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Molecular docking and *in vitro* antibacterial activity of chiral phthalimide on ESBL producing gram negative bacteria

Momin Khan[1], Muhammad Aatasam Hanif[2], Khunsa Rehman[3], Mehwish Arif[4], Humera Nazir[5], Sher Wali Khan[6], Aleyna Kangal[7], Rimsha Abid[8], Dunia A Al Farraj[9] Mohamed S Elshikh[10] and Hazrat Bilal[11]*

[1]Khyber Medical University, Peshawar, Pakistan
[2]Sharif Medical and Dental College, Lahore, Pakistan
[3]Sims Services Institute of Medical Sciences, Lahore, Pakistan
[4]Institute of Pathology and Diagnostic Medicine, Peshawar, Pakistan
[5]Department of Microbiology & Molecular Genetics, Bahauddin Zakariya University, Multan, Pakistan
[6]Department of Chemistry, Shaheed Benazir Bhutto University, Sheringal, Khyber Pakhtunkhwa, Pakistan
[7]School of Arts and Sciences, New Brunswick-Piscataway area campus of Rutgers University, USA
[8]Department of Medical Microbiology, Peshawar, Pakistan
[9]Department of Botany, College of Science, King Saud University, Riyadh, Saudi Arabia
[10]Department of Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[11]Department of Dermatology and Venereology, The Second Affiliated Hospital of Shantou University Medical College, China

Abstract: Antibiotic resistance is tricky enemy that challenges our healthcare system. It is a stealthy, adaptive and ever evolving opponent, which can take years to develop but can spread like wildfire. In this study, derivatives of chiral phthalimides were developed with this aim to control the growth of resistant strains of *Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa* by targeting their resistance causing proteins and explore their binding interaction focal points through computational docking. Total 8 novel chiral phthalimides were synthesized and its antibiogram analysis was done on Muller-Hinton Agar by disc diffusion method. Cytotoxicity studies were made to check efficacy of tested compounds on human RBCs and monitor release of hemoglobin absorbance at 540nm. By using *in silico* molecular approach, crystal structure of target protein was retrieved from Protein Data Bank and docked through Autodock vina and PyRx. The obtained results revealed that seven out of eight compounds have active inhibitory effects against virulent strains. Minimum Inhibitory Concentration (MIC) was measured for most potent compounds i.e., 2-(1,3-dioxoisoindolin-2-yl)-3-(4-hydroxyphenyl) propanoic acid (compound 7) and 3-(1,3-dioxoisoindolin-2-yl) propanoic acid (compound 8). Docking studies displayed a report of highest affinity binding points i.e., amino acids LYS315, ALA318, TYR150, THR262, HIS314 and ARG148 for compound 7 while ALA 318, LYS 315, ARG14 and ILE291 for compound 8.

Keywords: *Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa,* chiral phthalimides, molecular docking, MIC, cytotoxicity.

INTRODUCTION

Antibiotic resistance is a growing concern in global healthcare system. Many factors regarding resistance contribute to severity of infections that lead to many more death rates each year and by the end of 2050 this increased rate cause 150 million premature deaths if it will not have controlled. Research is needed to combat infections and slow down resistance mechanisms (Majumder *et al*., 2020). Gram negative bacteria including *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia* are becoming increasingly resistant to antibiotics, leading to increased morbidity and mortality (Morris and Cerceo, 2020). These organisms' survival becoming possible by resisting many cephalosporin antibiotics due to the secretion of special enzyme Beta Lactamase having potential to hydrolyze the beta lactam ring and also the monobactam and making them unable to reach their target (Mojica *et al*., 2022, Shafiq *et al*., 2022). Among beta lactamase enzymes, AmpC beta lactamase is a relevant class with a growing challenge in resistance mechanism. Gene encodes for AmpC lactamase present in many species of *Enterobacteriaceae* family and neutralizes the effects of ESBL inhibitors (Tamma *et al*., 2022). The world is experiencing major challenges by the adverse effects of drug-resistant bacteria. According to the World Health Report, third-generation cephalosporins are no longer effective against *K. pneumonia, E. coli* and *P. aeruginosa* (Kresken *et al*., 2023, Bilal *et al*., 2021a).

Phthalimide is a valuable class of drug with antibacterial, anti-inflammatory, analgesic, fungicide, pesticides and herbicides, antipsychotics and anticancer activities (Asghar *et al*., 2022). In organic chemistry, synthetic aromatic cyclic phthalimide $C_8H_5NO_2$ compounds are heterocyclic scaffolds that have a structural basis of two

**Corresponding author:* e-mail: bilal.microbiologist@yahoo.com

carbonyl groups linked to an amine moiety (-C=O-NH-C=O-) (Hoque *et al*., 2021). They can pass any biological barrier due to hydrophobic and neutral nature of phthalimide ring and key intermediate for the production of bio-active energetic therapeutic synthons (Das, 2021). These derivatives are also able to form hydrogen bonds with one carbonyl group and one NH group in their structure and are evidenced to have beneficial effects equal or greater than identified drug molecules and thus gain attention in biomedical research (Liu *et al*., 2022)

The main focus of contemporary research science has been computational biology for many years in order to understand the connection between molecular structure and activity. Molecular docking is frequently used to comprehend biological functions at the molecular level and to find new drugs.

