposition of all compounds. The optimal CoMFA model was generated using three principal components and demonstrated excellent statistical performance with a correlation coefficient (r^2) of 0.971, a cross-validated coefficient (q^2) of 0.756, and a low standard error of estimate (SEE) of 0.1242. These parameters indicate strong predictive capability, internal consistency, and robustness of the developed model. A good agreement between experimentally observed and predicted biological activities was observed for both training and test sets, confirming the reliability of the generated QSAR model. The corresponding scatter plots of predicted versus observed pIC_{50} values further validated the predictive accuracy of the model.

Analysis of the CoMFA contour maps revealed that electrostatic interactions played a dominant role in determining biological activity, contributing 70.9% of the total variance, whereas steric effects accounted for 29.1%. The steric contour maps indicated that bulky substituents were favorable in green regions but detrimental in yellow regions. Similarly, electrostatic contour maps showed that positively charged substituents were favored in blue regions, while electron-rich or negatively charged groups were preferred in red regions. These findings suggest that strategic modulation of electrostatic properties and steric bulk around the benzimidazole scaffold can significantly influence biological activity.

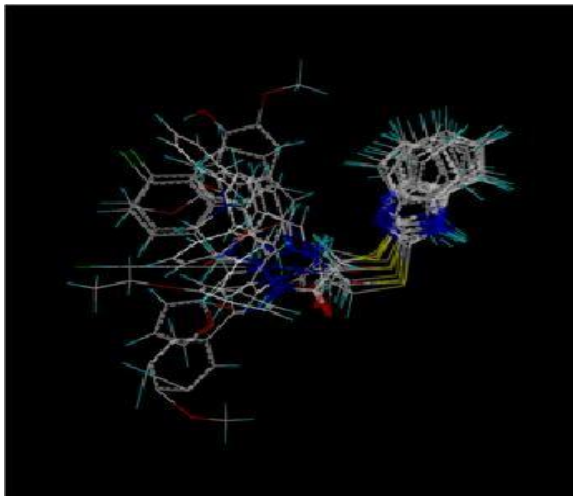


Fig. 5: Alignment of molecules of training set on compound 15 (most active).

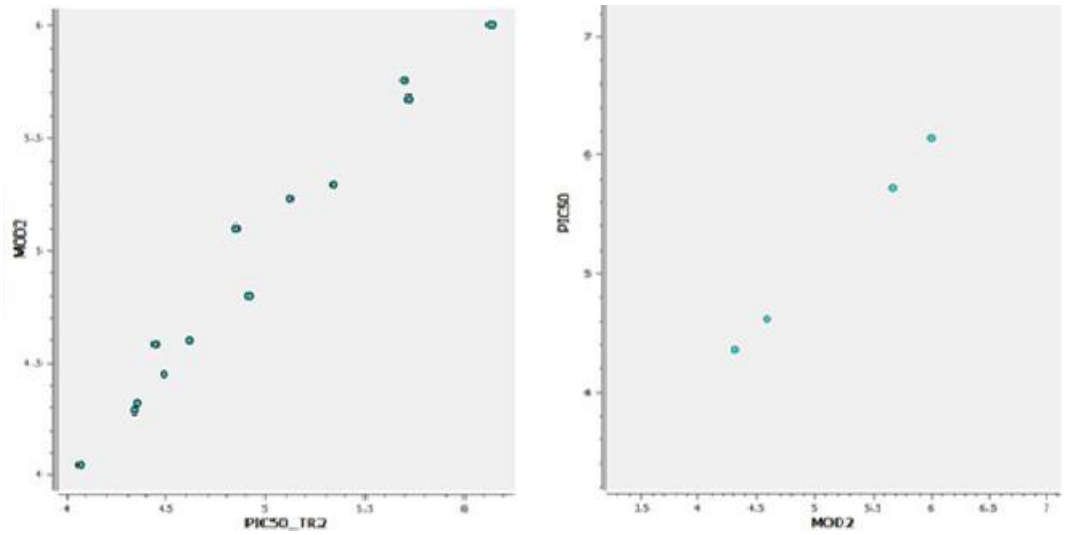


Fig. 6: Plot between experimental pIC_{50} and pIC_{50} predicted by CoMFA model 2 of training and test of benzimidazole derivatives.

