EFFECT OF METHYLENE BLUE, EOSIN METHYLENE BLUE AGAR, AND COMBINATIONAL ANTIBIOTIC ON CARBAPENEM RESISTANCE OF *ACINETOBACTER BAUMANNII* VIA IN VITRO AND IN SILICO APPROACH

ASHRAF, A.[1] – ZAHRA, N.[1] – SADIQQUI, F.[1] – ZEESHAN, B.[2] – SARWAR, A.[3] – ULLAH, N.[3] – AZIZ, T.[4*] – ALHARBI, M.[5] – ALSHAMMARI, A.[5] – ALASMARI, A. F.[5]

[1]*Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan*

[2]*Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, 90905 Sandakan, Sabah, Malaysia*

[3]*Food & Biotechnology Research Lab, PCSIR Complex, Lahore, Pakistan*

[4]*Department of Agriculture, University of Ioannina, Arta, Greece*

[5]*Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*

**Corresponding author*
e-mail: iwockd@gmail.com

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Abstract. *Acinetobacter baumannii* (AB) is a gram-negative bacterium and a main source of nosocomial infection that mainly affect immunocompromised individuals. Methylene blue (MB) is commonly used for staining in microbiology but in this article MB is used as resisting agent against AB. The main aim of this study is to find the most possible cure against *A. baunannii*. Isolated strains of *A. baumannii* were sub-cultured on Muller-Hinton agar (MHA) containing MB and sub-cultured on eosin methylene blue (EMB). Then antibiotic susceptibility testing (AST) and minimum inhibitory concentration (MIC) were determined. The *A. baumannii* isolates were highly inhibited by EMB medium but showed growth on MHA plates containing MB. The results infer that *A. baumannii* shows sensitivity against Meropenem. In-vitro studied effect of methylene blue shows inhibition against carbapenem resistance *A. baumannii* growth was also inhibited by EMB agar. AB strains showed sensitivity against synergistic combinational drugs (Meropenem + Amikacin), (Meropenem + Ciprofloxacin) (Imipenem + Tobramycin), these strains showed resistance against additive combinational drugs (Meropenem + Cefepime) and (Imipenem + Tobramycin), (Ciprofloxacin), (Cefepime) as compared with individually.
Keywords: *Acinetobacter baumannii (AB), carbapenem resistance (CR), combinational antibiotic, methylene blue, eosin methylene, blue agar*

Introduction

The rise of carbapenem refusal in *Acinetobacter baumannii* leads to difficulty in the therapeutic management of infections (Ramirez et al., 2020: Na et al., 2021). *Acinetobactor baumannii* is an oxidase-negative bacterium. Also, it is gram-negative, coccobacillus, non-motile, aerobic, catalase positive and multidrug-resistant. *A. baumannii* is a hospital-associated bacterium that can cause multiple infections. It mostly causes infection in immune-compromised persons such as ICU patients (Kim et al., 2020; Nureen et al 2023). This bacterium is frequently isolated from the hospital environment and hospital patients. This bacterium is cultured from the wounds, sputum, urine, and respiratory secretion. *A. baumannii* can be alive for a long period that is why it can spread easily. According to a study this bacterium can survive on a dry surface for

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about five months. This multidrug-resistant bacterium can cause multiple serious infections like bloodstream infection, nosocomial infection, urinary tract infection, bacteremia, pneumonia, septicemia, meningitis, and also infection in the surgical wound site. This organism directly targets immunocompromised individuals (Wang Y et al., 2023; Loose et al., 2019; Morris et al., 2019).

For the last 20 years, *A. baumannii* is one of the most trouble-causing pathogens all over the world in healthcare institutions, and it is all because of its exceptional capability of resistance and upgrading that is why new *A. baumannii* and its strains are resistant to all types of antibiotics. From 1991 to still now the biggest challenge for scientists of health institutes is to control its high antibiotic resistance. Scientists are trying to resolve this problem because *A. baumannii* is becoming more serious day after day. The main thing is *A. baumannii* have efflux pump genes which help this to resistance against antibiotics and because of this resistance against all type of antibiotics. This pathogen gets a lot of attention globally. One of the last therapeutic options is colistin used for ventilator-associated patients but now resistance of this bacteria against colistin is also described. Nowadays this multidrug-resistant pathogen has become a serious concern among the medical fraternity. Information from the WHO antimicrobial resistance is one of the major issues that physical health facing (Elham and Fawzia, 2019; Son et al., 2020).