In this research, synthesis of eight novel Phthalimide compounds with substitution of different amino acid reported as the antibacterial agent that are capable of inhibiting the growth of ESBL producing gram negative bacteria by targeting their enzymes. Furthermore, to confirm the inhibition process computational methods were carried out to find out its target inside bacteria.

MATERIALS AND METHODS

This experimental study was conducted at Microbiology department of Institute of Basic Medical Sciences (IBMS), Khyber Medical University (KMU), Peshawar, Pakistan.

General procedure for synthesis of phthalic anhydride (isobenzofuran-1, 3-dione)

60 mmol, 10 grams of -ortho phthalic acid and 120 mmol 11ml of freshly redistilled acetic anhydride were taken in a 200-ml round bottom flask. The mixture was refluxed on oil bath with occasional shaking. Reaction mixture was then allowed to cool, then diluted with anhydrous ether and was left for cooling overnight under nitrogen atmosphere in freezer. Crystals of phthalic anhydride separated out which were filtered and washed with anhydrous ether (2 x 20 ml) and dried under vacuum.

Synthesis of chiral phthalimides

Phthalic anhydride, amino acids, glacial acetic acid, and nitrogen were added to a round beaker for the synthesis of amide or imide and the mixture was stirred continuously for at least one hour. Glacial acetic acid was subsequently extracted using ethyl acetate or chloroform and water. Once a crude product was made, it was later purified by column chromatography. For each compound, the following amino acids react with phthalic anhydride.

Compound 1: 2-(1, 3-dioxoisoindolin-2-yl)-3-phenylpropanoic acid

2-(1, 3-dioxoisoindolin-2-yl)-3-phenylpropanoic acid compound was made when L-phenyl alanine reacts with phthalic anhydride.

Compound 2: 2-(1, 3-dioxoisoindolin-2-yl)-4-methylpentanoic acid

Reaction of phthalic anhydride and L-leucine gave the final product of 2-(1, 3-dioxoisoindolin-2-yl)-4-methylpentanoic acid.

Compound 3: 2-(1, 3-dioxoisoindolin-2-yl)-4-(methylthio) butanoic acid

Final product of 2-(1, 3-dioxoisoindolin-2-yl)-4-(methylthio) butanoic acid was made by reaction of amino acid L-methionine and pthalic anhydride.

Compound 4: Synthesis of 2-(1,3-dioxoisoindolin-2-yl)-3-methylpentanoic acid

Product of 2-(1, 3-dioxoisoindolin-2-yl)-3-methylpentanoic acid was synthesized by the reaction of phthalic anhydride and L-isoleucine.

Compound 5: *Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-3-methylbutanoic acid*

Product compound 2-(1, 3-dioxoisoindolin-2-yl)-3-methylbutanoic acid was prepared when both phthalic anhydride and L-valine reacts with each other.

Compound 6: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid

Compound 2-(1, 3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid was synthesized by the reaction of phthalic anhydride and L-cysteine.

Compound 7: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-3-(4hydroxyphenyl) propanoic acid

Compound 2-(1, 3-dioxoisoindolin-2-yl)-3-(4-hydroxyphenyl) propanoic acid was synthesized by the reaction of phthalic anhydride and L-tyrosine.

Compound 8: Synthesis of 3-(1, 3-dioxoisoindolin-2-yl) propanoic acid

Compound 3-(1, 3-dioxoisoindolin-2-yl) propanoic acid was synthesized by the reaction of phthalic anhydride and β-alanine.

Sample collection

All bacterial isolates were obtained from different hospitals in sterile container and stored under sterile conditions in KMU Microbiology Laboratory.

Bacterial culturing and identification

E. coli, K. pneumonia, and *P. aeruginosa* were cultured on Nutrient Agar, MacConkey Agar, Blood Agar, and MHA. A well-known Gram staining as well as the biochemical tests i.e., triple sugar iron Test, urease Test, oxidase Test, catalase Test and simon citrate Test were used to identify these gram-negative virulent strains.

Double disc synergy test for ESBL detection

ESBL detection was done by placing amoxicillin/clavunate disc (30ug) at the center of the

MHA plate while ceftazidime (30ug) and ceftriaxone (30ug) placed on each side of central disc. After incubation at 37°C for 24 hours, zone of inhibition towards amoxicillin/clavunate disc was considered a positive test for ESBL production (Bilal *et al*., 2021b).

Antibiogram analysis by disc diffusion

All synthesized compounds were dissolved in DMSO and final concentration was made of 10mg, 5mg, 2.5mg, 1.25 mg and 0.6 mg/ml, after that 10ul of each test compound dilution were loaded to paper discs and dried and then placed on MHA plates. Imipenem and ceftazidime discs were used as control (Hameed *et al*., 2019).

In vitro cytotoxicity assay

Eight amino acid derivatives were exposed to react with human RBCs in order to check its compatibility and hemolysis. RBCs were collected by centrifugation at 3000 rpm for 3mins and a pallet containing RBCs was washed several times to get debris free RBCs. Triton-x (0.5%) and normal saline used as positive and negative control respectively. After incubation again centrifugation was carried out and this time supernatant was collected and dispensed in cuvettes for spectrophotometer (Nassar *et al*., 2023).

