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Antibacterial Screening and Molecular Docking of 2-Chloro/ Nitrophenyl Benzimidazole Derivatives

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The multidrug-resistant (MDR) bacteria have increased at an alarming rate and caused serious health problems throughout the world. The lack of newly introduced antibiotics prompts researchers to design and develop efficient antimicrobials to combat this issue. Application of benzimidazole as a precursor in synthesis is one of many approaches to the discovery of new antibacterial compounds. Fifteen benzimidazole derivatives bearing chlorophenyl and nitrophenyl groups were screened using 96-well plate microdilution against eight bacteria strains; *Bacillus cereus* (ATCC 11778), *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923) and *Micrococcus luteus* (IIUM), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603) and *Salmonella typhimurium* (IMR S 974/05 B). Norfloxacin was used as a positive control, incorporated with resazurin dye to indicate bacterial growth. All compounds showed inhibition against Gram-positive and Gram-negative bacteria albeit with low activity. Molecular docking of selected compounds was also conducted to analyse their interactions with the protein targets of *E.coli* (PDB ID:4KFG) and *S.aureus* (PDB ID:4URM). Most of the synthesised compounds showed better binding affinities than norfloxacin. The solubility of the compounds in the in vitro analysis may contribute to the low antimicrobial activity results.

Keywords: Benzimidazole; antibacterial; Resazurin dye; 96-well plate microdilution

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Benzimidazole is one of the heterocyclic compounds known to exhibit antimicrobial properties. Because its structure is similar to the building block of nucleotides, principally, benzimidazole derivatives could mimic purine and thus could influent the lifecycle of the bacteria by inhibiting the synthesis of nucleic acids and proteins inside the bacterial cell wall, which would affect its growth and can even kill the bacteria [1]. Benzimidazole attributes its chemical features to the presence of an aromatic ring, which can be decorated with various substituents that could contribute to interactions such as hydrogen bonds, ion-dipole, hydrophobic and also van der Waals forces with wide-range of targets. Thus, the benzimidazole scaffold has a lot of potential in the design of effective compounds possessing therapeutic value.

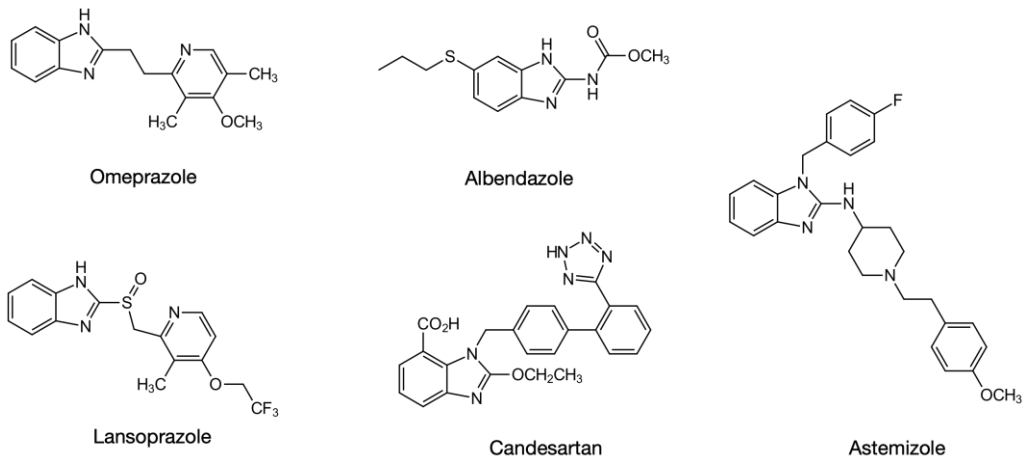

Figure 1. Commercially available benzimidazole drugs [9-12].

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Since the discovery of pharmaceutical properties of benzimidazoles in 1943 by Goodman and Nancy Hart, the compounds have been widely tested for various biological activities [2], including antibacterial [3], anticancer [4], antihypertensive [5], anticonvulsant [6], anti-inflammatory [7] and antioxidant [8]. Some of these compounds have been used commercially in a broad range of diseases including omeprazole, astemizole, albendazole, lansoprazole, and candesartan (Figure 1) [9-12].

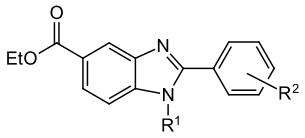
However, the rise of antibiotic deficiency has emerged as one of the most serious problems in infection therapy due to an increase in multidrug-resistant (MDR) bacteria cases, resilient pathogens, as well as the emergence of novel microorganisms [13]. The situation is more serious given that no new classes of antibiotics were discovered in the last 30 years, with the newest class of antibacterial drugs against Gram-positive bacteria being introduced in the 2000s [14].