This organism *Acinetobacter baumannii* and some of those who belong to this genus are present in nature we can recover them from water surface samples, soil, and usually from all types of environments (Ayenew et al., 2021). Not all organisms that belong to the *Acinetobacter baumannii* genus are present in the natural environment but most of them are. As this pathogen *A. baumannii* like moist habitat it directly targets the wet part of the immunocompromised patient body like target moist tissues, respiratory tract, mucous membrane, any injured part of the body, the part of the skin that disrupter it mostly attacks these areas if the injured part is left untreated it will cause septicemia and cause death (Chai et al., 2023; Wang H et al., 2023; Peng et al., 2022; Qin et al., 2022; Dabholkar et al., 2021: Wei et al., 2018; Lee et al., 2017)

Infections that are mostly this bacterium causes pneumonia, bacteremia, lungs infection, meningitis, bloodstream infection, respiratory tract infection, patients with open wound surgery, patients with a long period stay at the hospital, cause infection in intensive care unit patients (Nureen et al., 2023: Vázquez-López et al., 2020). *A. baumannii* is also involved in the cause of skin infections, and soft tissue infection, it most commonly causes infection in countries with a hot and humid climate. These infections also occur in persons who are heavy smokers or drink alcohol in an excessive amount. The antimicrobial-resistant pathogens are named ESKAPE pathogens from all these Carbapenem resistance bacterias *A. baumannii* is the number one threatening pathogen according to the World Health Organization (Said et al., 2021; Rangel et al., 2021). After being resistant to all types of antibiotics, this bacterial infection becomes untreatable. It has many different species according to the taxonomy species like *A. baumannii, A. nosocomiallis, A. pittii,* etc. these three are the most common hospital isolated species. In the beginning, these bacteria affect Europe, America on a large scale but now it causes trouble all over the globe. Many scientists work on this bacterium to find out the proper treatment for the infection it causes. They wrote many articles on different species of *A. baumannii*. the extent of imipenem resistance of *Acinetobacter baumannii* raised from 31.0% in 2005 to 70.7% in 2017 in China (Ning et al., 2017). A study showed that the patients with carbapenem resistance *A. baumannii* infection had a markedly greater risk of mortality correlated to the individuals

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with the carbapenem immune *A. baumannii* infection usual Gram-negative bacteria involved family of *Pseudomonas aeruginosa. Acinetobacter baumanii* is correlated with record fatality within the ICU owing to its suppressed multiple drug resistant quality. MDR bacteria are generally recognized in HAI and are correlated with notable fatality (Naveed et al., 2023; Uppalapati et al., 2020: Abouelfetouh et al., 2019). A research notice that about twenty percent of all notified infectious agent show multiple drug-resistant guides. known infectious agent along with carbapenem opposed the species of *Acinetobacter* drug-resistant *Pseudomonas* and various *aeruginosa*. HAI hit lots of patients all over the globe, leading to raised fatality and economic effects on medical management systems (Ayenew et al., 2021). At the same time present international duty of health-related germs is still undiscovered because of the absence of good information and observation systems (Tosato et al., 2020). The tale of the family *Acinetobacter* comes first to the beginning 20th century. when a Dutch microbiologist, Beijerinck, in 1911 reported a microbe which was isolated from soil named *Micrococcus calco-aceticus.* Which contains calcium. Related microbes were named fifteen different species, *Moraxellalwoffi, Bacteriumanitratum, Moraxell lwoffi* ver etc. In the Systematic Bacteriology 1974 edition, the *Acinetobacter* was placed, as well as information of a particular type, *Acinetobacter calcoaceticus.* In the "confirmed List of Bacterial Names," 2 separate species, *A. lwoffii* and *A. calcoaceticus*, were covered, based on the conclusion that some *acinetobacters* were capable of pH changing glucose (peleg et al., 2007). The primary goal of this study is to find the most possible cure against *A. baunannii*.

Methodology

Sample collection

Samples of *Acinetobacter baumannii* were collected directly from the Pathology lab of The University of Lahore Teaching Hospital by using Clinical and Laboratory Standards Institute (CLSI) laboratory protocols.