Calculation was carried out through following formula by putting OD values.

$$\%\text{Hemolysis} = \frac{\text{Sample} - \text{negative control}}{\text{Positive Control} - \text{Negative Control}} \times 100$$

Computational analysis

Selection of compound

Corina software created a three-dimensional structure from the two-dimensional structures of these chosen compounds, which was then displayed on the discovery studio visualizer.

Retrieval of target

Possible target prediction for these compounds was done using different web data bases including Similarity Ensemble Approach (SEA), ChemProt and Polypharmacology Browser (PPB).

Molecular docking

Molecular docking is a way to explore binding affinities for existing drugs or drug-like molecules. This bioinformatics-based approach has the ability to predict intermolecular interactions at some points in ligand-receptor complex and can alter the chemical behavior of target protein. To explore binding mode and interaction points of the Phthalimide compound inside target protein, docking analysis was performed. For this purpose docking tool i.e., Autodock 4.2 and PyRx was used.

STATISTICAL ANALYSIS

For data tabulation, Microsoft Excel was used for statistical analysis and graphical representation of data, latest version of GraphPad Prism was used.

RESULTS

Biochemical characterization

Table 2 summarizes all of the pure culture findings.

ESBL Screening

Extension of zone of inhibition around disc containing ceftazidime 30ug and cefotaxime 30ug towards amoxicillin/clavulanic acid disc shows that all the test organisms were positive to ESBL production (figs. 1, 2 and 3).

Fig.1: ESBL screening of *P. aeruginosa*

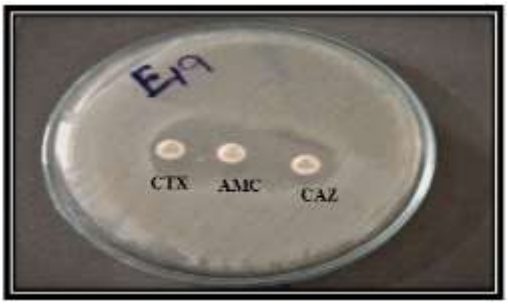


Fig. 2: ESBL screening of *E. coli*

Fig. 3: ESBL screening of *K. pneumonia*

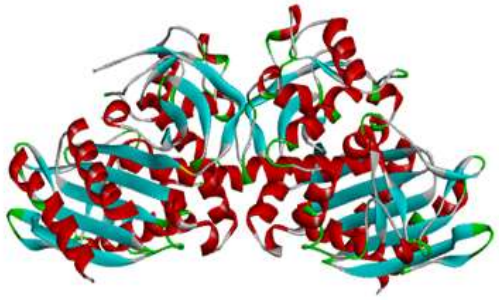


Fig. 4: 3D structure of beta lactamase AmpC

Antibacterial Susceptibility Testing and MIC Detection

Compound 7 and 8 were found to be efficacious against three bacterial isolates, namely *E. coli, P. aeruginosa* and *K. pneumonia* (table 3). Imipenem and ceftazidime discs were used as positive control. MIC was done by disc diffusion method and the results were listed in table 4.

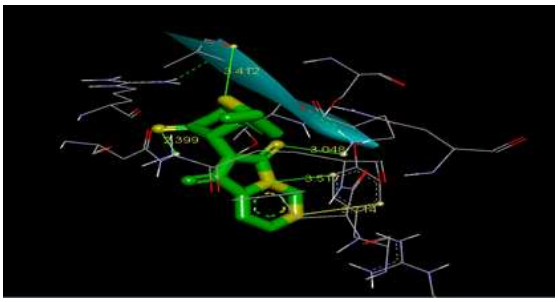


Fig. 5: 3D interaction of AmpC beta lactamase chain A with compound 7

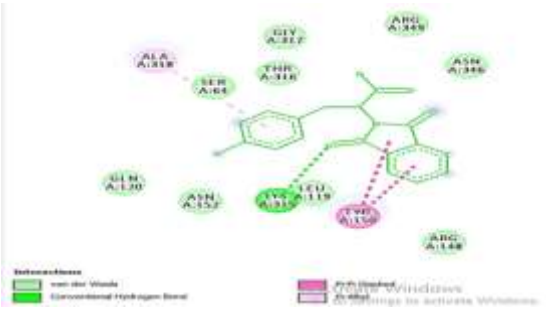


Fig. 6: 2D interaction of AmpC beta lactamase chain A with Compound 7.

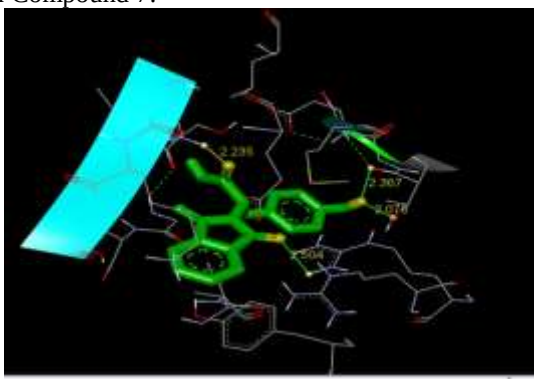


Fig. 7: 3D interaction of AmpC beta lactamase chain B with compound 7.

