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In-Vitro Antibacterial and Antifungal Evaluation of 2-Mercaptobenzimidazole Mannich Based Derivatives: Structure Activity Relationship and MIC Analysis against clinically relevant pathogens

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ABSTRACT

The appearance of multi-drug resistant microbial pathogens requires the need for developing new antimicrobial agents with enhanced efficacy and spectrum. In the current research work, a set of 2-mercaptobenzimidazole Mannich bases was synthesized and tested for their antibacterial and antifungal properties against clinically significant pathogens. Antimicrobial activity was determined by MIC and microplate absorbance of the synthesized compounds using roxithromycin as a reference standard. The microbiological study showed that the compounds possess promising antibacterial properties. All the title compounds were tested for their in vitro preliminary antimicrobial properties against six bacterial strains, *Staphylococcus aureus* ATCC- 6538, *Bacillus subtilis* ATCC-6633, *Escherichia coli* ATCC-25922, *Klebsiella pneumoniae* ATCC-1705, *Pseudomonas aeruginosa* ATCC-15442, *Resistant coli* ATCC-BAA2452, and four fungal strains, *Aspergillus fumigatus* FCBP-066, *Mucor species* FCBP-0300, *Fusarium solani* FCBP#0291, *Aspergillus flavus* FCBP-0064 by well plate method. The title compounds exhibited moderate to high specific antifungal activity. Clotrimazole was used as a reference standard. The synthesized compounds showed varying but promising antimicrobial activities, with increased potency against Gram-positive bacteria over Gram-negative bacteria. Compounds AK7 and AK9 showed strong antifungal activity against *A. fumigatus* with MIC of 12.5 and 10.37 µg/disc, respectively, similar to clotrimazole. The results of the structure-activity relationship (SAR) study showed that electron-withdrawing groups and optimal lipophilicity were essential in increasing the antimicrobial activity. The results of this study emphasize the potential of 2-mercaptobenzimidazole Mannich compounds as promising lead compounds for further optimization.

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a recent global health threat, making current treatments for common infections ineffective and common medical procedures difficult. According to the World Health Organization (WHO), bacterial AMR was directly linked to 1.27 million deaths in 2019 and accounted for 4.95 million deaths. According to the surveillance data of 2023, "one in every six laboratory-confirmed bacterial infections is now resistant to antibiotic treatment, and more than 40% of the pathogen-drug pairs being tracked have seen an increase in resistance" (WHO 2023). The global distribution of the disease burden due to AMR also varies, with the highest resistance rates found in the South-East Asian and Eastern Mediterranean regions, with one in every three infections being resistant, compared to one in every five in the

African region (WHO GLASS 2024). Microbial resistance has emerged as a global health threat, making conventional antibiotics and antifungal drugs less effective (WHO 2025). Gram-positive bacteria, Gram-negative bacteria, and opportunistic fungal pathogens such as *Aspergillus*, *Fusarium*, and *Mucor species* are major contributors to hospital-acquired infections and morbidity in immunocompromised patients. Despite extensive research on benzimidazole derivatives, limited studies have systematically explored 2-mercaptobenzimidazole Mannich bases against multidrug-resistant Gram-negative and filamentous fungal pathogens.

Some of the properties of Benzimidazole made it very much significant as scaffold these properties include water-solubility, the essential dipole moment of 4.08D, internal chelation, less melting point hence substitution occurs easily (Abbasi *et al.,* 2021) (Shahnaz *et al*., 2018). Less absorption is beneficial which is obtained through its limited water solubility (Hennessy, 1993). It is revealed that 5,6-dimethyl Benzimidazole is one of the components of B12 hence it has a significant role as an antimetabolite and antibacterial activity (Mobasher *et al.,* 2025) (Hodgkin *et al*., 1955). The incorporation of a mercapto group at the 2-position enhances biological reactivity, while Mannich base modification improves solubility and pharmacokinetic behavior (Wazir *et al.,* 2025) (Ali Kharl *et al.,* 2025). These structural modifications may enhance microbial membrane permeability and interaction with intracellular targets.