The design of benzimidazole derivatives for various applications could be done by adding a variety of functional groups into the benzene or/and imidazole moieties of the heterocyclic [15, 16]. A substantial number of studies have verified the effectiveness of benzimidazole derivatives against various strains of microorganisms. Incidentally, benzimidazole-containing drugs available commercially such as albendazole, mebendazole, thiabendazole and ridinalazole consist of 2-substituted benzimidazoles, and this is probably why many of the expansion of the benzimidazole structure always take place at C2.

For instance, in a series of benzimidazole derivatives reported by Ayhan-Kilcigil et al., compound **1** was shown to show significant antimicrobial activity against *B. subtilis* and *P. aeruginosa* with good MIC values, comparable to ampicillin [17]. In another study, benzimidazole-5-carboxylic acid alkyl esters, **2(a-c)**, were found to show antibacterial activity against methicillin-resistant *E. coli*, MRSA, *S. aureus*, *S. faecalis*, MRSE and *C. albicans* [18]. Benzimidazoles containing the hydrazone moiety, **3(a-b**), had also been synthesised and found to be significantly effective against the Gram-negative bacterial strains *P. vulgaris*, *S. typhimurium*, *K. pneumoniae* and *P. aeruginosa* [19] (Figure 2). Besides substitution at position 2, the presence of electron donating and/or withdrawing groups could also affect the antibacterial activity. For example, 2-phenylbenzimidazoles bearing fluorine and the chlorine substituents were shown to give high activity toward *E. coli, B. subtilis* and MRSA bacterial strains, while the presence of electron releasing and withdrawing groups at certain positions could affect the antimicrobial activity potency of 1,2-substituted benzimidazoles [20, 21]. However, the role of these substituents in the potency of the bioactivity is still unclear.

In light of the above, we report the antibacterial activity of 15 benzimidazole derivatives containing chloro- and nitrophenyl groups attached to C2 of the benzimidazole scaffold. The synthetic work of the compounds has been described elsewhere, and the structures of the compounds were confirmed by various spectroscopic methods [22]. In silico molecular docking of the compounds against bacterial DNA gyrase enzyme of *E.coli* and *S.aureus* were also performed to evaluate the ligand activity of the compounds towards the proteins.

Figure 2. Some of the reported benzimidazole derivatives with antimicrobial activity [17-19].



Compound	R^1	R^2
D1	*sec*-butyl	4-Cl
D2	*sec*-butyl	2,6-Cl
D3	*sec*-butyl	4-NO_2
D4	*sec*-butyl	2,4-NO_2
D5	*sec*-butyl	3,5-Cl
D6	*tert*-butyl	2-Cl
D7	*tert*-butyl	3-Cl
D8	*tert*-butyl	4-Cl
D9	*tert*-butyl	2,4-Cl
D10	*tert*-butyl	2,6-Cl
D11	*tert*-butyl	2-NO_2
D12	*tert*-butyl	4-NO_2
D13	*tert*-butyl	2,3-Cl
D14	*tert*-butyl	2,5-Cl
D15	*tert*-butyl	3,5-Cl

Figure 3. The structures of benzimidazoles derivatives used in the antibacterial analysis.

EXPERIMENTAL

Test Microorganisms

Bacterial strains of four Gram-positive bacteria *Bacillus cereus* (ATCC 11778), *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923) and *Micrococcus luteus* (IIUM), and four Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 700603) and *Salmonella typhimurium* (IMR S 974/05 B) were received from the Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia.

Synthesis of Benzimidazole Derivatives

The syntheses of 15 benzimidazole derivatives (Figure 3) used in this study were reported in previous work [22]. All the derivatives were characterised and confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry.

Preparation of Reagents and Standardised Inoculum

Each compound was prepared in 20 mg/mL of DMSO. Norfloxacin was prepared in 4.0 mg/mL. Resazurin was prepared at 0.015% in distilled water, vortexed and filter sterilised (0.22 μm filter). The bacteria were subcultured in nutrient agar plates for 24 hours at 37ºC for 7 days consecutively before being inoculated with Mueller-Hinton broth. Then, the cultured broths were adjusted to 0.5 McFarland turbidity or 10^8 CFU/mL using the spectrophotometer.