Cytotoxicity evaluation

Biocompatibility test was assessed on human RBCs to check their cytotoxic behavior. Table 5 summarizes the results based on MIC concentrations of test compounds varying from 100 to 200ug/mL. Triton-x (0.5%) taken as positive control and Phosphate Buffer Saline as negative control.

Retrieval of target protein by different server

Target report cards were acquired from various servers, including SEA, PPB and ChemProt and are shown below, where two servers provide the same results for two tested compounds. The UniProt ID of target protein is P00811 and the protein name is Beta lactamase AmpC (PDB ID: 1FSY). Detail reports on the proteins that our compound of interest targets were shown in table 6, 7 and 8.

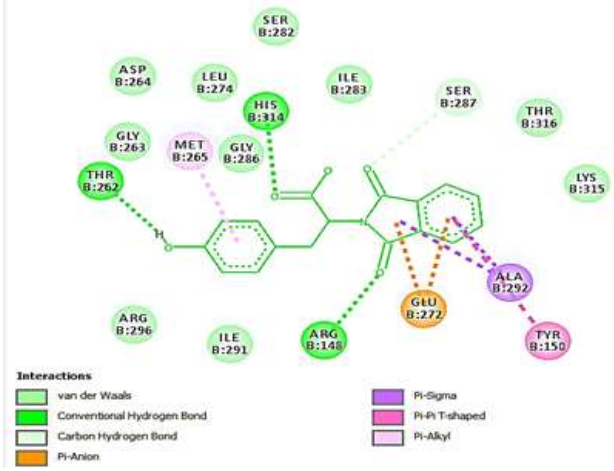


Fig. 8: 2D interaction of AmpC beta lactamase chain B with compound 7

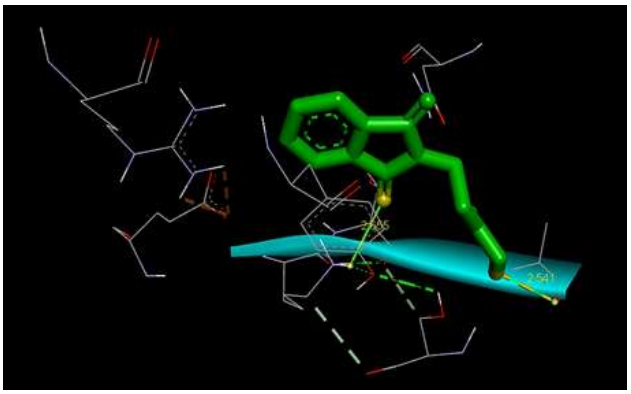


Fig. 9: 3D interaction of AmpC beta lactamase chain A with compound 8.

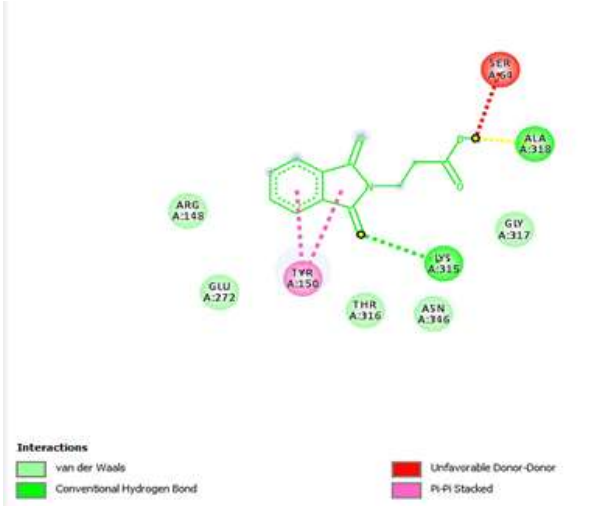


Fig. 10: 2D interaction of AmpC beta lactamase chain A with compound 8.

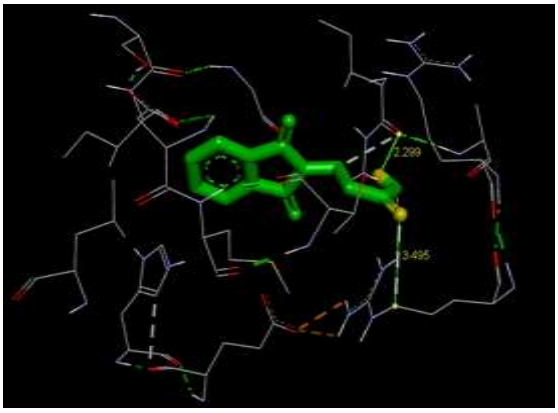


Fig. 11: 3D interaction of AmpC beta lactamase chain B with compound 8

Preparation of protein and ligand for docking

The cloxacillin boronic acid inhibitor and beta-lactamase AmpC were present in a complex in the protein that was downloaded from the RSCB website. Active amino acids involved in making complex with Ampicillin Beta Lactamase were investigated and Cloxacillin boronic acid inhibitor was removed from the grids using DS visualizer.