By Aminomethylation of different substrates, an impressive number of the compound showed antibacterial activity (Lóránd *et al*., 2001). Mannich bases of triazoles with ciprofloxacin were synthesized and most of the compounds showed increased activity than parent drug (V Patel and Won Park, 2013). Mannich bases of sulfonamides and tetracycline were synthesized and showed higher activity than parent molecules. Many Mannich bases derivatives lead to the discovery of new drugs e.g Telavancin is discovered from N-decyl aminoethyl vancomycin (Bérdy, 2012).

Over the last decade's occurrence of fungal infections was increased due to which it causes morbidity and mortality resulting in a major problem for mankind, this is very much problematic for a patient with compromised immune systems (Pfaller and Diekema, 2010). Now priority for the pharmaceutical industry is to discover novel antifungal agents to which minimum resistance can take place resulting in ineffective use of it. A few Mannich bases of 2,3-dihydro-1,3,4-oxadiazole-2-thiones were found equally potent to that of standard drug ciclopiroxolamine against several fungal strains (Manjunatha *et al*., 2010).

In this study, 2-mercaptobenzimidazole-based Mannich derivatives were synthesized and systematically evaluated for antibacterial and antifungal potential. Comparative assessment with standard drugs including roxithromycin, and clotrimazole was performed to establish therapeutic relevance and structure activity correlations. Therefore, this study aimed to synthesize and evaluate 2-mercaptobenzimidazole-based Mannich derivatives for their in-vitro antibacterial and antifungal activities and to establish preliminary SAR correlations

MATERIALS AND METHODS

Chemicals and Reagents

Methanol, Dimethylsulfoxide (DMSO), n-Hexane, Ethyl Acetate, Ethanol, Acetone, were purchased from Sigma-Aldrich, Germany, and Tween-20 from Merck- Schuchardt, USA. Sabouraud Dextrose agar was purchased from Oxoid, England), Phosphate buffer, Nutrient agar, Standard antibiotic (Roxithromycin), standard antifungal (Clotrimazole).

Apparatus and equipment

Glass funnels, Plastic funnels, Tripod stand, Pasteur pipette, Micropipette Sartorius France, Beakers, Round bottom flask, Freezer (Dawlance, Pakistan), Sonicator (Sweep Zone technology, USA), Centrifuge (B.Bran, Germany), CO_2 incubator MCO-17AIC (Sanyo, Japan), Microplate reader ELX 800 (Biotek, USA), Water bath (Biosan, Lativa), 96 well plates (SPL life science, Korea), Analytical weighing balance (Sartorius, France) and vernier caliper.

Microbial Strains

Antimicrobial activities against six bacterial strains, *Staphylococcus aureus* ATCC- 6538, *Bacillus subtilis* ATCC-6633, *Escherichia coli* ATCC-25922, *Klebsiella pneumoniae* ATCC-1705, *Pseudomonas aeruginosa* ATCC-15442, *Resistant coli* ATCC-BAA2452, and four fungal strains, *Aspergillus fumigatus* FCBP-066, *Mucor species* FCBP-0300, *Fusarium solani* FCBP#0291, *Aspergillus flavus* FCBP-0064 by well plate method.

Synthesis of 2-Mercaptobenzimidaole Mannich Based Derivatives (AK1-AK12)

All analytical grade chemicals and reagents were procured from recognized commercial suppliers and used without further purification. A series of twelve 2-mercaptobenzimidazole Mannich derivatives (AK1-AK12) were synthesized via aminomethylation reactions under controlled conditions as previously reported (Kharl *et al.,* 2026).

Antibacterial Assay

Antibacterial activity was assessed using the broth microdilution method in 96-well plates. Bacterial inocula were prepared in sterile nutrient broth and adjusted to 0.5 McFarland turbidity standard. Stock solutions of synthesized compounds (4 mg/mL) were prepared in DMSO, and serial dilutions were performed to obtain concentrations ranging from 100 to 3.70 µg/mL. After incubation at 37°C for 24 hours, optical density was measured at 600 nm using a microplate reader. The percentage inhibition was calculated relative to positive and negative controls. Minimum inhibitory concentration (MIC) was defined as the lowest concentration showing significant growth inhibition.

Stock Solutions

For the preparation of stock solution dissolve 4mg of the compound in 1ml of DMSO. Standard antibiotic stock solution was also prepared 4mg/ml to be used as a positive.

Inoculum Preparation

A colony was picked from the stock culture with the help of a sterile wire loop added to 10ml of sterile nutrient

broth. It was then incubated for 24 hours at 37°C. Finally, turbidity was adjusted to McFarland 0.5 turbidity standard.