Preparation of nutrient media

For all the strains culturing Nutrient Agar Media were prepared. The media were prepared 13.2 g Nutrient agar/386.8 ml distilled water. Then autoclaved under standard conditions of pressure, time and temperature which is 250℉, time 30-60 min. After autoclave, media was poured in sterilized glass petri dishes then allowed to solidify. Plates were prepared after solidification. Bacterial samples were applied by Streak Plate method on prepared plates of Nutrient agar media. The plates were then incubated for 48 h at 37°C in an incubator. The colony morphology was observed after 48 h.

Isolation of samples

Names and date label on the plates. Use a sterilized loop for striking, refer to an aseptic protocol procedure. Sterilize your loop and drug the loop into the samples. Touch your loop to the agar surface on petri plates and apply the striking method. Repeat the same procedure the second time. Keep those plates in the incubator and leave them for 48 h. After 48 h check those plates and observe the growth of bacteria. Also the bacteria were identified at the species level by using biochemical testing method. WHO guidelines were followed during performing whole procedure.

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Growth of Acinetobacter baumannii on MB and EMB media

Methylene Blue (MB) and Eosin Methylene Blue (EMB) Agar were prepared for checking the antimicrobial activity against *Acinetobacter baumannii.* The media were prepared and autoclaved under standard Temperature and pressure. Then media were poured in petri dishes and leaved for solidification. Bacterial strains applied on culture plats by streak plate method and placed in incubator at 37°C for 48 h. After 48 h growth were observed. *Acinetobacter baumannii* shows growth on MB but on EMB no growth observed even after 72 h incubation.

MHA broth preparation

Mueller-Hinton agar (MHA) 5.32 g dissolved in 134.68 *ml* distilled water then autoclaved at standard temperature 121°C for 60 min. Then MHA broth poured in glass test tubes for inoculation of bacteria. After inoculation of bacteria broth tubes placed in incubator for 24 h. Next day turbidity was checked.

Preparation of discs

Discs were made by using Whattman filter paper, for performing AST. After making discs sterilized them in pre heated oven at 160°C.

Antibiotic susceptibility testing (AST)

Antibiotic susceptibility test is a method used to check resistance of antibiotics. Multiple methods use for AST, the method I used here is disc diffusion. MHA media were prepared for AST. After preparing media plates, the Lawn culture method which were used to inoculate MHA plats with the help of cotton swabs. After swabbing very carefully to cover whole plate, antimicrobial discs applied manually on inoculated MHA plates. Carefully placed them in incubation at 37°C for 24 h. Next day zones of inhibition were observed.

AST of Meropenem on A. baumannii culture

Meropenem antibiotic were applied to check the antimicrobial effect on *Acinetobacter baumannii* by using MHA medium.

AST of synergistic and additive drugs

Combinational drugs (Meropenem + Amikacin) and (Meropenem + Ciprofloxacin) Both synergistic drugs were used to check the effect of against bacterial culture. In additive drugs the combination which were used are (Meropenem + cefepime) and (Imipenem + Tobramycin). MHA medium used for the process.

Zone of inhibition

The zone of inhibition was measured with the help of ruler in millimeters (mm). Zone measured from the center of the disc to the end area of the zone. Total of the zone was equal to the diameter of the zone.

(In every step the experiments were repeated twice to avoid mistakes and flukes.)

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In-silico analysis

Compounds selection

In in-silico process the very first thing was the selection of compounds. Compounds were selected by the literature study. Different proteins like OmpA, CarO, OXA-24, and OccAB3 were selected by multiple articles study from the UniProtKB. These proteins were selected because they are the main outer membrane protein of organism *Acientobacter baumannii* and helps to become resistance against antibiotics.

Ligand formation

These four proteins OmpA, CarO, OXA-24 and OccAB3 were chosen on the basis of their function as an OM protein. Their SDF files were transferred into computer from PubChem database. Then after that 2D structure was sketched on Chemsketch, and then in PBD file format saved. Their structures were prepared with the help of PyMol. PyMOL software was used for 3D visualization of protein ultrastructure.

Binding sites predication

Active site is the area or region which bind with the substrate molecule for making a strong bonding. For finding of active site depth residue was used (http://cospi.iiserpune.ac.in/depth/).

Grid box generation.

Grid generation is as every ligand have specific binding sites with the proteins where ligand binds. For all ligand specific amino acids were selected that were required for the grid generation. Grid a square shaped box that consist on some specific amino acids and ligand. Active sites of receptor were surrounding by cubic box for docking process.