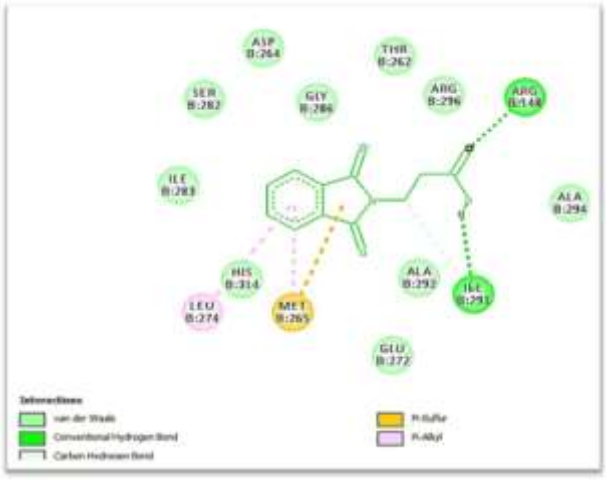


Fig. 12: 2D interaction of AmpC beta lactamase chain B with compound 8

AmpC beta lactamase consist of two chains, A and B. HIS186, ALA318, GLY320, TYR221, TYR150, ASN152, GLN120 and SER64 were the active sites found in chain A while LYS219, SER64, LYS67, GLN120, TYR150, ASN152, TYR221, ASN 289, ALA318, THR319 and GLY320 were found in chain B.

Molecular docking of compound 7 with beta lactamase AmpC

Chain A docking results

Compound 7 was docked with target protein AmpC beta lactamase associated with *E. coli*. Compound 7 interacted with active amino acid residues of chains A and B, as shown in table 9. fig. 5 and 6 depicts the 3D and 2D interaction of AmpC beta lactamase chain A with Compound 7. LYS315 interact with O23 at the distance of 3.048, ALA318 interact with C12 at the distance of 3.412 and TYR150 interact with C21 and C19 at the same time making distances of 3.515 and 3.717 respectively.

Chain b docking results

THR262, ARG148 and HIS314 are chain B amino acids engaged in interactions with compound 7, as shown in fig. 7, where H30 molecule interacts with THR262 at two points with distances of 2.078 and 2.367 and ARG148 interacts with O15 at a distance of 2.504. At a distance of 2.235, HIS 314 interacts with O3.

Molecular docking of compound 8 with beta lactamase AmpC

Chain a docking results

Compound 8 was docked with target protein AmpC beta lactamase associated with *E. coli*. table 10 shows all docking results of compound 8 with chain A and B of AmpC beta lactamase.

Chain b docking results

Compound 8 was docked with chain B of target protein AmpC Beta Lactamase associated with *E. coli*. ILE291 and ARG148 interacted with H17 and O3 making distances of 2.299 and 3.495 respectively.

DISCUSSION

Gram negative pathogenic bacteria have a greater level of resistance to currently available antibiotics, leading to the failure of empirical treatments and the need to hydrolyze previously effective antibiotics.

This resistance mechanism is mediated by the production of resistant enzymes called ESBLs (Bilal *et al*., 2020). The main goal of this study is to create novel drugs that can specifically target the proteins of bacteria and withstand the process of hydrolyzing by enzyme.

Phthalimide compounds were widely used as powerful inhibitors of many antimicrobials and were thought to have a wide range of therapeutic and pharmaceutical uses in science and medicine (Islam *et al*., 2022). Due to its structural diversity and heterocycle element, which serves as the initial synthon for the production of numerous pharmacophores, phthalimide has become a significant target for the synthesis of novel drugs over the past two decades (Yadav *et al*., 2023).

It is helpful in the pharmaceutical industry due to its wide range of applica-tions (Asadi *et al*., 2023). Numerous studies have looked into its characteristics in conjunction with other important intermediates, including Schiff bases, amino acids, N-substituted alky, -alkyloxy and many other chemical structures (Hadi *et al*., 2022).