Procedure

All experiments were performed in triplicate and results expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA ($p < 0.05$ considered significant). Different antibacterial strains including *E.coli*, *R.coli, B.subtilis, S.aureus*, *K.pneumonia, P.aeruginosa* were utilized to assess the antibacterial potential of newly synthesized compounds by using 96 well plate method.

Freshly prepared nutrient agar is autoclaved to remove any microorganisms, 10µl of sample and 190µl of Inoculum was poured in each well, Cefixime as positive control and DMSO as negative control is used. It was then incubated for 24 hours at 37°C. After that reading is taken by using a microplate reader at 600nm.

MIC

The micro broth dilution method was further used to analyze the compounds showing sufficient antibacterial activity against any strain. Further dilutions were made from 100µg/ml to three folds so that final concentration of 100, 33.33, 11.11, 3.70µg/ml is obtained, now add 10µl of sample and 190µl of inoculums in each well. The plate was then incubated at 37°C for 24 hours. Absorbance was measured at 600nm after 30min that serve as zero time reading and after 24hours of incubation that act as a final reading. Calculate %inhibition by using this formula:

%Inhibition= (Abs- Abn)/(Abp-Abn)*100

Abs= Absorbance of sample Abn= Absorbance of negative Abp= Absorbance of positive control.

Antifungal Assay

For antifungal assays, spore suspensions were made in a 0.02% Tween-20 solution. Fungal suspensions were inoculated into sterile Sabouraud Dextrose Agar plates, and discs containing synthesized compounds were placed. The plates were incubated at 27°C for 24-48 hours, and the zones of inhibition were measured in millimeters. The MIC of active compounds was determined using serial dilution techniques. All experiments were done in triplicate, and data were expressed as mean ± standard deviation.

Stock Solutions

For the preparation of stock solution dissolve 4mg of the synthesized compound in 1ml of DMSO. Clotrimazole is employed as a positive control; additionally, 4mg/ml of DMSO acts as a stock solution.

Inoculum Preparation

5ml of 0.02% v/v Tween 20 is taken in a test tube add 2 to 3 loops of strains from the standard plates.

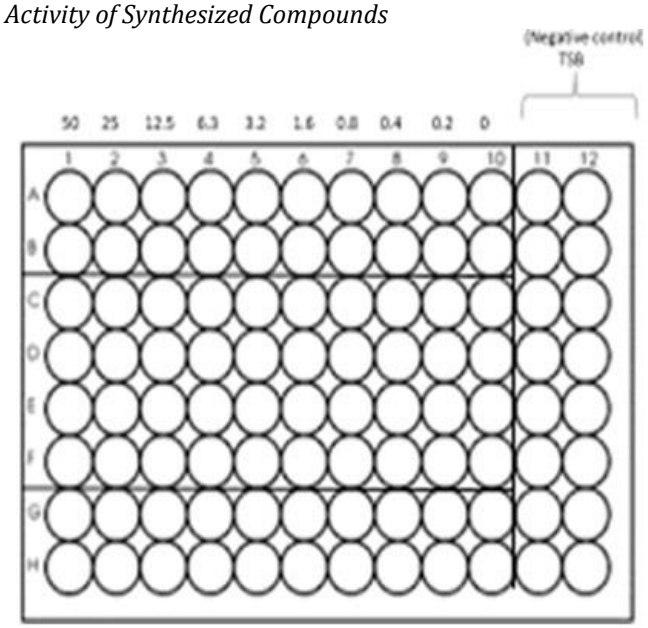
Procedure

The procedure reported by (Zahra *et al*., 2017) was used to perform an antifungal assay. Sterile SDA media was poured in Petri plates, add 100µl of spore suspension for every strain from already prepared Tween 20 solution and swabbed by using a sterile cotton bud. 5µl of the sample was applied to the sterile filter paper disc and placed in Petri plates. Clotrimazole (5µl) and DMSO (5µl) were used as positive and negative control respectively.

These plates are then incubated for 24-48 hours at 27°C, after that by using vernier caliper zone of inhibition was measured in mm. Samples having a zone of inhibition less than 7mm are ineffective, those having 7-9mm are moderately effective and those having more than 9mm are effective but further screening is only done for those samples having a zone of inhibition more than 12mm, their MIC is determined by using two-fold serial dilutions and further their zone of inhibition is calculated by using vernier caliper in mm, tabulate the result against each strain.

