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Original Article

DESIGN, SYNTHESIS AND EVALUATION OF PYRAZOLE SUBSTITUTED BENZIMIDAZOLE AS AN ANTI-TUBERCULAR, ANTI-FUNGAL AND ANTI-MICROBIAL AGENT

VIJAY BABU A.[1], PRIYA D.[2*], KATHIRAVAN M.K.[2]

[1]Department of Pharmaceutical Chemistry, SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu 603203, Tamil Nadu, India, [2]Dr. APJ Abdul Kalam Research Lab, SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulathur, Chengalpattu 603203, Tamil Nadu, India
Email: priyad@srmist.edu.in

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ABSTRACT

Objective: A series of pyrazole substituted benzimidazole derivatives were subjected to *in silico* studies to identify a new lead for anti-tubercular, antimicrobial and antifungal activity.

Methods: Docking studies was carried out against Mycobacterium tuberculosis InhA bound with ETH-NAD adduct-PDB ID: 2H9I and Crystal structure of *S. aureus* TyrRS in complex with SB-239629-PDB ID: 1JIJ.

Results: Based on the binding interactions, binding energies, and ADMET predictions, the most active compounds were produced, consisting of a para-halo phenyl substitution at the pyrazole nucleus that was connected to benzimidazole. The synthesized compounds were evaluated for tuberculostatic activity using microplate Almar blue assay method, and anti-microbial and anti-fungal activity by disc diffusion method.

Conclusion: Compound 5c with chloro substituted phenyl ring on the pyrazole showed moderate anti-tubercular, mild antifungal and anti-microbial activity. This compound may thus represent a novel, multi target molecule having a selective class of anti-tubercular, anti-fungal and anti-microbial activity.

Keywords: Pyrazole, Docking, ADMET, Anti-tubercular, Anti-Fungal, Anti-microbial

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INTRODUCTION

Microbial infections cause a number of prevalent and serious diseases that have been treated with the discovery of new antibacterial and antifungal agents. Several azole derivatives have been found to exhibit antifungal activity in addition to antimycobacterial activity [1]. Tuberculosis (TB) is a historical disease which has harmed humans for over 4,000 y [2]. About 1/4th of the world's population has been affected by latent tuberculosis [3]. There is a lifetime risk of 5 to 15% of TB infected people being seriously affected by the disease. Globally, 5.8 million people with TB were newly diagnosed and notified in 2020 [4]. According to WHO in 2020, more than 10 million individuals are estimated to have been infected with TB while 1.3 million people lost their lives as a result of the disease including 2,14,000 HIV-positive people.

TB is caused by the bacillus Mycobacterium tuberculosis and is readily transmitted from an infected individual to others via the air. This disease may affect the lungs (known as pulmonary TB) as well as other parts of the body (extrapulmonary TB). When pulmonary tuberculosis is present, some of the signs and symptoms that may be present include a persistent cough, chest discomfort, weakness, loss of weight, an increase in fever, and night sweats [5]. Those who smoke, as well as those whose immune systems are already impaired due to conditions such as HIV, malnutrition, or diabetes, have an increased likelihood of being unwell.

M. tuberculosis bacilli may survive for a longer period by persisting in a nonreplicating or slowly replicating state. In the case of asymptomatic conditions, the lesions can be cured in a period of 6 to 8 w (fig. 1). Whereas in the symptomatic condition, the bacteria spreads throughout the lungs [6].

Treatment for TB has avoided more than 60 million lives since 2000. There are 10 medications now authorised by the U. S. FDA for treating TB. 22 medications, combination regimens, and 14 vaccine candidates were in clinical trials as of August 2020. In this context, various possible innovative anti-tuberculosis treatments have been introduced to current main-line therapies, such as Rifampicin, isoniazid, pyrazinamide and ethambutol. Current first line medications may induce severe liver damage, GIT bleeding, lack of appetite, or yellowing of your skin or eyes [7].

In medicinal chemistry, benzimidazole derivatives are fashionable structures used to identify pharmaceuticals. Due to their therapeutic qualities, they have aroused a lot of attention and are a pharmacophore of choice for building anti-tubercular [8-11], antibacterial [12, 13], and antifungal agents [14-16]. On the other hand, pyrazole derivatives too, possess a wide spread of chemotherapeutic activities and recently it has been reported that functionalized side chain around the benzimidazole with the pyrazole nucleus provides newer opportunities in designing compounds with synergistic activity as potent antimicrobial, antifungal and antitubercular agents [17].