Table 1: Characteristics of synthesized compounds

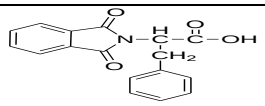
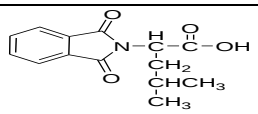
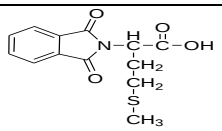
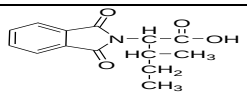
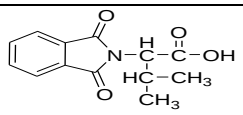
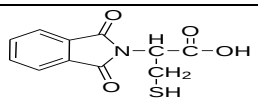
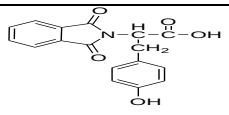
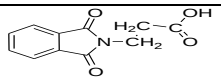
Compound 1: Synthesis of 2-(1,3-dioxoisoindolin-2-yl)-3-phenylpropanoic acid		
Yield	74% crystalline solid	 $C_{17}H_{13}NO_4$ Mol. Wt.: 295.29
Melting point	160-164 °C	
$[\alpha]_D^{25}$	21.2 (c = 24 mg/2 ml CH_2Cl_2)	
FTIR (KBr)	3250-2615 (-OH), 2943 (-CH stretching), 1742 (CO imide), 1671 (CO acid) cm^{-1}.	
Compound 2: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-4-methylpentanoic acid		
Yield	77% white crystaline solid	 $C_{14}H_{15}NO_4$ Mol. Wt.: 261.27
Melting point	80-86 °C	
$[\alpha]_D^{25}$	20.8 (c = 24 mg/2 ml CH_2Cl_2).	
FTIR (KBr):	3251-2614 (-OH), 2942 (-CH stretching), 1740 (CO imide), 1670 (CO acid) cm^{-1}.	
Compound 3: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-4-(methylthio) butanoic acid		
Yield	74% white crystaline solid	 $C_{13}H_{13}NO_4S$ Mol. Wt.: 279.31
Melting point	130-134 °C	
$[\alpha]_D^{25}$	20.2 (c = 24 mg/2 ml CH_2Cl_2)	
FTIR (KBr)	3250-2615 (-OH), 2943 (-CH stretching), 1742 (CO imide), 1671 (CO acid) cm^{-1}.	
Compound 4: Synthesis of 2-(1,3-dioxoisoindolin-2-yl)-3-methylpentanoic acid		
Yield	74%. White crystalline solid	 $C_{14}H_{15}NO_4$ Mol. Wt.: 261.27
Melting point	113-115 °C	
$[\alpha]_D^{25}$	20.2 (c = 24 mg/2 ml CH_2Cl_2).	
FTIR (KBr)	3250-2615 (-OH), 2943 (-CH stretching), 1742 (CO imide), 1671 (CO acid) cm^{-1}	
Compound 5: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-3-methylbutanoic acid		
Yield	79 %. White crystalline solid	 $C_{13}H_{13}NO_4$ Mol. Wt.: 247.25
Melting point	125-128 °C	
$[\alpha]_D^{25}$	21.5 (c = 24 mg/2 ml CH_2Cl_2).	
FTIR (KBr)	3250-2615 (-OH), 2943 (-CH stretching), 1742 (CO imide), 1671 (CO acid) cm^{-1}.	
Compound 6: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-3-mercaptopropanoic acid		
Yield	77 %. White crystalline solid	 $C_{11}H_9NO_4S$ Mol. Wt.: 251.26
Melting point	135-139 °C	
$[\alpha]_D^{25}$	20.4° (c = 24 mg/2 ml CH_2Cl_2).	
FTIR (KBr)	3250-2615 (-OH), 2943 (-CH stretching), 1742 (CO imide), 1671 (CO acid) cm^{-1}.	
Compound 7: Synthesis of 2-(1, 3-dioxoisoindolin-2-yl)-3-(4-hydroxyphenyl) propanoic acid		
Yield	76 %. White crystalline solid	 $C_{17}H_{13}NO_5$ Mol. Wt.: 311.29
Melting point	above 300 °C decompos	
$[\alpha]_D^{25}$	20.3° (c = 24 mg/2 ml CH_2Cl_2).	
FTIR (KBr)	3253-2613 (-OH), 2941 (-CH stretching), 1742(CO imide), 1671 (CO acid) cm^{-1}.	
Compound 8: Synthesis of 3-(1, 3-dioxoisoindolin-2-yl) propanoic acid		
Yield	74 %. White crystalline solid	 $C_{11}H_9NO_4$ Mol. Wt.: 219.19
Melting point	122-128 °C	
$[\alpha]_D^{25}$	20.6° (c = 24 mg/2 ml CH_2Cl_2).	
FTIR (KBr)	3250-2615 (-OH), 2943 (-CH stretching), 1742 (CO imide), 1671 (CO acid) cm	

Table 2: Biochemical characterization of gram-negative bacteria

Biochemical test	Reaction on tested orgnaisms	Interpretation
Escherichia coli		
Urease test	No reaction	The bacterium doesn't produce urease enzyme thus no production of pink color and the test is negative.
Triple Sugar Iron Test	Turns to yellow color, No blackening off color. Bubbles have been seen in slants.	This bacterium doesn't produce H2S gas thus there is no indication of black color at the butt of slant. It produces gas, so bubbles and cracks in slants show positive results. Appearance of yellow color indicates the consumption of three sugars.
Simon Citrate Test	No reaction	This bacterium doesn't consume citrate as carbon source thus no appearance of blue color and the test is negative.
Oxidase Test	No color changes.	This show that organism does not uses aerobic respiration and lack the cytochrome-oxidase-system
Catalase Test	Bubbles production seen.	Catalase enzyme catalyze the oxygen from hydrogen peroxide hence the test is positive for the test organism.
Pseudomonas aeruginosa		
Urease test	Slant color is yellow; no color changes have been seen.	Test is negative for Urease enzyme production.
Triple Sugar Iron Test	Red slant, red butt, no bubbles production and no blackening have been seen.	Results indicate that glucose is not fermented. No gas and H_2S production seen
Simon Citrate Test	Color changes to blue.	This bacterium consumes citrate as carbon source thus color appeared as blue and the test is positive.
Oxidase Test	Minor color changes have been indicated	The organisms have cytochrome-oxidase-system.
Catalase Test	Bubbles production has been seen.	Test is positive as production of catalase enzyme, catalyzes the oxygen from hydrogen peroxide.
Klebsiella pneumonia		
Urease test	Pink color of slant has been seen.	Organism produces urease enzyme that split urea thus color is changes.
Triple Sugar Iron Test	Turns to yellow color, no blackening occurs and No bubbles production.	Results indicate that there is no gas production, no H_2S production and glucose is fermented.
Simon Citrate Test	Color changes to blue	This bacterium consumes citrate as carbon source thus color appeared as blue and the test is positive.
Oxidase Test	No color changes have been seen	Test organism has not oxidase-system and the test is negative.
Catalase Test	Bubbles production has been seen.	Test is positive as production of catalase enzyme, catalyzes the oxygen from hydrogen peroxide.