Figure 1

Representative 96-Well Microplate Showing Antibacterial Activity of Synthesized Compounds



RESULTS

Antibacterial Assay

Microwell plate method is utilized and absorbance is measured. Six different bacterial strains including *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli,* and *Resistant coli* were used and antibacterial activity of synthesized compounds was evaluated. Different compounds show different antibacterial activity against different strains, significant activity against *S.aureus* and *K.pneumoniae*, moderate activity against *R.coli* and *E.coli*, no activity against *B.subtilis* and *P.aeruginosa*.

The synthesized Mannich derivatives exhibited moderate to strong antibacterial activity. A comparative trend was observed (Table 1)

- Gram-positive bacteria showed higher susceptibility.
- Gram-negative bacteria demonstrated relatively lower sensitivity.

Several derivatives produced inhibition zones comparable to standard drug against Gram-positive strains. MIC values indicated enhanced potency for compounds bearing electron-withdrawing substituents.

Gram-Positive vs Gram-Negative Comparison

Greater efficacy against Gram-positive organisms may be attributed to their simpler peptidoglycan-rich cell wall structure. Reduced activity against Gram-negative bacteria may be due to the presence of an outer membrane

acting as a permeability barrier.

Figure 2

Antibacterial Activity of Synthesized 2-Mercaptobenzimidazole-Based Mannich Derivatives (AK1–AK12) Against Gram-Positive and Gram-Negative Bacterial Strains.