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

The MIC assay was carried out in triplicate by using the microdilution method on a 96-well agar plate [23]. 50 μL of Muller-Hinton broth was dispersed using a multichannel micropipette into the wells of columns 1-11, and 100 μL of Muller-Hinton broth into column 12 as a control medium. The serial dilution was carried out by mixing 50 μL of compounds solution and norfloxacin (positive control) into the first well and 50 μL was carried to the next well until the 10th well and the last 50 μL solution was removed. 50 μL of DMSO was added and mixed into column 11 and then 50 μL of the solution (negative control) was removed. Then, cultured broth of 0.5 McFarland turbidity standard was diluted by 1:100 in Muller-Hinton broth resulting in approximately 10^6 CFU/mL. After that, 50 μL of diluted cultured broth was added in all wells except for the control broth in column 12. Columns 1-10 contained serial dilution of compounds and norfloxacin, column 11 was dispersed with 100 μL of diluted standard inoculum with solvent DMSO (approximately 25% v/v), while column 12 contained 100 μL of Muller-Hinton broth (control medium). The plates were incubated for 24 hours at 37 ºC. After that, 30 μL of 0.015% of resazurin was added into each well and continued for incubation for 4 hours. Upon completion of the incubation, the bacteria growth was observed by colour

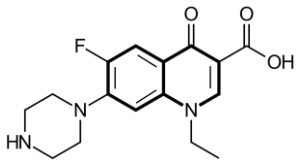
changes of resazurin. The MIC is the minimum concentration of blue resazurin colour that remained unchanged. The MBC was determined by subculturing 50 μL of each culture tube that showed no bacteria growth into the sterilised Muller-Hinton agar plate. The agar plates were incubated for 24 hours at 37ºC and analysed for microbial growth.

Molecular Docking

The molecular docking simulations were performed using AutoDock Vina version 1.2.3 software (The Scripps Research Institute, La Jolla, San Diego, USA) and visualised by Discovery Studio Biovia 2021 (Dassault Systèmes, San Diego, California, USA). The benzimidazole analogues and norfloxacin were docked at the predicted binding site. The protein grid box, which is the active site for docking was set up using AutoDock Tools to enclose the aforementioned residues. The protein targets of *E.coli* DNA-Gyrase B (PDB ID:4KFG) and *S.aureus* DNA-Gyrase B (PDB ID:4URM) were downloaded from RCSB-Protein Data Bank (PDB). The grid box dimension at 0.375 Å for 4KFG was 56 x 60 x 56 and centred at 22.922 x 21.556 x -6.044, while for 4URM was 68 x 68 x 80, centred at 34.991 x 0.250 x 28.695. The docking simulations were then performed using AutoDock Vina, where the docking scores (in kcal/mol) were generated. The fragmented ligand post-dockings were corrected using the *AssignBondOrdersFromTemplate* function within RDKit [24]. The complexation regions were visualised with Pymol version 2.5.2 whereas Biovia Discovery Studio Visualizer 2021 was employed to analyse the interactions within the complexes.

RESULTS AND DISCUSSION

The work presented here is a continuation of our previous studies on 1-*tert*-butyl and 1-*sec*-butyl benzimidazole analogues bearing various chlorophenyl and nitrophenyl substituents at C2 and ester moiety at C5 (Figure 3). The synthesis and characterisation of the derivatives are already mentioned elsewhere [22]. The compounds were evaluated for antimicrobial activity using the 96-well plate microdilution method. Resazurin dye was used to assist in the observation of bacterial growth, indicated by the change of resazurin colour from blue to pink. The well without bacteria growth would remain blue. Four Gram-positive and four Gram-negative bacteria were chosen for this screening. Norfloxacin (Figure 4), which has a similar scaffold to benzimidazole was used as the positive control. This antibacterial agent was known to show excellent activity with broad-spectrum inhibition against both Gram-positive and Gram-negative bacteria [25].



Norfloxacin

Figure 4. Structure of Norfloxacin.

The inhibition activity results of the benzimidazole derivatives with are summarised in Table 1 and Table 2. In general, all 15 compounds varied in their activities from the absence of inhibition to fair activity but much lower antibacterial activity compared to norfloxacin. All the compounds exhibited inhibition activity against *S. pyogenes* with **D9** and **D12** presented as the most potent compounds (0.3 mg/mL). Although the MIC values of the compounds against *S. pyogenes* were lower compared to other bacteria, none of the compounds is bactericidal against *S. pyogenes* even at the highest concentration. The inhibition activity of benzimidazole of the derivatives against *S. aureus* was in the range of 0.3-1.3 mg/mL with **D3,** demonstrating the most potent compound. However, **D5** and **D12** exhibited the lowest value for MBC (2.5 mg/mL).