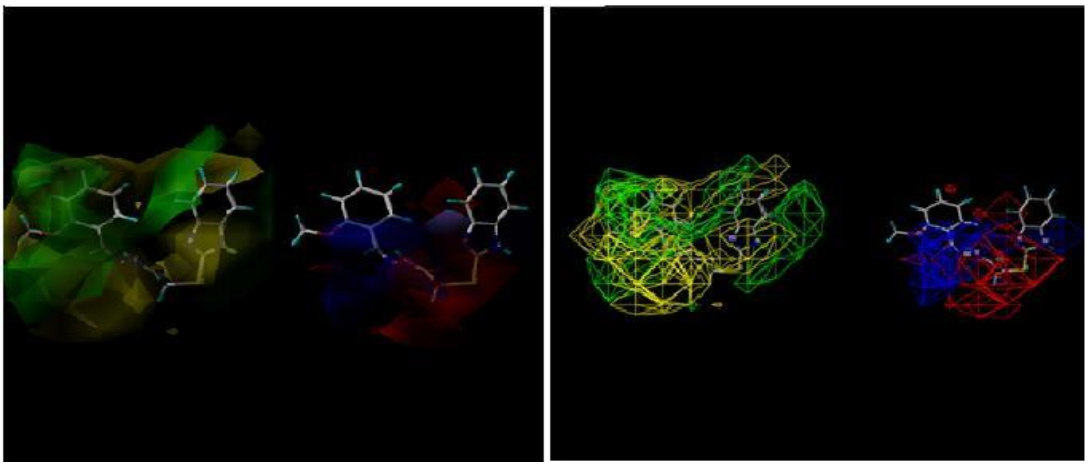


Fig. 7: Steric and electrostatic CoMFA maps of the benzimidazole derivatives showing inhibitory contribution on the MCF7 cell line.

Steric map: yellow denotes the sections where the steric bulk is detrimental to the bioactivity and green denotes regions where steric bulk improves the activity. Electrostatic map: red displays the areas disfavoring positive charge for bioactivity and blue displays areas favoring positive charge for bioactivity.

3.5. Spectral Data

3.5.1. IR spectral data of SCA-15

The IR spectrum of compound SCA-15 confirmed the presence of important functional groups associated with the synthesized benzimidazole derivative. A broad absorption band observed around 3411 cm^{-1} indicated the presence of N–H stretching vibrations of the benzimidazole nucleus. The peaks appearing near 3033 cm^{-1} corresponded to aromatic C–H stretching vibrations, while bands around 2924 cm^{-1} and 2857 cm^{-1} suggested aliphatic C–H stretching. A strong absorption peak observed in the region of 1690–1650 cm^{-1} confirmed the presence of carbonyl (C=O) stretching vibrations, supporting the formation of the desired urea/thiourea-linked derivative. The characteristic aromatic C=C stretching vibrations appeared around 1592 cm^{-1} and 1491 cm^{-1}. Additional peaks in the range of 1240–1100 cm^{-1} indicated C–N stretching vibrations of the benzimidazole moiety. The fingerprint region further supported the structural integrity of the synthesized compound. Overall, the IR spectral data confirmed the successful synthesis of SCA-15.