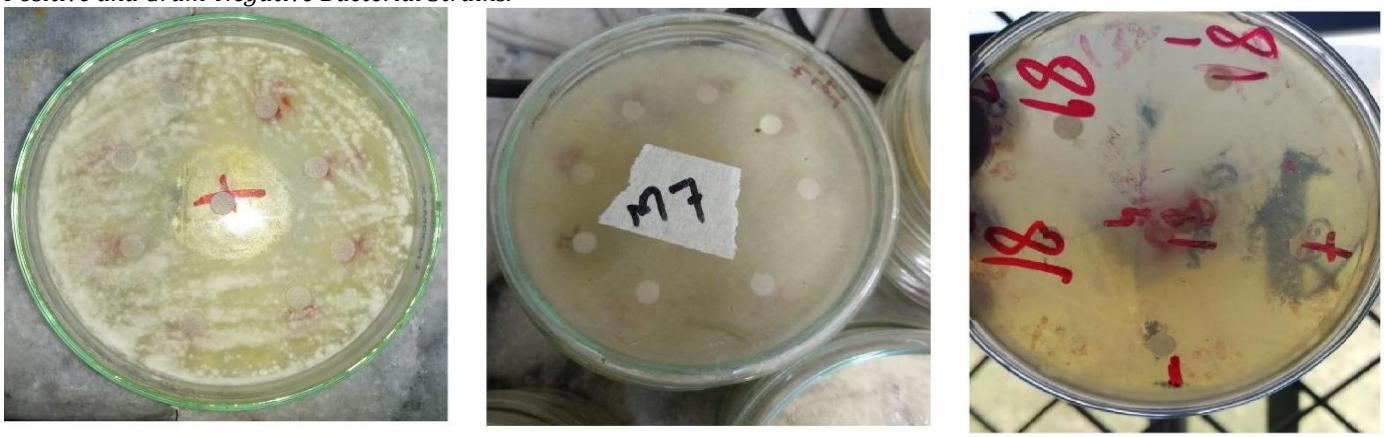


Table 1

Antibacterial Activity of Synthesized Compounds

Sample	*E.coi* (Abs)	MIC µg/ml	*R.coli* (Abs)	MIC µg/ml	*B.subtilis* (Abs)	MIC µg/ml	*S.aureus* (Abs)	MIC µg/ml	*K.pneumonia* (Abs)	MIC µg/ml	*P.aeruginosa* (Abs)	MIC µg/ml
AK1	0.523	-	0.739	-	0.945	-	0.637	-	0.432	100	0.2	-
AK2	0.514	-	0.449	100	0.858	-	0.422	100	0.645	-	0.12	-
AK3	0.592	-	0.583	33.3	1.096	-	0.466	33.3	0.456	100	0.357	-
AK4	0.598	-	0.924	-	0.894	-	0.333	100	0.621	-	0.112	-
AK5	0.647	-	0.48	100	0.754	-	0.43	100	0.484	33.3	0.148	-
AK6	0.672	-	0.51	100	0.787	-	0.676	-	0.497	33.3	0.124	
AK7	0.677	-	0.5	100	0.848	-	0.42	100	0.43	100	0.111	-
AK8	0.812	-	0.755	-	0.785	-	0.801	-	0.537	33.3	0.124	-
AK9	0.765	-	0.755	-	0.795	-	0.586	33.3	0.494	33.3	0.181	-
AK10	0.871	-	0.598	33.3	0.851	-	0.518	33.3	0.468	100	0.493	-
AK11	0.215	33.3	0.763	-	0.685	-	0.401	100	0.55	33.3	0.114	-
AK12	0.482	-	0.681	-	0.709		0.452	33.3	0.511	33.3	0.102	

Antifungal assay

Synthesized compounds were further analyzed for their antifungal activity; four different fungal strains were used *Mucor sp. F.solani, A.fumigatis,* and *A.flavus*. Different compounds show different activities against different strains. AK7 and AK9 show effective activity against *A.fumigatis,* other all compounds show mild to moderate activity. None of the synthesized compounds showed any activity against *F.solani*. AK9 and AK12 show effective activity against *A.flavus,* other all compounds show mild to moderate activity. AK6 and AK12 show effective activity against *Mucor,* other all compounds show mild to moderate activity. Table 3.4 shows antifungal results of synthesized compounds.

Significant antifungal activity was observed against *Aspergillus* species (most sensitive), *Fusarium* species (moderate sensitivity), *Mucor* species (comparatively resistant). Some derivatives demonstrated activity approaching that of clotrimazole, particularly against Aspergillus species (Table 2)

Figure 3

Antifungal Activity of Synthesized 2-Mercaptobenzimidazole Mannich Derivatives Against Fungal Strains

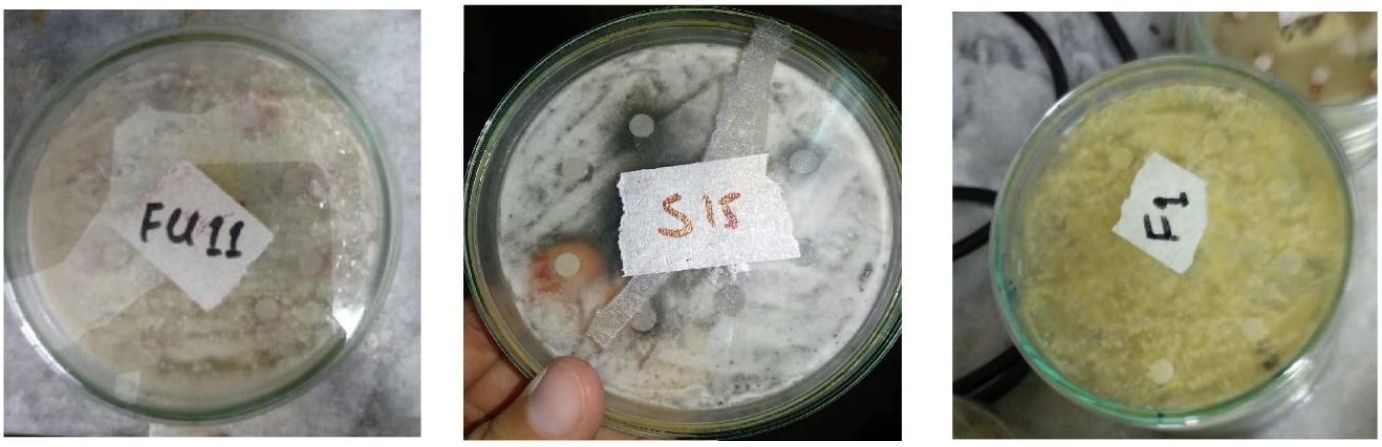


Figure 4

Heatmap Representation of Minimum Inhibitory Concentration (MIC) Values of Synthesized Derivatives Against Tested Bacterial and Fungal Strains