All compounds showed inhibition of *M. luteus* in the range of 0.6-1.3 mg/mL, with **D3**, **D5** and **D8** exhibiting bactericidal properties at 5.0, 2.5, and 5.0 mg/mL, respectively. Hence, **D5** possessed the most potent antibacterial properties with MIC and MBC values of 0.6 and 2.5 mg/mL, respectively. On the other hand, the benzimidazole derivatives showed less inhibition against *B. cereus* compared to the other Gram-positive bacteria. All compounds gave MIC value of 2.5 mg/mL except for **D1**, with MIC value of 1.3 mg/mL. **D5** and **D10** showed the lowest MBC value (2.5 mg/mL), while others possessed MBC value of 5.0 mg/mL except for **D2** and **D3** which showed non-bactericidal properties at the highest concentration (5.0 mg/mL).

The benzimidazoles in general demonstrated lower inhibition potency of Gram-negative bacteria except for *K. pneumoniae* (MIC = 0.6-1.3 mg/mL), with **D5** and **D12** exhibiting bactericidal properties at 2.5 mg/mL (Table 2). All compounds gave similar MIC value with *E. coli* (2.5 mg/mL) and appeared to be bactericidal with MBC values in the range of 2.5-5.0 mg/mL. Except for **D6**, all compounds also inhibited *S. typhimurium* (2.5-5.0 mg/mL), while **D5** was the most potent compound against *P. aeruginosa* (MIC = 1.3 mg/mL; MBC = 2.5 mg/mL).

Table 1. MIC and MBC screening of benzimidazole derivatives against Gram-positive bacteria strains.

Compound	Bacteria concentration (mg/mL)							
	B. cereus		*S. pyogenes*		*S. aureus*		*M. luteus*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
D1	1.3	5.0	0.6	-	0.6	5.0	1.3	-
D2	2.5	-	0.6	-	0.6	-	0.6	-
D3	2.5	-	0.6	-	0.3	5.0	1.3	5.0
D4	2.5	5.0	0.6	-	0.6	5.0	0.6	-
D5	2.5	2.5	0.6	-	0.6	2.5	0.6	2.5
D6	2.5	5.0	0.6	-	0.6	5.0	0.6	-
D7	2.5	5.0	0.6	-	0.6	-	0.6	-
D8	2.5	5.0	0.6	-	0.6	5.0	0.6	5.0
D9	2.5	5.0	0.3	-	1.3	5.0	0.6	-
D10	2.5	2.5	0.6	-	1.3	5.0	0.6	-
D11	2.5	5.0	0.6	-	1.3	-	0.6	-
D12	2.5	5.0	0.3	-	0.6	2.5	0.6	-
D13	2.5	5.0	0.6	-	1.3	-	1.3	-
D14	2.5	5.0	0.6	-	1.3	5.0	1.3	-
D15	2.5	-	0.6	-	1.3	5.0	1.3	-
norfloxacin	0.032	0.128	0.002	0.064	0.002	0.064	0.002	0.128

Table 2. MIC and MBC screening of benzimidazole derivatives against Gram-negative bacteria strains.

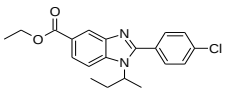
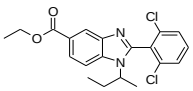
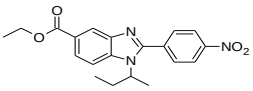
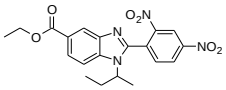
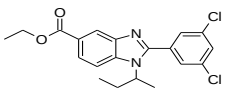
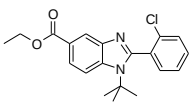
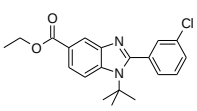
Compound	Bacteria concentration (mg/mL)							
	E. coli		*P. aeruginosa*		*S. typhimurium*		*K. pneumoniae*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
D1	2.5	2.5	2.5	5.0	5.0	-	0.6	-
D2	2.5	5.0	2.5	2.5	2.5	-	0.6	-
D3	2.5	2.5	2.5	5.0	2.5	5.0	0.6	5.0
D4	2.5	5.0	2.5	-	2.5	-	0.6	-
D5	2.5	2.5	1.3	2.5	2.5	2.5	0.6	2.5
D6	2.5	2.5	2.5	2.5	-	-	0.6	5.0
D7	2.5	5.0	2.5	-	5.0	-	0.6	-
D8	2.5	2.5	2.5	2.5	2.5	5.0	0.6	5.0
D9	2.5	5.0	2.5	2.5	2.5	5.0	1.3	5.0
D10	2.5	5.0	2.5	-	2.5	5.0	0.6	-
D11	2.5	5.0	2.5	5.0	2.5	-	0.6	5.0
D12	2.5	2.5	-	-	2.5	2.5	0.6	2.5
D13	2.5	2.5	5.0	-	2.5	2.5	0.6	-
D14	2.5	2.5	-	-	2.5	-	1.3	5.0
D15	2.5	2.5	2.5	2.5	2.5	2.5	0.6	5.0
norfloxacin	0.002	0.064	0.002	0.004	0.004	0.128	0.002	0.128