Protein ligand docking

After the preparation of all the proteins and ligand the docking procedure was done by using PyRx. Single ligand which was carbapenem was run for all four proteins. Their binding energies and patterns were observed, and their graphs were also produced.

Results

Invitro effect of methylene blue and eosin methylene blue agar on carbapenem and combinational drug resistance *Acinetobacter baumannii* research work was done in the Department of "Institute of Molecular Biology and Biotechnology". About a hundred samples were collected and observed in microbiology lab. All activities and results were done and observed on time (*Figs. 1* and *2; Tables 1, 2 and 3*). In *Table 1* the serial no. shows the sequence or increases incrementally with each row. And the codes mean the id no. from samples.

Assessment of protein structure

One of *Acinetobacter baumannii's* outer membrane proteins is OccAB3. This outer membrane protein helps bacteria to be resistant to antibiotics. This protein has a

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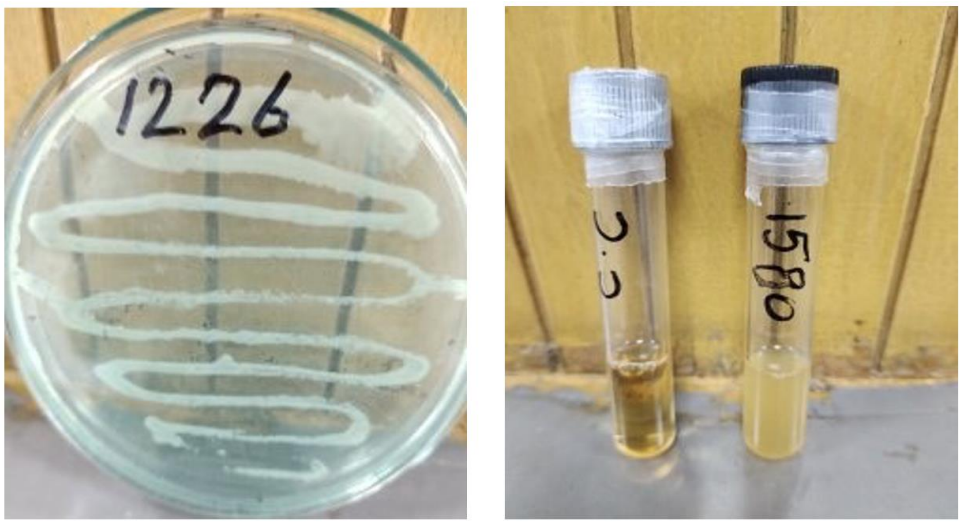
Molecular Mass 50.62 kDa. The Ramachandran Plot and the values of OccAB3 show that 94.25% of protein amino acids were present in the preferred region, and in the allowed region the residues were 4.75%. OmpA (outer membrane protein A) is the main membrane protein. Helps to mediate bacterial biofilm formation, play a roll in antibiotic resistance. Its Molecular rang is about 28 kDa to 36 kDa. OmpA is belongs to the family surface-exposed proteins. The Ramachandran plot and the value of OmpA indicated the 98.32% of protein amino acids were in the preferred region and 1.68% of residues were present in allowed region (*Fig. 3*).

Table 1. *Collected samples observation*

Serial No.	ID Number	Sample observation
1	29245	Pus
2	1226	Pus
3	1330	Pus
4	1236	Pus
5	11327	Pus
6	17306	Pus
7	30895	Pus
8	288	Pus
9	8245	Pus
10	10245	Pus
11	1167	Pus
12	10140	Pus
13	1220	Pus
14	25324	Pus
15	1251	Pus
16	1381	Pus
17	24112	Pus
18	1580	Pus
19	1592	Pus



(A) **(B)**

Figure 1. *(A) Growth on MB and (B) MHA broth*

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Table 2. *AST results of Meropenem drug on the growth of Acinetobacter baumannii*