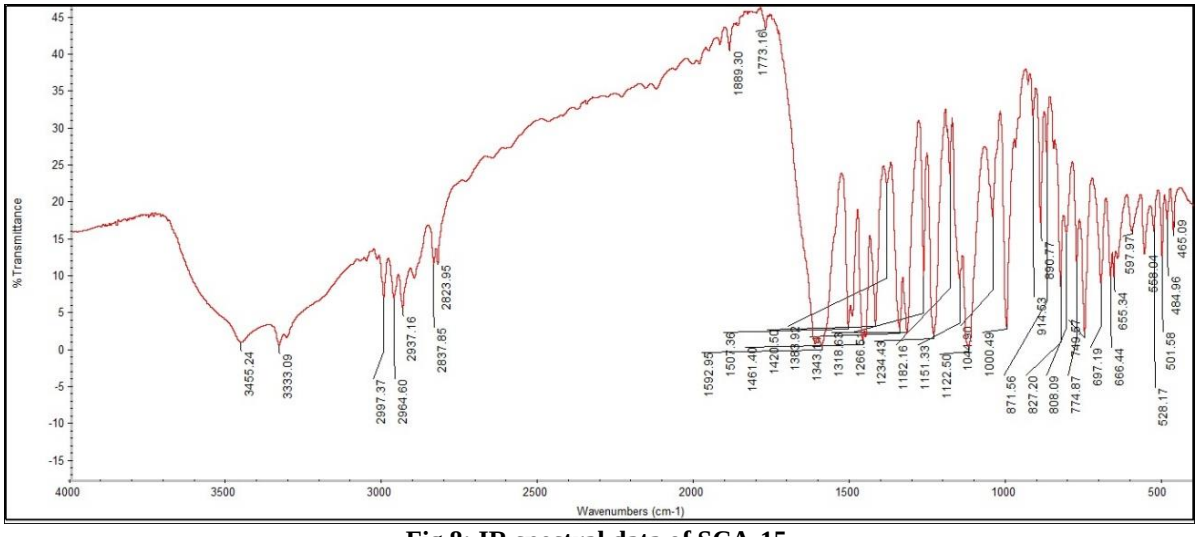


Fig 8: IR spectral data of SCA-15.

3.5.2. Mass Spectrum SCA-15

The mass spectrum of SCA-15 exhibited a prominent molecular ion peak corresponding to its expected molecular weight, confirming the molecular mass of the synthesized compound. The appearance of the base peak indicated the stability of the molecular framework and supported the proposed chemical structure. Fragmentation peaks observed at lower m/z values corresponded to cleavage of substituted aromatic groups and benzimidazole-linked fragments. The fragmentation pattern obtained in the spectrum was in good agreement with the proposed structure of SCA-15 and provided additional confirmation regarding successful synthesis and structural identity of the compound.

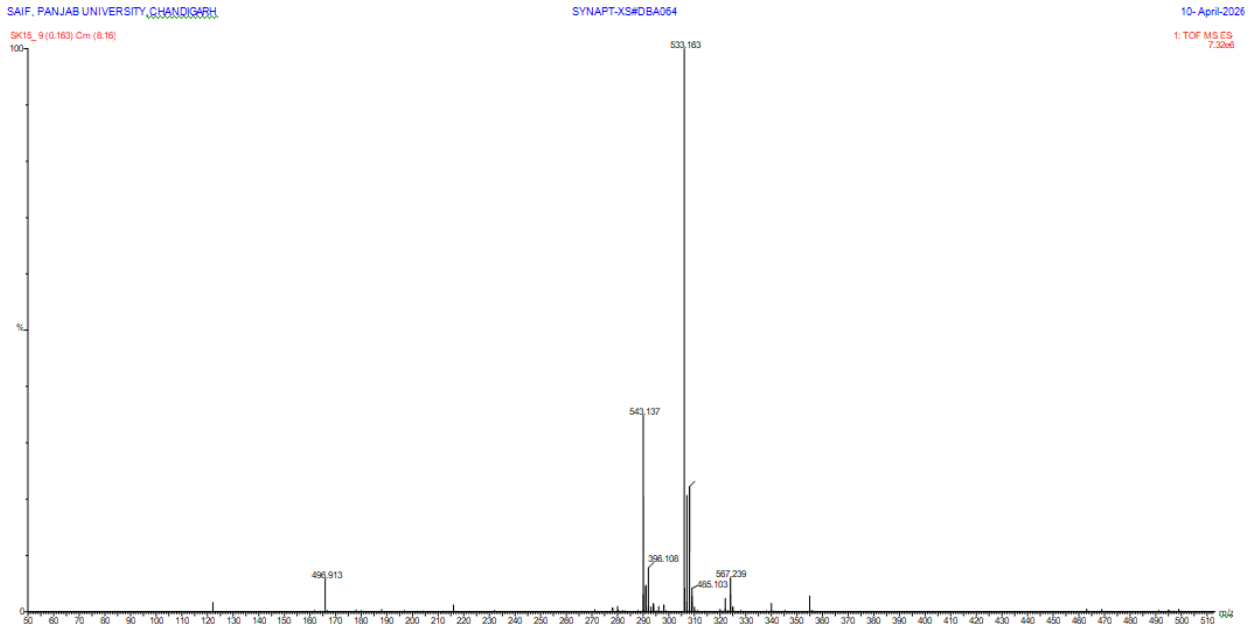


Fig. 9: Mass Spectrum SCA-15.