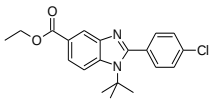
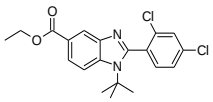
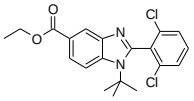
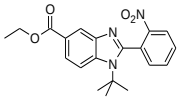
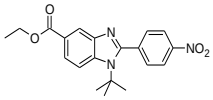
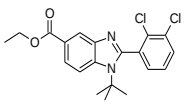
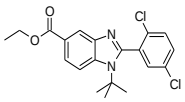
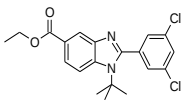
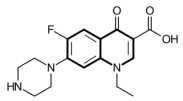
Studies showed that benzimidazoles could show better antibacterial properties towards certain types of strains than others [26, 27]. Despite low inhibition results, it could be seen that the tested compounds have the potential to become broad-spectrum anti-bacterial agents as they are able to preferably inhibit Gram-positive bacteria; *S. pyogenes*, *S. aureus* and *M. luteus* and Gram-negative bacteria, *K. pneumonia*. The low inhibition value could also probably be due to the low solubility of the compounds. Thus, further modifications are required to increase the solubility of the compounds, such as converting the ester moiety to

carboxylic acid. Higher stability, bioavailability and significant biological activity have been reported with benzimidazoles bearing fluorine, propylene and tetrahydroquinoline [28]. Besides that, changing the alkyl group at N1 benzimidazole to the electron withdrawing group may also increase the solubility, as well as increase the activity of the compounds [20].

The molecular docking study was conducted to analyse the binding interactions of the compounds with the binding pockets of *E.coli* (PDB ID:4KFG) and *S.aureus* (PDB ID:4URM), and compared to the reference drug, norfloxacin. The binding activity results are tabulated in Table 3.

Table 3. The binding affinities of the synthesised benzimidazoles and norfloxacin with the active site of the *E.coli*) and *S.aureus* receptors.

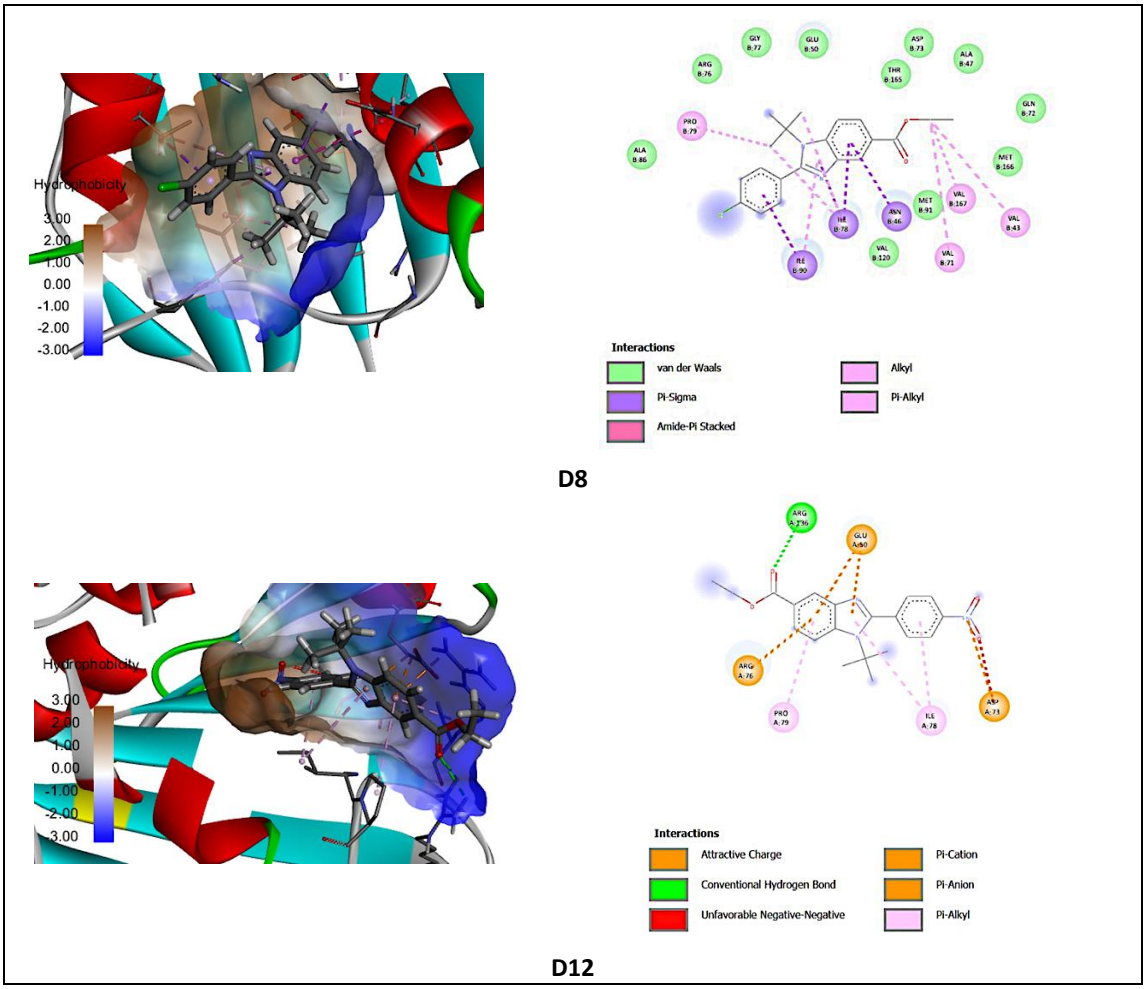
Compound	Binding affinity (kcal/mol)	
	4KFG (*E.coli*)	**4URM** *(S.aureus)*
 **D1**	-7.7	-7.6
 **D2**	-7.4	-7.1
 **D3**	-7.5	-7.8
 **D4**	-7.5	-7.7
 **D5**	-7.7	-7.3
 **D6**	-7.6	-7.4
 **D7**	-7.6	-8.0