Sr. No.	ID Number	Antibiotics	Working	Zone of inhibition	Susceptible	Zone of inhibition
1	29245	Meropenem	25 mm	Sensitive	19.5 mm	Resistant
2	1226	Meropenem	35.5 mm	Sensitive	26.5 mm	Sensitive
3	1330	Meropenem	33 mm	Sensitive	26 mm	Sensitive
4	1236	Meropenem	36 mm	Sensitive	21 mm	Resistant
5	11327	Meropenem	30 mm	Sensitive	27.5 mm	Sensitive
6	17306	Meropenem	30 mm	Sensitive	23 mm	Sensitive
7	30895	Meropenem	38.5 mm	Sensitive	35.5 mm	Sensitive
8	288	Meropenem	34.5 mm	Sensitive	25.5 mm	Sensitive
9	8245	Meropenem	31.5 mm	Sensitive	15 mm	Resistant
10	10245	Meropenem	37.5 mm	Sensitive	27.5 mm	Sensitive
11	1167	Meropenem	35.5 mm	Sensitive	30.5 mm	Sensitive
12	10140	Meropenem	37 mm	Sensitive	24.5 mm	Sensitive
13	1220	Meropenem	35 mm	Sensitive	28.5 mm	Sensitive
14	25324	Meropenem	36.5 mm	Sensitive	25 mm	Sensitive
15	1251	Meropenem	40 mm	Sensitive	29.5 mm	Sensitive
16	1381	Meropenem	33.5 mm	Sensitive	24 mm	Sensitive
17	24112	Meropenem	36 mm	Sensitive	24 mm	Sensitive
18	1580	Meropenem	35 mm	Sensitive	29 mm	Sensitive
19	1592	Meropenem	15 mm	Resistant	15.5 mm	Resistant

Table 3. *AST results of combination drug on the growth of Acinetobacter baumannii*

Sr. No	ID Number	Antibiotics	Zone of inhibition	Sensitive	Resistance
1	29245	Meropenem	25	S	
		Amikacin	23	S	
		Amikacin	20	S	
2	1226	Meropenem	35.5	S	
		Amikacin	29.5	S	
		Meropenem + Amikacin	29	S	
3	1330	Meropenem	33	S	
		Amikacin	28.5	S	
		Meropenem + Amikacin	27	S	
4	1236	Meropenem	36	S	
		Amikacin	30	S	
		Meropenem + Amikacin	24	S	
5	11327	Meropenem	30	S	
		Amikacin	27	S	
		Meropenem + Amikacin	26	S	
6	17306	Meropenem	30	S	
		Amikacin	29.5	S	
		Meropenem + Amikacin	24.5	S	
7	30895	Meropenem	38.5	S	
		Amikacin	31.5	S	
		Meropenem + Amikacin	27	S	
8	288	Meropenem	34.5	S	
		Amikacin	29	S	
		Meropenem + Amikacin	28.5	S	

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Sr. No	ID Number	Antibiotics	Zone of inhibition	Sensitive	Resistance
9	8245	Meropenem	31.5	S	
		Amikacin	32	S	
		Meropenem + Amikacin	23	S	
10	10245	Mero-penem	37.5	S	
		Amikacin	28.5	S	
		Meropenem + Amikacin	29.5	S	
11	1167	Meropenem	35.5	S	
		Amikacin	30	S	
		Meropenem + Amikacin	31	S	
12	10140	Meropenem	37	S	
		Amikacin	33	S	
		Meropenem + Amikacin	30	S	
13	1220	Meropenem	35	S	
		Amikacin	26.5	S	
		Meropenem + Amikacin	26	S	
14	25324	Meropenem	36.5	S	
		Amikacin	36	S	
		Meropenem + Amikacin	35	S	
15	1251	Meropenem	40	S	
		Amikacian	34.5	S	
		Meropenem + Amikacin	32	S	
16	1381	Meropenem	33.5	S	
		Amikacin	29	S	
		Meropenem + Amikacin	28	S	
17	24112	Meropenem	36	S	
		Amikacin	27	S	
		Meropenem + Amikacin	25	S	
18	1580	Meropenem	35	S	
		Amikacin	26.5	S	
		Meropenem + Amikacin	26	S	
19	1592	Meropenem	15		R
		Amikacin	0		R
		Meropenem + Amikacin	0		R
1	1167	Meropenem	21.5	S	
		Cefepime	0		R
		Meropenem + Cefepime	15.5		R
2	1226	Meropenem	19.5		R
		Cefepime	0		R
		Meropenem + Cefepime	15		R
3	17306	Meropenem	16		R
		Cefepime	0		R
		Meropenem + Cefepime	11		R
4	1580	Meropenem	23	S	
		Cefepime	14.5		R
		Meropenem + Cefepime	17.5		R