Hence, in the present study, we have used a hybrid approach where two or more pharmacophores are fused into a single molecule, thus exhibiting more potent activity when compared to a single moiety, further it can also reduce undesirable side effects. By using this approach, we developed an effective agent with less GI toxicity using the benzimidazole scaffold fused to substituted pyrazole. The substituted pyrazole moiety at C-2 of the benzimidazole ring was studied for its anti-tubercular, antimicrobial and antifungal activity.

Experimental section

The chemicals and reagents of analytical grade were procured from Sisco laboratories and are used for the study. IR was performed using SHIMADZU IRTracer-100 FTIR spectrophotometer. Determination of melting points were performed by Digital SMP 202 apparatus. Determination of thin layer chromatography were performed on silica gel plates (Silica Gel 60 GF254) and visualized under UV chamber (254 nm). Determination of Mass spectroscopy by Shimadzu Lab solutions. Anti-Tubercular activity assay was performed by using Almar blue dye method, Anti-microbial and Anti-fungal activity was performed by disc diffusion method.

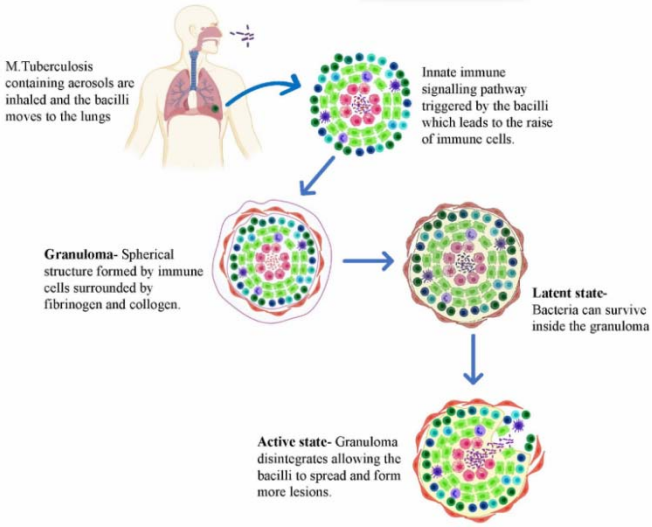

Fig. 1: Tuberculosis pathogenesis and disease progression

Molecular docking

Autodock 4.2.6 conducted molecular docking. ChemDraw Professional 16.0 was used to create two-dimensional structures of Pyrazole-substituted benzimidazole, while Avogadro 1.2.0 was used to minimise energy. Mycobacterium Tuberculosis InhA bound to ETH-NAD (PDB ID: 2H9I) and S. aureus TyrRS bound to SB-239629 (PDB ID: 1JIJ) were retrieved from the protein data bank (RCSB). Docking energy, score, and active site of contacts were used to find the optimum protein-ligand complex.

Admet prediction

The Osiris Property Explorer [18] an open-source software, was used for the toxicity prediction which determines the features of mutagenicity, carcinogenicity, irritability, and reproductive impact. An online admetSAR-2.0 web server was used for predicting adsorption, distribution, metabolism and elimination. AMES toxicity, carcinogenicity, acute oral toxicity, and human gene inhibition was evaluated for all 10 compounds.

Physiochemical property

Physiochemical property was calculated using Mol inspiration, which provides information whether the designed compounds follow Lipinski's rule of five (RO5). The Lipinski rule violation causes compounds with less absorption. Hydrophobicity, electronic distribution, hydrogen bonding properties, molecule size, and flexibility are all key molecular factors that go into the drug score [19].

Synthesis

Step 1: Synthetic procedure for compound 3

50.8 mmol of Phenyl hydrazine was dissolved in10 ml of glacial acetic acid and 10 ml of water and later added to 34.3 mmol of acetophenone in 20 ml of glacial acetic acid in a boiling tube. The mixture was cooled in ice and shaken for 5 min, hydrazone precipitates out. The precipitated hydrazone (3) was filtered, washed with dilute acetic acid and water to yield hydrazone as colourless crystals. The product was recrystallized using ethanol.