 **D8**	-7.8	-8.0
 **D9**	-7.7	-8.0
 **D10**	-7.3	-7.6
 **D11**	-7.4	-7.3
 **D12**	-7.6	-7.7
 **D13**	-7.3	-7.6
 **D14**	-7.3	-7.8
 **D15**	-6.8	-7.6
 norfloxacin	-7.2	-7.5

Compound **D8** shows the best binding affinity in both protein receptors. The binding affinities of all compounds with 4KFG are higher than norfloxacin except for **D15**. In general, chlorophenyl benzimidazoles gave better binding affinities than nitrophenyl analogues, and the *sec*-butyl and *tert*-butyl moieties in the benzimidazole structure do not seem to affect the results. The chlorophenyl benzimidazoles also showed better binding affinities in 4URM active sites, independent of the position of the attachment on the phenyl substituent.

Based on the results, the interactions of a few derivatives are selected for discussion. Figure 5 shows the 3D and 2D binding modes of **D8**, **D12** and norfloxacin in the 4KFG active sites, while Figure 5 for **D8**, **D3** and norfloxacin in the 4URM active sites. Compound **D8** interacts with the 4KFG binding sites mainly by several alkyl and π-sigma interactions, while **D12** interacts through a single hydrogen bond and π-alkyl and a few attractive charges (Figure 4). The charge transfer due to π-sigma interactions may help in the intercalation of the ligand in the receptor's binding site [29]. There is also an unfavourable negative-negative interaction between NO_2 and Asp73. Only one hydrogen bond interacted between norfloxacin and the Arg136 of the 4KFG's protein active site. However, the interactions of fluorine atom with Gly72 and Arg76 of the protein's binding sites may explain its potency as antimicrobial activity.

Compound **D8** is associated with 4URM mainly via alkyl and π-alkyl interactions, which interact with the chloro- substituent, phenyl and *tert*-butyl alkyl groups (Figure 6). There is also a hydrogen bond interaction between N3 of benzimidazole with Glu58. Meanwhile, the presence of NO_2 substituent in compound **D3** induced the phenyl group orientation that allows the amide-π stacking with Asn54. This interaction is relevant for structure-based drug design because it enables the less polar π surface of protein amides to be accessible for ligand interactions in the binding sites [30]. The position also allows the ester group to interact with Arg144 and Arg84 via hydrogen bonds. On the other hand, although norfloxacin showed lower binding affinity, there are three hydrogen bond interactions exerted by the carboxylic acid moiety with Asp81 and Asn54, which probably contributed to its excellent antimicrobial activity.