These studies have revealed that it is an effective anti-cancer, anti-inflammatory, anti-fungal, anti-convulsant, and anti-bacterial agent (Tok *et al*., 2023). However, no research has looked into how its hybridization with an amino acid produces a new compound with potent antibacterial action that specifically targets the bacterial Beta Lactamase enzyme.

In this study, a group of eight amino acid derivative Phthalimide compounds were tested against gram-negative bacteria strains that produce the beta lactamase enzyme. This research documented its cytotoxicity towards human RBCs, antibacterial activity and interactions with the enzyme AmpC beta lactamase. Using the disc diffusion method on Muller-Hinton Agar plates, all of the newly synthesized compounds demonstrated low to intermediate sensitivity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. While other compounds are only marginally active against bacterial isolates, compound 3 has shown no action on any of the tested strains. At a dose of 10 mg/ml, which is quite high, compound 7 and 8 demonstrated higher activity than the other six compounds, with zones of inhibition for *E. coli, P. aeruginosa* and *K. pneumonia* measuring 12mm, 14mm and 14mm, respectively.

Table 3: Zone of inhibition for test compounds at different concentrations

Standard Control		*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*
Ceftazidime (10ug)		05	12	9.5
Imipenem (10ug)		18	12	17
CONC. mg/ml				
C1	(10)	9	9	0
	(5)	8	8	0
	(2.5)	7	7	0
	(1.25)	0	0	0
C2	(10)	10	11	11
	(5)	8	7	08
	(2.5)	7	0	7
	(1.25)	0	0	0
C3	(10)	0	0	0
	(5)	0	0	0
	(2.5)	0	0	0
	(1.25)	0	0	0
C4	(10)	7	0	9
	(5)	7	0	8
	(2.5)	0	0	7
	(1.25)	0	0	0
C5	(10)	10	11	10
	(5)	8	10	7
	(2.5)	8	9	7
	(1.25)	7	7	0
C6	(10)	8	8	8
	(5)	7	7	8
	(2.5)	0	0	7
	(1.25)	0	0	0
C7	(10)	13	13	14
	(5)	10	9	9
	(2.5)	8	8	8
	(1.25)	7	7	7
C8	(10)	12	14	14
	(5)	9	11	11
	(2.5)	8	9	9
	(1.25)	7	7	7

Table 4: Minimum Inhibitory Concentration (µg/mL)

Compounds	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumonia*
1	250	250	-
2	250	5	250
3	-	-	-
4	500	-	250
5	125	1.25	250
6	500	500	250
7	125	125	125
8	125	125	125

The MIC values at concentrations of 125-1000ug/ml was assessed and it was found that only two compounds, compound 7 and 8, had MICs obtained at least at concentration, i.e., 125 g/ml, while other compounds did not inhibit the strains below 250ug/ml. Imipenem and ceftazidime, two common antibiotics, were used as the susceptibility test's control. Results showed that for compounds 7 and 8, inhibitory activity was relatively greater than ceftazidime and slightly lower than imipenem.

Table 5: Cytotoxicity assay

Test Compound 100-200ug/mL	% Hemolysis Standards		
	Non-Hemolytic <2%	Slightly Hemolytic 2-5 %	Hemolytic >5%
C1	-	4%	-
C2	-	5%	-
C3	-	-	-
C4	-	3.7%	-
C5	-	3%	-
C6	2%	-	-
C7	2%	-	-
C8	1.2 %	-	-

Table 6: Similarity Ensemble Approach

Compound 7					
Target key	Q79MP6_PSEAI+5	Q6TUJ4_PSEAI+5	HELD_ECOLI+5	TYSY_ECOLI+5	BLA1_STEMA+5
Target	blaIMP-1	blaIMP-1	helD	thyA	N/A
Organism	*P. aeruginosa*	*Pseudomonas aeruginosa*	*E. coli*	*E. coli*	*Stenotrophomonas maltophilia (Pseudomonas maltophilia) (Xanthomonas maltophilia)*
Target Protein:	Beta-Lactamase	Elastase	DNA Helicase IV	Thymidylate synthase	Metallo-beta-lactamase L1 type 3
UniProt ID:	Q79MP6	P14756	P15038	P0A884	P52700

Compound 8			
Target key:	MAP1_ECOLI+5	HELD_ECOLI+5	ELAS_PSEAE+5
Target name:	Map	helD	blaIMP-1
Organism:	*Escherichia coli*	*Escherichia coli*	*Pseudomonas aeruginosa*
Target Protein:	Methionine aminopeptidase	DNA Helicase IV	Elastase
UniProt ID:	P0AE18	P15038	P14756