Step 2: Synthetic procedure for compound 4

Cold stirred 0.0437 mole of DMF solution [Dimethyl Formamide] and 3.62 ml of phosphorous oxychloride was added to the conical flask and kept in ice bath for suppression of the fumes, to this 3 gm of compound (3) was added slowly and refluxed at 70-80 °C for 6 to 7 h and later cooled at room temperature. Saturated aqueous sodium bicarbonate was added to neutralize the solution and the obtained solid was filtered. The filtered product (4) was washed using excess of cold water, dried and recrystallized using ethanol.

Step 3: Synthetic procedure for compounds 5

0.92 mmol of ortho-phenylene diamine and 0.92 mmol of substituted carbaldehyde with 4 ml of ethanol was added to 0.15g of NH_4Cl. The mixture was refluxed at 80°C for 2 h, and on cooling, the product (5) precipitates out. It was filtered, washed, dried and recrystallized with ethanol. Thin layer chromatography [ethyl acetate: n-hexane, 1:2 v/v] was used identify the end product [20]. Synthesis of novel amide derivative was prepared as depicted in Scheme (fig. 2). Different substitution used in the synthesis are listed in table 1.

Anti-tubercular activity

The compounds were evaluated against M. tuberculosis using Microplate Alamar Blue Assay (MABA) for anti-tubercular activity [21]. 96 wells plates containing deionized sterile water (200µl) and Middlebrook 7H9 broth (100µl) were diluted directly on the plate and were sealed using paraffin. It was then incubated at 37 °C for 5 d. The plates were filled with freshly prepared Almar blue reagent (25µl) and 10% tween 80 in the ratio of 1:1, and were kept in incubator for 24 h. The pink colour indicates bacterial growth, whereas the blue colour was regarded as no bacterial growth. The minimum inhibitory concentration was used to determine the least drug concentration that prevents the colour shift from blue to pink [22].

Table 1: Different substitution used in the synthesis

Compound	R_1	R_2	R_3	R_4
a	Br	H	H	H
b	F	H	H	H
c	Cl	H	H	H
d	OCH_3	H	H	H
e	I	H	H	H
f	NH_2	H	H	CF_3
g	Cl	Cl	H	H
h	Cl	NO_2	NO_2	H
i	Br	H	CF_3	H
j	I	H	CF_3	H

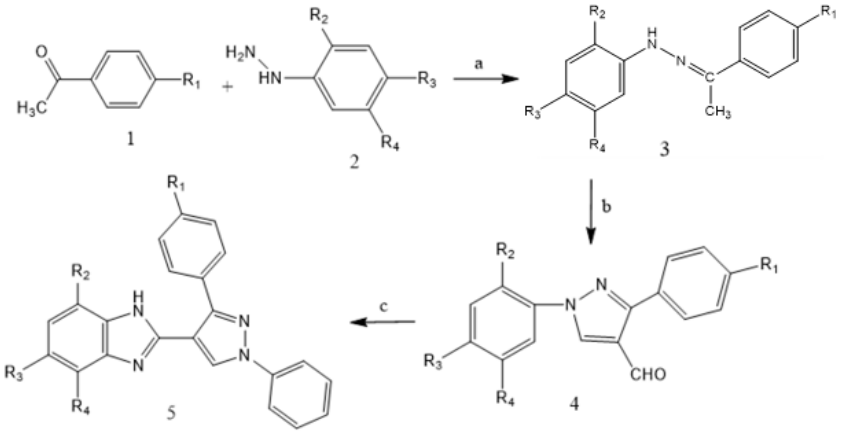

Fig. 2: Synthetic scheme for the preparation of substituted 2-(1,3-diphenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole 5(a-j) Reagents and conditions: (a) CH3COOH, in ice bath (b) DMF, POCl3, 6-7 h reflux at 70-80 ˚C (C) Benzene-1,2-diamine, CH3OH, NH4Cl, 2 h reflux at 80 ˚C