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Sr. No	ID Number	Antibiotics	Zone of inhibition	Sensitive	Resistance
5	11327	Meropenem	22	S	
		Cefepime	0		R
		Meropenem + Cefepime	16.5		R
6	1592	Meropenem	0		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
7	24112	Meropenem	19		R
		Cefepime	0		R
		Meropenem + Cefepime	16.5		R
8	1236	Meropenem	15.5		R
		Cefepime	0		R
		Meropenem + cefepime	0		R
9	30895	Meropenem	15.5		R
		Cefepime	16.5		R
		Meropenem + Cefepime	20		R
10	8245	Meropenem	17.5		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
11	10245	Meropenem	19.5		R
		Cefepime	0		R
		Meropenem + Cefepime	18		R
12	25324	Meropenem	19		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
13	1330	Meropenem	14.5		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
14	1251	Merpenem	19		R
		Cefepime	0		R
		Meropenem + Cefepime	17.5		R
15	1220	Meropenem	20		R
		Cefepime	0		R
		Meropenem + Cefepime	19.5		R
16	1381	Meropenem	17.5		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
17	288	Meropenem	16.5		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
18	29245	Meropenem	16.5		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
19	10140	Meropenem	15.5		R
		Cefepime	0		R
		Meropenem + Cefepime	0		R
1	1381	Imipenum	12		R
		Tobramycin	18.5		R
		Imipenum + Tobramycin	15.5		R

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Sr. No	ID Number	Antibiotics	Zone of inhibition	Sensitive	Resistance
2	11327	Imipenum	10		R
		Tobramycin	16.5		R
		Imipenum + Tobramycin	15		R
3	30895	Imipenem	0		R
		Tobramycin	22.5	S	
		Imipenem + Tobramycin	19.5	S	
4	1167	Imipenem	0		R
		Tobramycin	20	S	
		Imipenem + Tobramycin	15.5		R
5	1236	Imipenem	0		R
		Tobramycin	19	S	
		Imipenem + Tobramycin	15		R
6	29245	Imipenem	0		R
		Tobramycin	15.5		R
		Imipenem + Tobramycin	13		R
7	24112	Imipenem	10		R
		Tobramycin	21	S	
		Imipenem + Tobramycin	16		R
8	10245	Imipenem	13		R
		Tobramycin	18		R
		Imipenem + Tobramycin	15		R
9	1330	Imipenem	16.5		R
		Tobramycin	31.5	S	
		Imipenem + Tobramycine	25	S	
10	1580	Imipenem	0		R
		Tobramycine	32.5	S	
		Imipenem + Tobramycine	23.5	S	
11	17306	Imipenem	20		R
		Tobramycine	27	S	
		Imipenem + Tobramycine	22.5	S	
12	1226	Imipenem	0		R
		Tobramycine	24	S	
		Imipenem + Tobramycine	19.5	S	
13	1592	Imipenem	17.5		R
		Tobramycin	16.5		R
		Imipenem + Tobramycin	14		R
14	1251	Imipenem	10		R
		Tobramycin	20.5	S	
		Imipenem + Tobramycin	15		R
15	25324	Imipenem	14		R
		Tobramycin	31	S	
		Imipenem + Tobramycin	21	S	
16	1220	Imipenem	10		R
		Tobramycin	15		R
		Imipenem + Tobramycin	15.5		R
17	8245	Imipenem	0		R
		Tobramycin	20.5	S	
		Imipenem + Tobramycin	17		R