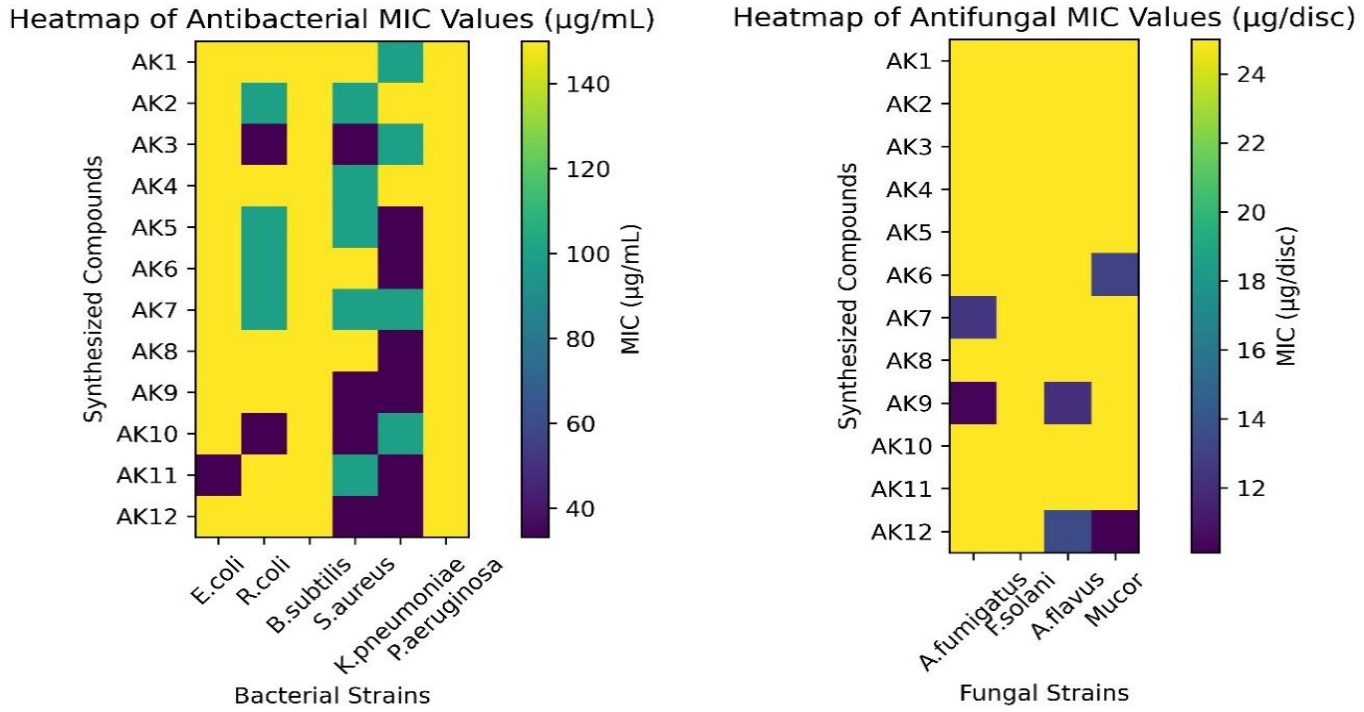


Table 2

Antifungal Activity of Synthesized Compounds with ±S.D

Samples	*A.fumigatis*	MIC µg/disc	*F.solani*	MIC µg/disc	*A.flavus*	MIC µg/disc	*Mucor*	MIC µg/disc
AK1	8±0.5	-	3±0.5	-	4±0.5	-	4±0.5	-
AK2	6±0.5	-	5±0.5	-	2±0.5	-	6±0.5	-
AK3	9±0.707	-	8±0.707	-	4±0.5	-	9±0.707	-
AK4	10±0.707	-	2±0.5	-	8±0.707	-	7±0.5	-
AK5	8±0.5	-	2±0.5	-	3±0.5	-	4±0.5	-
AK6	5±0.5	-	4±0.5	-	5±0.5	-	16±0.707	13.15
AK7	15±0.707	12.5	5±0.5	-	2±0.5	-	5±0.5	-
AK8	8±0.707	-	7±0.5	-	4±0.5	-	8±0.707	-
AK9	13±0.707	10.37	4±0.5	-	14±0.707	12.12	7±0.5	-
AK10	7±0.5	-	3±0.5	-	6±0.5	-	9±0.707	-
AK11	9±0.707	-	2±0.5	-	9±0.707	-	3±0.5	-
AK12	6±0.5	-	6±0.707	-	17±0.707	13.56	13±0.707	10.12