Anti-microbial activity

The *in vitro* study for anti-microbial activity was performed by disc diffusion method against bacterial strains such as *E. coli* and *S. aureus*. This method was used to assess if the compounds have any significant antibacterial activities [23]. As a comparator and positive control, the antibiotics streptomycin and kanamycin were used. Using agar cultures in sterile distilled water the bacterial suspensions were made and a sterile swab was used to evenly seed agar plates. For test compounds, each derivative (0.05g) was dissolved in 10 ml DMSO, from the above solution 50 mg/ml concentration was prepared and further drawn to 500µg/ml concentration. Each compound was filled from the stock solution onto 6-mm diameter hole made on the plate. They were allowed to dry. Each plate comprises of two impregnations one for the test compound and other for the control (tetracycline 5ug/ml) to compare the activity with that of the standard. The plates were incubated at 37 °C for 24 h and were checked for zone of inhibition at the end of the incubation period. Inhibition zone diameters were measured (in mm) and recorded. The anti-bacterial activity was expressed as the minimum inhibitory concentration in µg/ml [24, 25].

Anti-fungal activity

Antifungal activity was evaluated using the disc diffusion technique on Sabouraud dextrose agar medium. The petri dish was filled with Sabouraud dextrose agar [SDA] medium. The inoculums containing fungal suspension were distributed over the solid plate with sterile swab after the medium was solidified. Amphotericin B was considered as a positive control. 20µl of sample and amphotericin B were added in sterile discs and placed in SDA plates and were incubated for 24 h at 28 ˚C. The diameter of the zone of inhibition was then measured to evaluate antifungal activity [26, 27].

RESULTS AND DISCUSSION

Molecular docking

Molecular Docking was performed using AutoDock 4.2.6 for compounds 5(a-j) to determine the binding energies, and best interactions. Table 2 demonstrates binding energy for the compounds against InhA, and tyrosyl-tRNA synthetase target. The docking of the ligand molecules (a-j) indicates that all inhibitor compounds in the active pockets are bonded to one or more amino acids. The theoretical binding energies of all ten compounds ranged from-8.81 kcal/mol to-9.89 kcal/mol for InhA target and-6.16 kcal/mol to-9.68 kcal/mol for tyrosyl-tRNA synthetase target (table 2). Compound 5a, 5c, and 5i showed good binding energies when compared to the standard isoniazid and Clotrimazole. 3D View of the Binding Conformation of compounds was shown in fig. 3 and 4.

Compound 5a and 5c with InhA target exhibited hydrogen bonding interaction with GLY96, GLY14, SER94 with the benzimidazole attached to the pyrazole. A π-π stacking interaction on the phenyl ring attached to the pyrazole with PHE41 was formed. A π-σ stacking interaction on the phenyl ring attached to the pyrazole with ILE16 was formed and ALA198 with the bromine and chlorine attached to the phenyl ring.

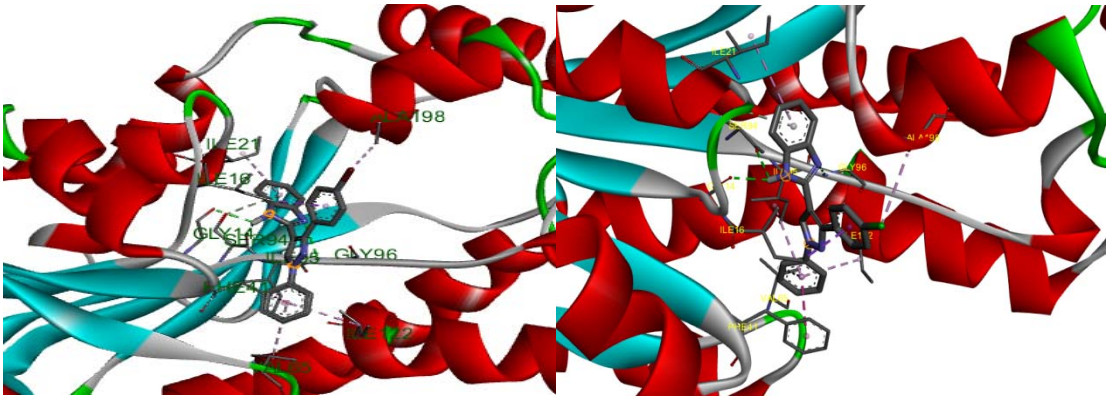

Fig. 3: 3D View of the binding conformation of compound 5a and 5c at the active site of InhA target

Compound 5a and 5c with tyrosyl-tRNA synthetase target exhibited hydrogen bonding interaction with LEU128, LEU173 with the benzimidazole attached to the pyrazole. A π-π stacking interaction on the phenyl ring attached to the pyrazole with SER132 was formed. A π-σ stacking interaction on the phenyl ring attached to the pyrazole with LEU133 was formed and PHE136 with the bromine and chlorine attached to the phenyl ring.