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Sr. No	ID Number	Antibiotics	Zone of inhibition	Sensitive	Resistance
18	288	Imipenem	0		R
		Tobramycin	17		R
		Imipenem + Tobramycin	15		R
19	10140	Imipenem	5		R
		Tobramycin	0		R
		Imipenem + Tobramycin	20	S	
1	8245	Meropenem	27.5	S	
		Ciprofloxacin	35	S	
		Meropenem + Ciprofloxacin	37	S	
2	10140	Meropenem	38	S	
		Ciprofloxacin	33.5	S	
		Meropenem + Ciprofloxacin	29.5	S	
3	29245	Meropenem	27	S	
		Ciprofloxacin	27.5	S	
		Meropenem + Ciprofloxacin	31	S	
4	1592	Meropenem	10		R
		Ciprofloxacin	10		R
		Meropenem + Ciprofloxacin	0		R
5	288	Meropenem	34	S	
		Ciprofloxacin	27	S	
		Meropenem + Ciprofloxacin	31.5	S	
6	1236	Meropenem	25	S	
		Ciprofloxacin	23	S	
		Meropenem + Ciprofloxacin	25	S	
7	1330	Meropenem	31.5	S	
		Ciprofloxacin	28	S	
		Meropenem + Ciprofloxacin	26.5	S	
8	1220	Meropenem	35	S	
		Ciprofloxacin	29	S	
		Meropenem + Ciprofloxacin	34.5	S	
9	25324	Meropenem	36.5	S	
		Ciprofloxacin	31.5	S	
		Meropenem + Ciprofloxacin	34	S	
10	1167	Meropenem	37.5	S	
		Ciprofloxacin	27.5	S	
		Meropenem + Ciprofloxacin	39.5	S	
11	10245	Meropenem	21	S	
		Ciprofloxacin	10		R
		Meropenem + Ciprofloxacin	21	S	
12	30895	Meropenem	25.5	S	
		Ciprofloxacin	23.5	S	
		Meropenem + Ciprofloxacin	21.5	S	
13	1580	Meropenem	23	S	
		Ciprofloxacin	20		R
		Meropenem + Ciprofloxacin	24.5	S	
14	24112	Meropenem	26.5	S	
		Ciprofloxacin	22.5	S	
		Meropenem + Ciprofloxacin	25	S	

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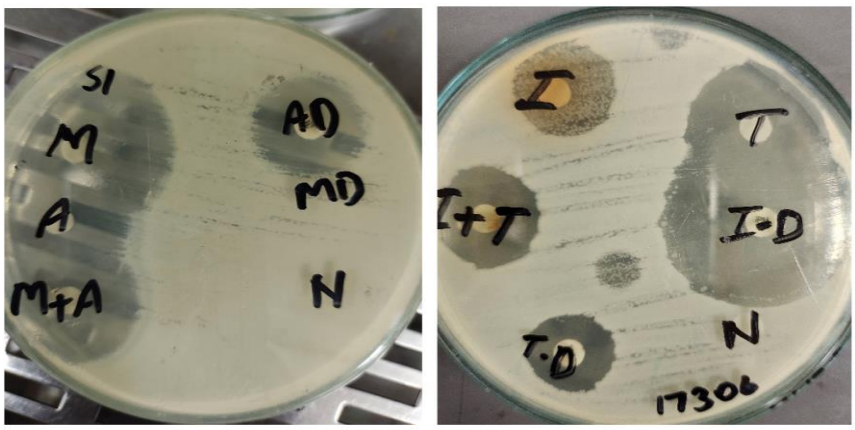
Sr. No	ID Number	Antibiotics	Zone of inhibition	Sensitive	Resistance
15	1381	Meropenem	23	S	
		Ciprofloxacin	19.5		R
		Meropenem + Ciprofloxacin	23.5		
16	17306	Meropenem	21.5	S	
		Ciprofloxacin	20		R
		Meropenem + Ciprofloxacin	22	S	
17	1251	Meropenem	25	S	
		Ciprofloxacin	21.5	S	
		Meropenem + Ciprofloxacin	25.5	S	
18	11327	Meropenem	20		R
		Ciprofloxacin	16		R
		Meropenem + Ciprofloxacin	19.5		R
19	1226	Meropenem	20		R
		Ciprofloxacin	17.5		R
		Meropenem + Ciprofloxacin	22	S	



(A) (B)

Figure 2. *(A) AST result (B) AST results*

OXA-24 is also an outer membrane protein. The molecular rang is 28.11 kDa it is a multi-domain protein (alpha and beta). This OM protein present in *A. baumannii* bacteria. The Ramachandran plot and the values of OXA-24 indicate the 93.80% of protein amino acids were present in preferred region and 4.55% residues were present in allowed region. CarO is a membrane protein of *Acinetobacter baumannii* having crystalline structure. The molecular range is 56.14 kDa. This protein have 2 chains A and B. The Ramachandran plot and the values of CarO indicated the 96.50% of protein amino acids were lied in preferred region and 2.50% of residues were in allowed region.