Table 7: Polypharmacology Browser

Compound 7		Compound 8
ID	CHEMBL2026	CHEMBL2026
Type	SINGLE PROTIEN	SINGLE PROTIEN
Preferred name	BETA LACTAMASE AmpC	Beta lactamase AmpC
Organism	*Escherichia coli*	*Escherichia coli*
Protein target classification	Enzyme hydrolase	Enzyme hydrolase
UniProt Accession	P00811	P00811

Table 8: ChemProt

Compound 7			Compound 8
Protein	AmpC Beta lactamase		AmpC Beta lactamase
UniProt ID	P00811		P00811
Organism	*Escherichia coli*		*Escherichia coli*
ChEMBL similar compounds	1318563	0262871	0079589
	1442461	1489884	1513688
	1528866	1552050	0396647
	1441268	1434845	1300699
	0405886	1436305	
	0258538	1394844	
	1591906	1396011	
	1611273	1395084	
	0428611	0260855	

Table 9: Docking results of compound 7 and beta lactamase AmpC

Compound	Protein		Amino acids involved in Chain A	Drug atom	Distance (Å)
7	Beta lactamase AmpC	Chain A	LYS315	O23	3.048
			ALA318	C12	3.412
			TYR150	C21	3.515
			TYR150	C19	3.715
		Chain B	THR262	H30	2.078
			THR262	H30	2.367
			HIS314	O3	2.235
			ARG148	O15	2.504

Table 10: Docking Results of Compound 8 and Beta lactamase AmpC

Compound	Protein		Amino acids involved in Chain A	Drug atom	Distance (Å)
8	Beta lactamase AmpC	Chain A	ALA 318	H17	2.541
			LYS 315	O16	2.655
		Chain B	ILE291	H17	2.299
			ARG148	O3	3.495

Propanoic acid, which exhibits strong antibacterial action, is present in compounds 7 and 8 of the phthalimide derivative series (Nasab *et al*., 2022). The compound of phthalimide and propionic acid was reported as good anti-inflammatory and analgesic agent (Wang and Yang, 2022). A previous study used a phthalimide-containing propanoic amino acid as an anti-HIV agent but was unable to produce any appreciable results for HIV prevention (Gattu *et al*., 2023). However, it is believed that bacterial strains respond best to this mixture of propanoic acid and phthalimide (Gattu *et al*., 2023).

Computational methodology was used to investigate the target of compounds 7 and 8 inside bacteria. Three online servers were used to obtain reports of specific targets of compounds containing propanoic acid based on their chemical similarity to compounds already present in the ChEMBL database. The docking results for compound 7 and beta lactamase AmpC are shown in Table 9**,** and they demonstrate that all of the active amino acids from chain A target protein, including LYS315, ALA318, TYR150, and TYR150, effectively bind to molecules O23, C12, C21, and C19 of compound at distances of 3.048, 3.412, 3.515, and 3.715 Å respectively, while THR262, ARG148, HIS314, and THR262 amino acids of chain B makes distances of 2.367, 2.504, 2.235 and 2.078 Å with compound molecule H30, O15, O3 and H30 respectively. When coupled with AmpC Beta Lactamase, Phthalimide Compound 8 displayed robust hydrogen bonding. Table 10 depicts the amino acid residues ALA318 and LYS315 connecting to drug molecules H17 and O16 at distances of 2.541 and 2.655 Å respectively, while chain B of target protein showed interaction of amino acid ILE291 and ARG148 with molecules H17 and O3 at the distance of 2.299 and 3.495 Å respectively.

For compound 7, Figs. 6 and 8 showed that the hydrogen molecules of the phthalimide compound with the shortest distance are joined by strong hydrogen bonds formed by the amino acids LYS 315, THR262, HIS315 and ARG148. Figs. 10 and 12 for Compound 8 showed that ALA 318, LYS 315 and ARG 148 were involved in creating strong hydrogen bonds with the shortest distances, i.e., below 4 Å to oxygen and hydrogen at various positions, demonstrating the strong bonding of ligand and protein as below or closer than 4 Å (Askarzadeh *et al*., 2022, Matore *et al*., 2023).

According to American Society for Testing and Material Designation standards, when the tested compounds were assessed for their biocompatibility towards human Red Blood Cells, they demonstrated moderate to less hemolysis (Hashem *et al*., 2023). Compounds 6, 7, and 8 failed to meet established criteria for hemolysis with less than 2% hemolysis, indicating that they are non-hemolytic. Compounds 1, 2, 4, and 5 exhibited hemolysis between 2 and 5%, indicating that they are only marginally hemolytic. No compound was hemolytic for human RBCs because none of the compounds displayed hemolysis higher than 5% (Tabassum *et al*., 2022).

CONCLUSION

The goal of this study was to investigate the anti-bacterial properties of newly synthesized compounds of phthalimide derivatives of amino acids in order to find novel therapeutic agents against gram-negative bacterial infections and to suspect its precise target within bacteria using molecular docking. A total of seven out of the eight compounds has the ability to inhibit three gram-negative bacterial strains. According to the study, minor adjustments are required to make these substances more efficient at lower concentrations in order to increase their antibacterial potential. Finding out its *in vivo* inhibition activity against AmpC beta lactamase, a potential target within the bacteria examined in this research, is crucial for overcoming the issue of drug resistance.

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