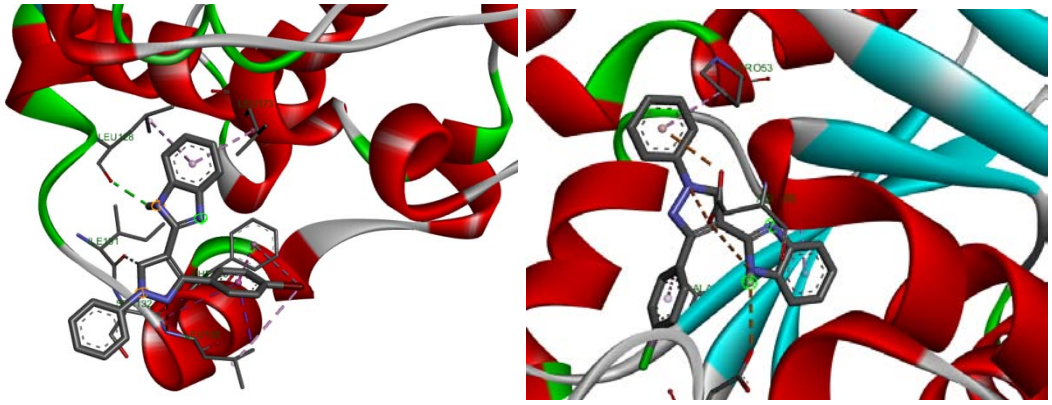

Fig. 4: 3D view of the binding conformation of compound 5a and 5c at the active site of tyrosyl-tRNA synthetase target

Table 2: Binding energies of pyrazole substituted benzimidazole calculated using autodock

S. No.	Compounds	Binding energy (Kcal mol-1) PDB ID: 2H9I	Binding energy (Kcal mol-1) PDB ID: 1JIJ
1	5a	-9.86	-9.14
2	5b	-9.19	-7.23
3	5c	-9.89	-9.68
4	5d	-9.4	-7.6
5	5e	-8.81	-7.64
6	5f	-9.07	-6.16
7	5g	-9.75	-8.12
8	5h	-8.92	-8.51
9	5i	-9.83	-9.09
10	5j	-9.69	-8.06
	Standard	Isoniazid -4.8	Clotrimazole -6.53

ADME properties

The designed ten compounds were subjected for ADMET prediction using AdmetSAR and when working with *in silico* toxicity models, overall toxic phenomena, such as carcinogenicity, mutagenicity and other models that contribute to toxicity manifestations are identified by using Osiris property explorer. All the 10 compounds were found to be non-toxic, non-carcinogenic and have good drug-like properties and the results of all the 10 compounds were tabulated in table 3.

Table 3: ADMET Parameters of pyrazole substituted benzimidazole by AdmetSAR and osiris property explorer

Compounds	HIA	BBB	CYP substrate/inhibition	Carcinogenicity	Mutagenicity	Drug-likeness	Reproductive effect
5a	86.29	0.77	Nonsubstrate/inhibitor	NT	NT	-1.00	NT
5b	87.26	0.76	Nonsubstrate/inhibitor	NT	NT	3.95	NT
5c	88.35	0.77	Nonsubstrate/inhibitor	NT	NT	5.68	NT
5d	88.29	0.52	Nonsubstrate/inhibitor	NT	NT	4.35	NT
5e	86.92	0.76	Nonsubstrate/inhibitor	NT	NT	4.54	NT
5f	85.36	-0.69	Nonsubstrate/inhibitor	NT	NT	4.39	NT
5g	86.33	0.55	Nonsubstrate/inhibitor	NT	NT	-2.68	NT
5h	87.33	0.68	Nonsubstrate/inhibitor	NT	NT	-5.46	NT
5i	86.75	0.628	Nonsubstrate/inhibitor	NT	NT	-6.61	NT
5j	87.39	0.63	Nonsubstrate/inhibitor	NT	NT	-4.71	NT