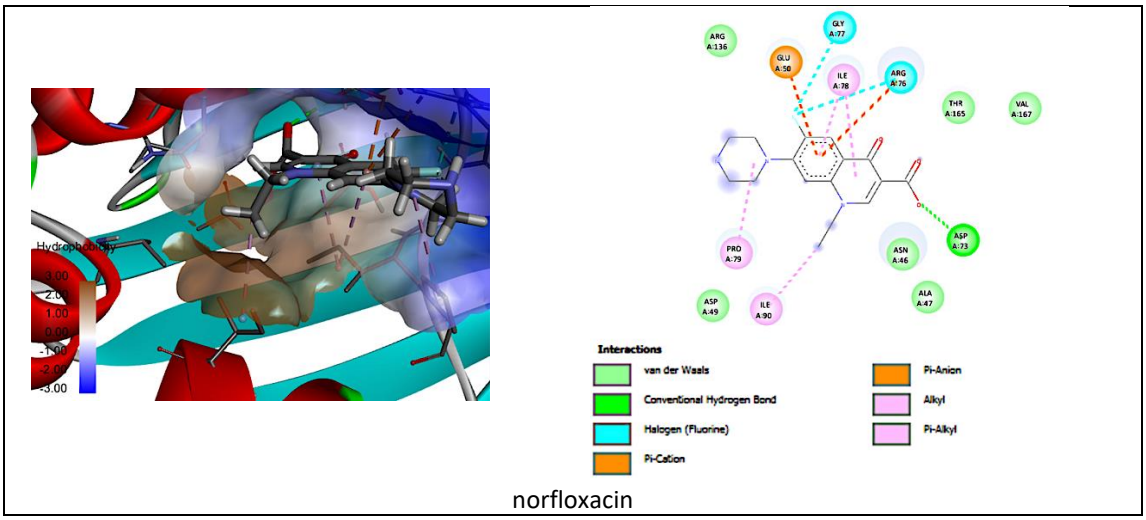
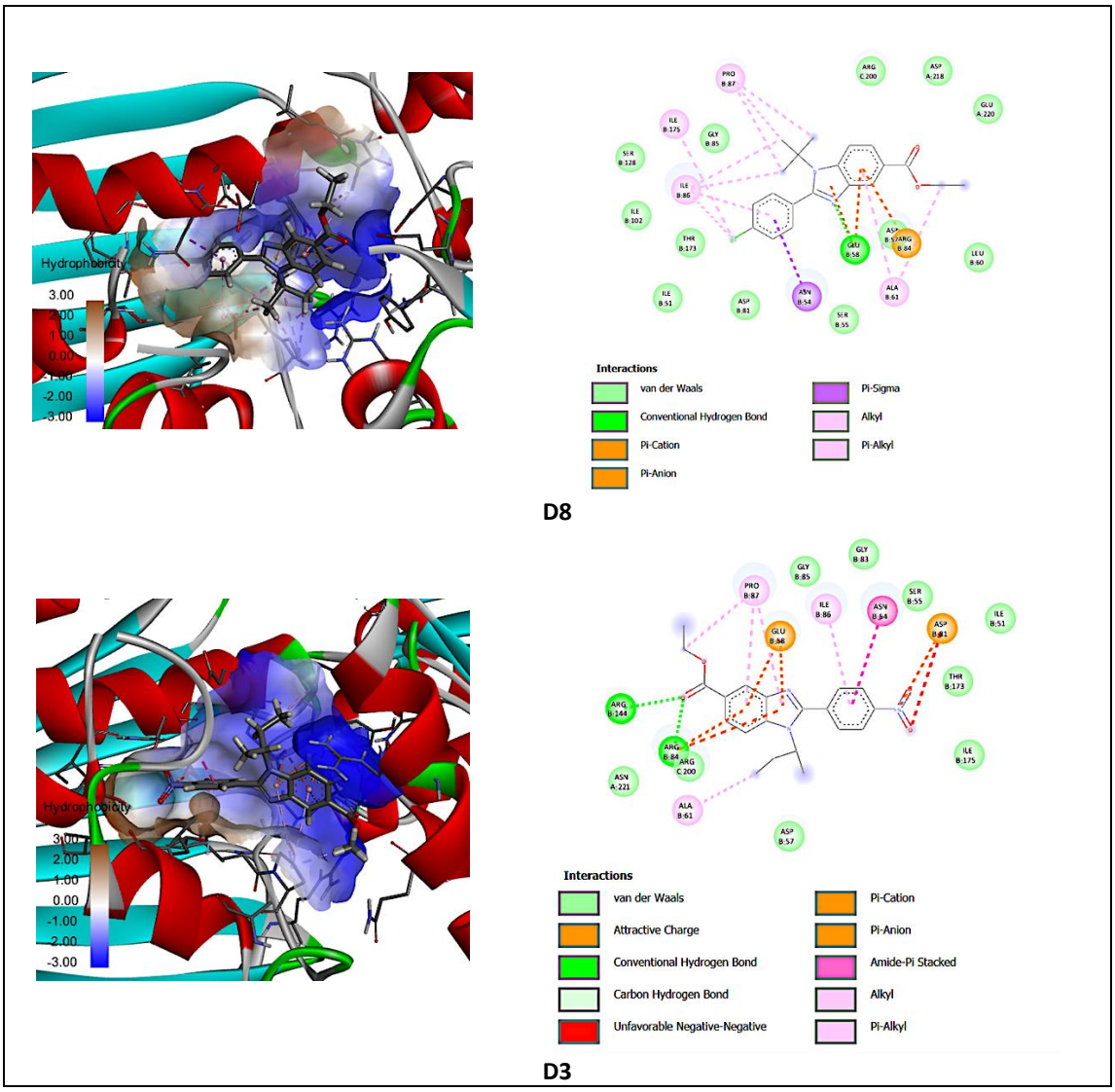