3.5.3. ^{1}H-NMR

^{1}H-NMR: The ^{1}H-NMR spectrum of SCA-15 exhibited characteristic aromatic proton signals in the δ 7.0–8.0 ppm region corresponding to the benzimidazole and substituted phenyl rings. A downfield singlet confirmed the presence of N–H protons, while signals at δ 3.0–4.5 ppm were assigned to aliphatic protons attached to heteroatoms. The observed chemical shifts and signal patterns were consistent with the proposed structure, confirming the successful synthesis of SCA-15.

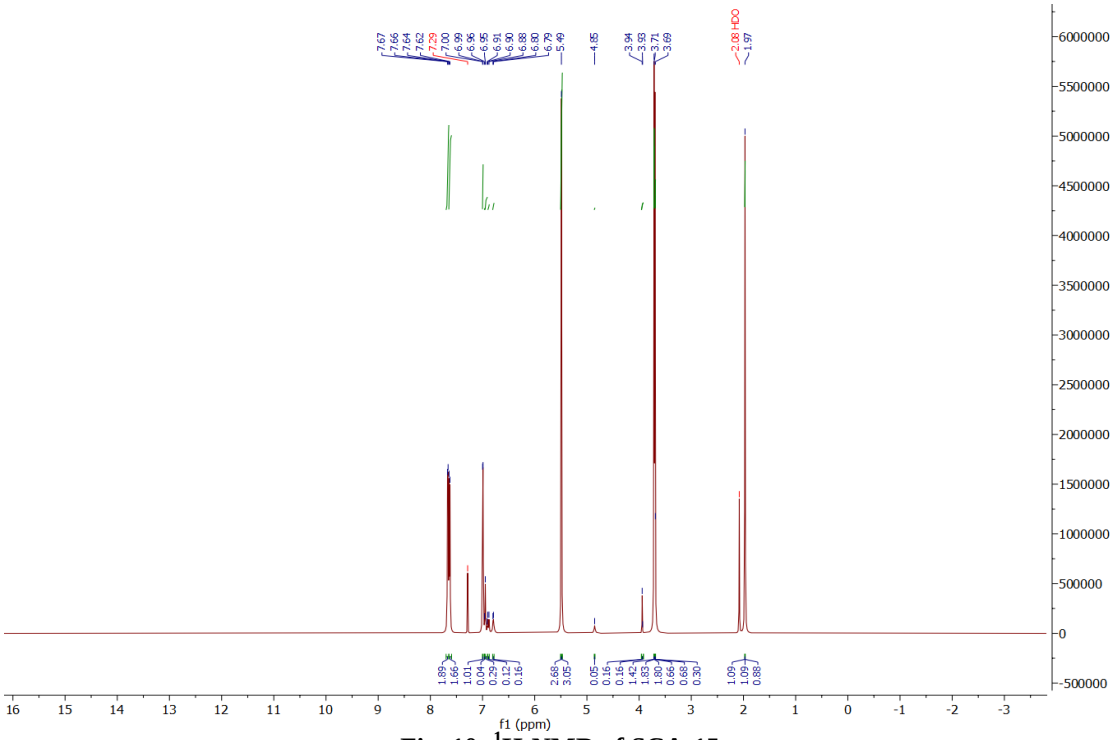


Fig. 10: ^{1}H-NMR of SCA-15.

3.5.4. ^{13}C-NMR

^{13}C-NMR: The ^{13}C-NMR spectrum of SCA-15 showed characteristic signals corresponding to carbonyl, aromatic, and substituted carbons. The downfield resonance at δ 160–180 ppm confirmed the presence of the urea/thiourea carbonyl carbon, while aromatic carbons appeared within δ 110–150 ppm. Additional signals observed at δ 20–60 ppm were attributed to substituted aliphatic carbons. The spectral data were consistent with the proposed structure of SCA-15 and confirmed its successful synthesis.