DISCUSSION

The antimicrobial activity observed in this study highlights the pharmacological potential of the 2-mercaptobenzimidazole scaffold. The enhanced efficacy against Gram-positive bacteria may be attributed to the absence of an outer membrane barrier, facilitating improved compound penetration. In contrast, reduced activity against Gram-negative organisms may result from limited permeability and efflux mechanisms. The antifungal activity, particularly against *Aspergillus* species, suggests possible interference with ergosterol biosynthesis or fungal enzymatic systems. The enhanced activity of certain derivatives suggests that the 2-mercaptobenzimidazole scaffold plays a vital role in antimicrobial action. The Mannich base modification likely improves lipophilicity and facilitates microbial membrane penetration. Irrational and excessive use of antimicrobials resulting in resistance which leads to prolonging hospital stay, provoke infections, ultimately lead to morbidity and mortality (Llor and Bjerrum, 2014). Based on the medicinal importance of benzimidazole and Mannich bases, we were anticipating that the synthesized compounds would be effective against various strains of microbes so that we can easily overcome the antimicrobial resistance. Six different bacterial strains including *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli,* and *Resistant coli* were used and antibacterial activity of synthesized compounds was evaluated. Different compounds show different antibacterial activity against different strains, significant activity against *S.aureus* and *K.pneumoniae*, moderate activity against *R.coli* and *E.coli*,

no activity against *B.subtilis* and *P.aeruginosa*.
Four different fungal strains were used *Mucor sp. F.solani, A.fumigatis,* and *A.flavus*. Different compounds show different activities against different strains. AK7 and AK9 show effective activity against *A.fumigatis,* other all compounds show mild to moderate activity. None of the synthesized compounds showed any activity against *F.solani*. AK9 and AK12 show effective activity against *A.flavus,* other all compounds show mild to moderate activity. AK6 and AK12 show effective activity against *Mucor,* other all compounds show mild to moderate activity.

Structure Activity Relationship (SAR)

Structure–activity relationship analysis indicated that derivatives bearing electron-withdrawing substituents exhibited enhanced antibacterial and antifungal potency. Increased lipophilicity likely contributed to improved membrane diffusion and intracellular accumulation. However, the activity remained moderate compared to standard drugs, indicating the need for further structural optimization. Limitations of the present study include the absence of cytotoxicity evaluation, mechanistic enzyme studies, and molecular docking validation, which are recommended for future investigations.

1. Electron-withdrawing substituents (e.g., halogens, nitro groups) enhanced antibacterial activity.
2. Electron-donating groups showed moderate activity.
3. Increased lipophilicity improved antifungal potency.
4. Optimal hydrophilic–lipophilic balance was critical for dual antibacterial–antifungal activity.

Compounds bearing para-halogen substituents (AK7, AK9) exhibited enhanced antifungal potency, possibly due to increased lipophilicity facilitating membrane penetration. The comparatively stronger activity against Gram-positive bacteria supports the hypothesis that cell wall permeability influences efficacy. The antifungal activity suggests possible interference with ergosterol biosynthesis or fungal enzyme systems. Overall, although standard drugs demonstrated superior potency, several synthesized derivatives showed promising activity within therapeutically relevant ranges.

CONCLUSION

The present study demonstrates that 2-mercaptobenzimidazole-based Mannich derivatives possess promising antibacterial and antifungal properties, particularly against Gram-positive bacteria and *Aspergillus* species. Compounds AK7 and AK9 show activity against *A.fumigatus* fungal strain, whereas AK 9 and AK12 shows activity against *A.flavus* strain and AK6 and AK12 show activity against *Mucor sp.* Significant antibacterial activity against *S.aureus, K.pneumonia,* and *R.coli* has been observed. Structural modifications significantly influenced antimicrobial potency, with electron-withdrawing substituents enhancing activity. While standard drugs such as cefixime and clotrimazole exhibited higher overall efficacy, selected derivatives approached comparable activity levels, indicating their potential as lead compounds for further structural optimization and drug development.

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