Figure 5. The 3D and 2D receptor-ligand interactions of compounds **D8**, **D12** and norfloxacin the in the active pocket 4KFG receptor.



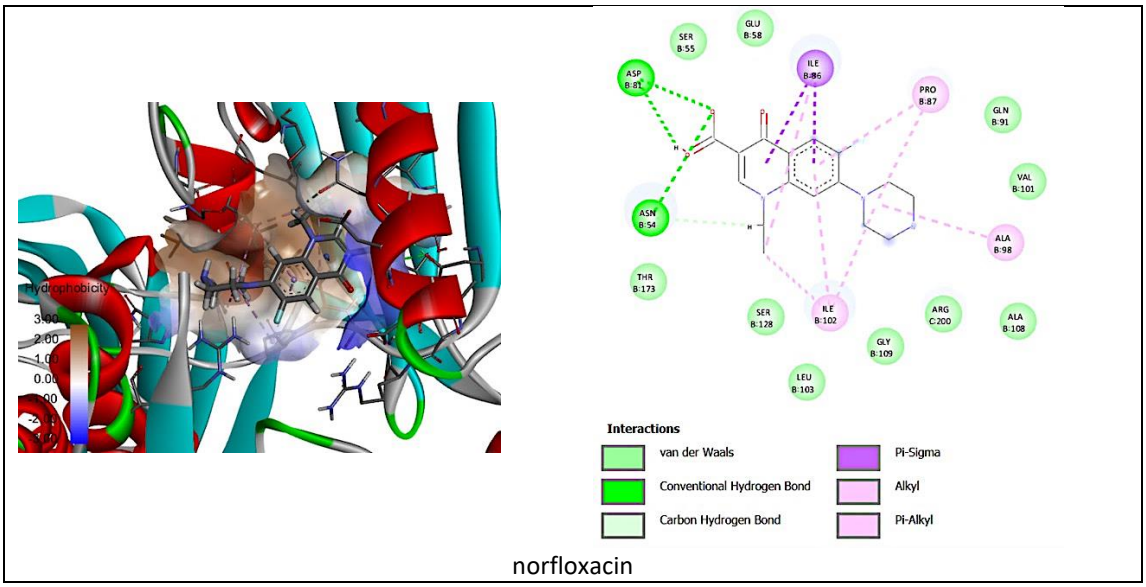


Figure 6. The 3D and 2D receptor-ligand interactions of compounds **D8**, **D3** and norfloxacin the in the active pocket 4URM receptor

CONCLUSION

This study focused on the antibacterial investigation of fifteen benzimidazole derivatives bearing chloro- and nitro-substituents at various positions on the phenyl moiety attached to C2 of the benzimidazole scaffold. Albeit low inhibitory activities were exhibited, the tested benzimidazole derivatives showed promising antibacterial activities towards Gram-positive and Gram-negative bacteria. The poor solubility of the compounds may contribute to the low inhibitory activity observed. In contrast to the bioassay results, molecular docking of the derivatives showed better binding affinities to the target proteins. The results indicate that modifications of the structures are necessary to increase their solubility and antibacterial activity. Efforts towards the study of more efficient synthetic methods, antimicrobial properties and structure-property relationship of this class of compounds must continue in the discovery of new antibacterial agents.

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