NT-Non-toxic, T-Toxic

Table 4: Physiochemical property of pyrazole substituted benzimidazole by molinspiration

Compounds	Log P	Molecular weight	No. of rotatable bonds	No. of hydrogen donors	No. of hydrogen acceptors	Violations
5a	4.55	415.29	3	1	4	0
5b	3.92	354.39	3	1	4	0
5c	4.43	370.84	3	1	4	0
5d	3.75	366.42	4	1	5	0
5e	4.26	462.29	3	1	4	0
5f	3.14	351.14	3	3	5	0
5g	5.88	473.28	4	1	4	1
5h	2.41	460.84	5	1	10	0
5i	5.39	483.29	4	1	4	1
5j	5.11	530.29	4	1	4	2

Based on the docking score, ADME property, toxicity profile, compounds 5a and 5c found to have better potency when compared to the standard and hence were synthesised and evaluated for antitubercular, antifungal and antimicrobial activities.

Physiochemical property

Physiochemical property findings by Molinspiration revealed that all of the designed compounds (5a-f) had high drug score values, indicating that they had good drug-like behaviour and may be used as drug candidates. The values predicted for all 10 compounds are given in the table 4.

Chemistry

Pyrazole substituted benzimidazole, 5a and 5c, were synthesised based on the *in silico* results. p-bromo acetophenone and p-chloro acetophenone on reaction with phenyl hydrazine yielded respective hydrazones following Vilsmeier-Haack (VH) reaction. Reddy *et al.*, reported the synthesis of a chloro pyrazolyl benzo[d]imidazole (5c) although via a different route using sodium metabisulphite [28]. This upon reaction with phosphorus oxychloride, cyclization takes place resulting in the formation of pyrazole based carbaldehydes, 4a and 4c, which on condensation with o-phenylene diamine and NH_4Cl yielded the final pyrazole substituted benzimidazole (5a and 5c). The completion of the synthesis was confirmed by the physical data like Rf value, melting point, TLC, IR, ^1H and ^{13}C NMR and Mass.

2-(3-(4-bromophenyl)-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (5a)

Crystalline yellow powder; yield: 68%; m. p: 110 to 112 °C; Rf: 0.37; Solubility: Chloroform and Methanol. IR (KBr): 3309 (ν N–H), 3030 (ν C–H), 1685 (ν C=N), 1495 (ν C=C),1753 (δ C–H), 827 (γ C–H), 594 (ν C–Br) cm–1; ^1H NMR (DMSO-d6, 400 MHz) spectrum δ, ppm: 5.08 (br. s, 1H, NH); 7.42–7.59 (m, 11H, ArH); 8.73–8.89 (m, 2H, ArH); 9.28 (s, 1H, ArH). ^{13}C NMR spectrum δ, ppm: 149.4; 146.7; 143.2; 140.5; 132.1; 131.6; 129.8; 128.3; 126.6; 126.2; 123.1; 120.6; 115.9; 109.3; HR-MS (ESI): *m/z* calcd. for $C_{22}H_{15}BrN_4$: 415.29; found: 415.3000 $[M+H]^+$.

2-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazole (5c)

Crystalline light yellow powder; yield: 60%; m. p: 115 to 117 °C; Rf: 0.58; Solubility: Chloroform and Methanol; IR (KBr): 3176 (ν N–H), 3014 (ν C–H), 1645 (ν C=N), 1593 (νC=C), 1697 (δC–H), 837 (γC–H), 680 (ν C–Cl) cm–1; ^1H NMR (DMSO-d6, 400 MHz) spectrum δ, ppm: 5.04 (br. s, 1H, NH); 7.42–7.60 (m, 11H, ArH); 8.78–8.87 (m, 2H, ArH); 9.26 (s, 1H, ArH). ^{13}C NMR spectrum δ, ppm: 149.7; 146.4; 143.7; 140.8; 133.6; 131.8; 131.7; 129.6; 129.7; 128.8; 127.6; 122.8; 120.4; 115.9; 109.4; HR-MS (ESI): *m/z* calcd. for $C_{22}H_{15}ClN_4$: 370.10; found: 370.1500 $[M+H]^+$.