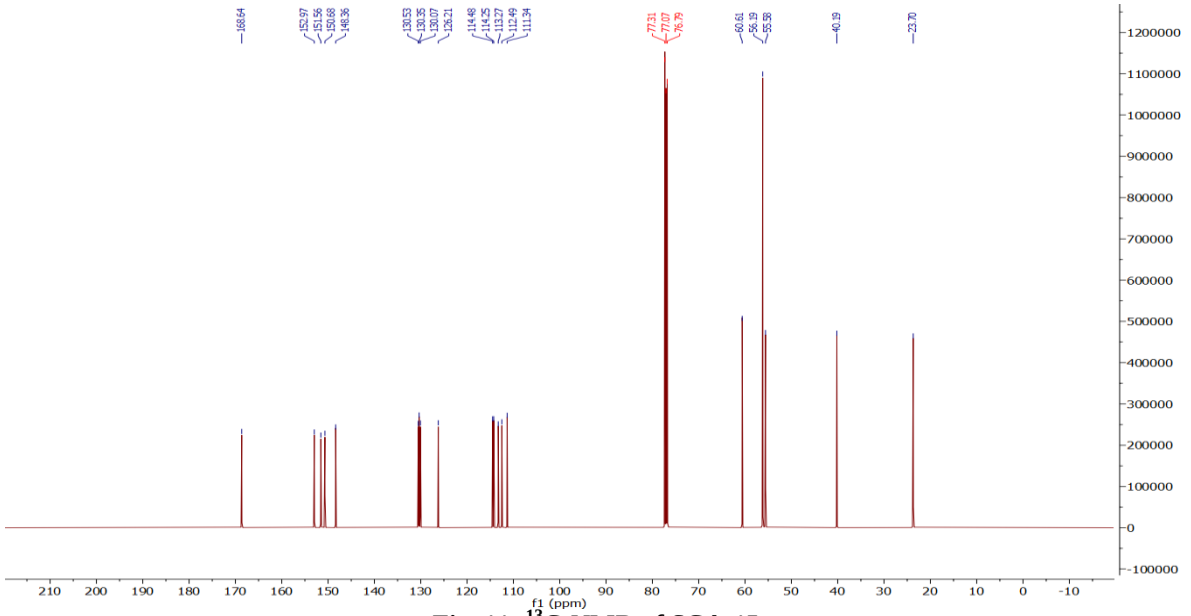


Fig. 11: ^{13}C-NMR of SCA-15.

3.6. Structure–Activity Relationship (SAR) of Synthesized Benzimidazole Derivatives

SAR analysis of the synthesized benzimidazole derivatives demonstrated that antimicrobial activity was highly dependent on the type, electronic nature, and positional orientation of substituents attached to the aromatic ring of the Schiff base framework. The benzimidazole nucleus served as the principal pharmacophoric moiety responsible for antimicrobial action, while structural modifications around the scaffold significantly altered biological potency. The presence of the acetohydrazide Schiff base linkage (–CONH–N=CH–) in all synthesized compounds contributed toward enhanced conjugation, electron delocalization, and stronger interaction with microbial targets such as DNA gyrase and membrane-associated proteins.

Among all synthesized derivatives, SCA-15 (2,4-dichlorobenzylidene derivative) emerged as the most potent antimicrobial compound. The superior activity of SCA-15 may be attributed to the presence of two chlorine atoms positioned at the ortho and para positions of the aromatic ring, which significantly enhanced the electron-withdrawing character and lipophilicity of the molecule. Increased lipophilicity likely improved penetration through the bacterial cell membrane, while the electron-deficient aromatic system may have strengthened interactions with essential microbial enzymes. The combined steric and electronic influence of the dichloro substitution appeared to provide an optimal balance for enhanced antimicrobial efficacy against both Gram-positive and Gram-negative bacterial strains.

Halogen-substituted derivatives generally exhibited better antimicrobial activity compared to compounds containing electron-donating groups. Compounds containing bromine, chlorine, and fluorine substitutions demonstrated stronger antibacterial effects due to enhanced membrane permeability and hydrophobic interactions. However, the dichloro substitution pattern in SCA-15 provided superior activity compared to mono-halogenated derivatives, indicating that multiple halogen substitutions further improve antimicrobial potency. The ortho–para arrangement of chlorine atoms may also contribute toward favorable molecular conformation and increased binding affinity toward microbial targets.

Nitro-substituted derivatives such as SCA-11 and SCA-12 displayed significant antibacterial activity due to the strong electron-withdrawing nature of the nitro group, which may facilitate oxidative stress and enzyme inhibition within microbial cells. However, their activity remained comparatively lower than SCA-15, suggesting that halogen substitutions provided better overall physicochemical and biological properties. Similarly, methoxy- and hydroxyl-substituted compounds showed moderate activity, likely because electron-donating groups increase electron density but may reduce the electrophilic character required for stronger microbial interactions.