Anti-tubercular activity

The compounds were tested for anti-tubercular activity using bacterial strain M. tuberculosis H37Rv ATCC 27294 (American Type Culture Collection) by Microplate Alamer blue assay (MABA) against the standard Pyrazinamide, Ciprofloxacin, Streptomycin. MIC (Minimum Inhibition Concentration) was determined on incubating for 5 d at 37 ˚C. Prafulla *et al.*, has identified that Pyrazole substituted benzimidazole showing potent anti-tubercular activity at concentration 100 µg/ml [10], while MIC value of compound 5a was found to be 50 µg/ml and 5c at 25 µg/ml (table 5). The compound 5c is more sensitive against the bacterial strains M. tuberculosis H37Rv ATCC 27294 when compared to compound 5a.

Table 5: Anti-tubercular activity pyrazole substituted benzimidazole by MABA

Sample	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
5a	S	S	R	R	R	R	R	R
5c	S	S	S	R	R	R	R	R

S–SensitiveR–Resistance

Anti-microbial activity

The pyrazole substituted benzimidazole derivative obtained from 5c was found to have a higher degree of zone of inhibition (in mm) of microbial growth (E. coli 25 S. aureus 20), than that of 5a (E. coli 24, S. aureus 19) against the standard tetracycline (E. coli 27, S. aureus 22). The results are shown in graph fig. 5. Padalkar *et al.*, identified 2-[substituted-1H-pyrazol-4-yl]-1H-benzimidazoles exhibiting good antibacterial activity against E. coli and S. aureus and good inhibitory growth in the case of Candida albicans [30].

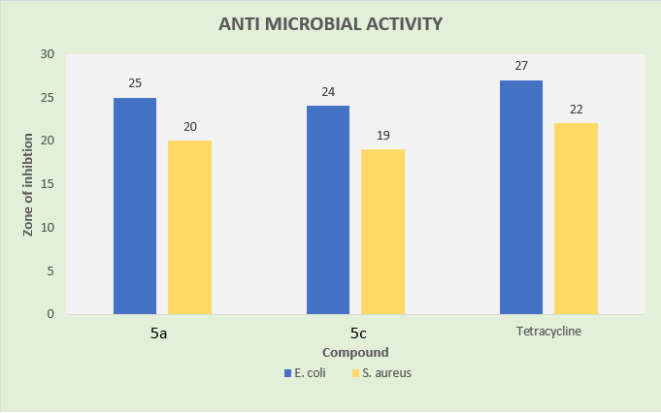

Fig. 5: Anti-microbial activity of pyrazole substituted benzimidazole derivative

Table 6: Anti-fungal activity pyrazole substituted benzimidazole derivative

Organisms	Compound	Zone of inhibition (mm)			Antibiotic (1 mg/ml)
		Concentration			
		1000 µg/ml	750 µg/ml	500 µg/ml	
Aspergillus niger	5a	12	11	11	12
	5c	11	9	9	13
Candida albican	5a	16	11	7	27
	5c	18	14	8	27

Anti-fungal activity

Saundane *et al.*, reported the synthesis of a series of benzimidazole–pyrazole compounds using a two-step strategy with intermediate chalcones and the compounds possessed good anti-fungal activity against S. aureus and A. niger [29]. Both the compounds 5a and 5c were screened for antifungal activity and it shows moderate activity against Aspergillus niger and Candida albican at 500µg/ml when compared to that of standard drug Amphotericin B (1 mg/ml) (table 6).

CONCLUSION

Due to the widespread biological property of pyrazole substituted benzimidazole, new compounds were designed and subjected to *in silico* studies like docking and Admet predictions. Based on the binding energy and Admet results, compounds with low binding energy and less toxic pyrazole substituted benzimidazole derivatives were synthesised and characterised by using melting point, TLC, IR, MASS and NMR spectra. Anti-tubercular, antimicrobial activities and antifungal activity for the synthesized compounds was evaluated. Both the synthesised compounds showed activity, of which the chloro substituted showed better activity than the compound with bromo substitution and hence the pyrazole substituted benzimidazole bearing chloro phenyl group can be considered for further analysis.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declared that none conflicts of interest concerning the authorship.

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