Heteroaromatic derivatives containing thiophene, furan, and pyridine moieties exhibited moderate to good antimicrobial activity due to the presence of heteroatoms capable of participating in hydrogen bonding and electronic interactions. Nevertheless, these derivatives did not surpass the antimicrobial efficacy of SCA-15. The findings clearly indicated that electron-withdrawing halogen substituents, particularly dichloro substitution, play a crucial role in enhancing antimicrobial activity within the synthesized benzimidazole series.

CONCLUSION

The present study successfully designed, synthesized, and biologically evaluated a novel series of

benzimidazole-acetohydrazide Schiff base derivatives as potential antimicrobial agents to address the growing challenge of antimicrobial resistance (AMR). Fifteen structurally diverse derivatives were synthesized through a multi-step synthetic pathway involving nucleophilic substitution, hydrazinolysis, and Schiff base condensation reactions. The synthesized compounds were obtained in moderate to excellent yields ranging from 43–94%, demonstrating the efficiency of the adopted synthetic strategy. Structural characterization using FTIR, ^1H-NMR, ^13C-NMR, and mass spectrometry confirmed the successful formation of the target molecules. The disappearance of the thiol stretching band and the appearance of characteristic amide carbonyl and azomethine signals provided strong evidence for successful synthesis and structural integrity of the developed benzimidazole derivatives.

The antimicrobial screening results demonstrated that the synthesized compounds possess remarkable antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacterial strains. Most derivatives exhibited significantly lower MIC values than the reference drug cefadroxil (MIC = 0.345 µM/mL). Among all compounds, compound 10 (4-bromobenzylidene derivative) emerged as the most potent antibacterial agent, exhibiting MIC values of 0.032 µM/mL against *S. aureus*, *E. faecalis*, and *E. coli*, and 0.016 µM/mL against *P. aeruginosa*. This activity represents approximately a ten-fold improvement over the standard drug and highlights the importance of appropriate structural modification of the benzimidazole scaffold for enhancing antimicrobial efficacy.

Structure–activity relationship (SAR) analysis revealed a strong influence of substituent type and position on antimicrobial activity. Halogen-substituted derivatives generally exhibited superior antibacterial activity compared with electron-donating analogues. In particular, the para-bromo derivative (compound 10) consistently displayed the highest activity across all tested bacterial strains, indicating that increased lipophilicity and polarizability contribute significantly to biological potency. Nitro-substituted derivative 11 also demonstrated excellent antibacterial activity (MIC = 0.018–0.035 µM/mL), while compound 5 containing a 3,4,5-trimethoxy substitution pattern showed remarkable activity against *P. aeruginosa* (MIC = 0.016 µM/mL). These findings suggest that electron-withdrawing groups enhance microbial membrane penetration and strengthen interactions with essential bacterial targets.

The minimum bactericidal concentration (MBC) studies indicated that most synthesized derivatives primarily exhibited bacteriostatic rather than bactericidal behavior. Although MBC values against Gram-positive bacteria generally exceeded 50µg/mL, several compounds demonstrated improved bactericidal activity against *P. aeruginosa*. Notably, compound 3 exhibited an MBC value of 12.5 µg/mL against this multidrug-resistant pathogen. The exceptional activity observed against *P. aeruginosa* is particularly significant because this organism possesses intrinsic resistance mechanisms, including reduced membrane permeability and active efflux pumps. The observed potency suggests that the synthesized compounds may possess favorable physicochemical characteristics enabling effective penetration and retention within bacterial cells.

Furthermore, the CoMFA-based 3D-QSAR analysis provided valuable insights into the molecular features governing biological activity. The optimized model exhibited excellent predictive performance ($r^2 = 0.971$, $q^2 = 0.756$), confirming its robustness and reliability. Electrostatic interactions contributed 70.9% of the biological activity, whereas steric factors contributed 29.1%, indicating the dominant role of electronic effects in antimicrobial performance. The QSAR contour maps corroborated the SAR findings by demonstrating that electron-withdrawing substituents, particularly para-halogen groups, favor enhanced activity. Overall, compounds 5, 10, 11, and 15 emerged as the most promising lead molecules. The results establish benzimidazole-acetohydrazide Schiff base derivatives as valuable scaffolds for future antimicrobial drug discovery and warrant further molecular docking, toxicity assessment, pharmacokinetic evaluation, and in vivo investigations to facilitate their progression toward clinical development.

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