Active sites of proteins

OccAB3

The active site of OccAB3 having 9 amino acids those are Phe 280, Gly 283, Thr 284, Ser 286, Pro 287, Asp 291, Phe 292, Met 293, Asp 296. These amino acids have probability higher than 0.2 to 0.6 (*Fig. 4*).

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OXA-24

The binding site of OXA-24 contained 6 amino acids which are Ser 81, Ser 128, Tyr 133, Thr 175, Glu 179, Thr 197. All these selected amino acids have probability greater than 0.4 (*Fig. 5*).

CarO

The binding site of CarO consist of 6 amino acids i.e. Lys 203, Tyr 214, Trp 216, Lys 221, Tyr 227, Phe 226. All these amino acids were selected for active site those having the probability higher than 0.2 to 0.3 (*Fig. 6*).

OmpA

The binding pocket of OmpA having 8 amino acids which are Leu 222, Met 228, Arg 231, Ser 239, Lys 251, Thr 270, Leu 278, Ser 283. All these selected amino acids have probability greater than 0.4 (*Fig. 7*).

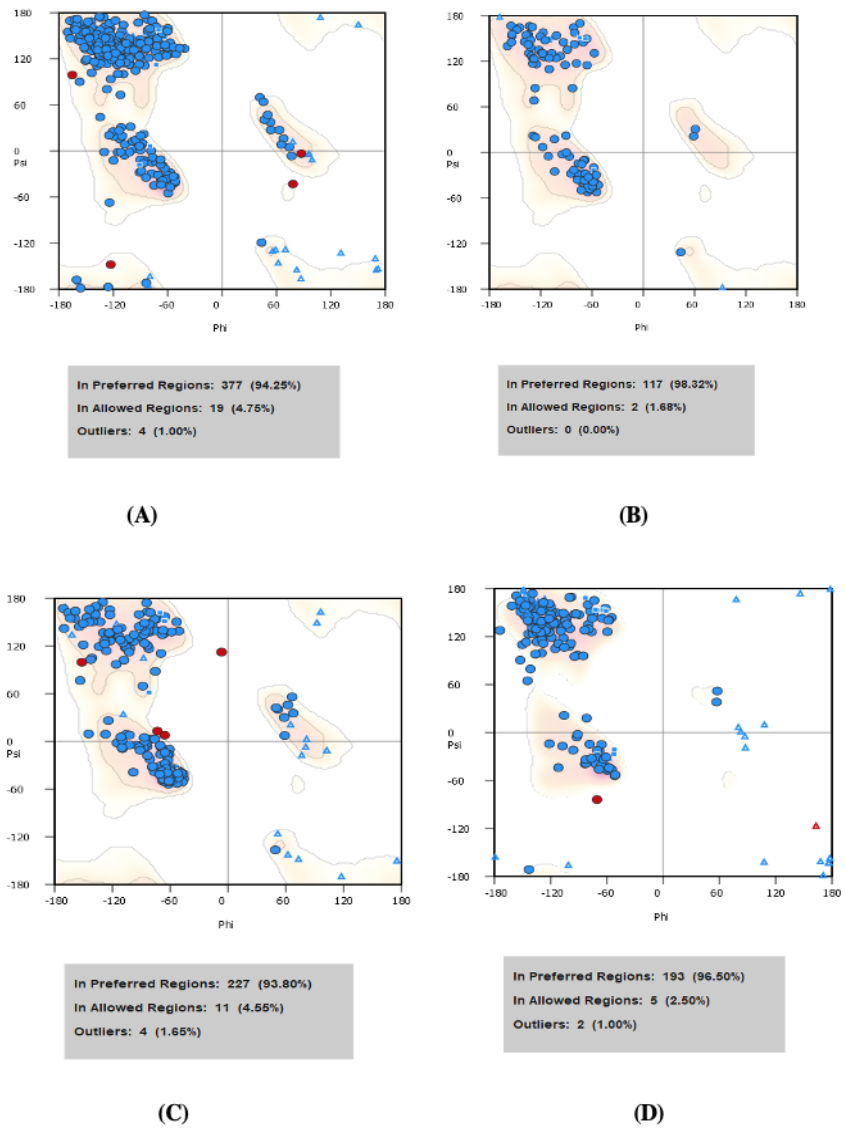

Figure 3. *Ramachandran Plot (A) OccAB3, (B) OmpA, (C) OXA-24, (D) CarO*

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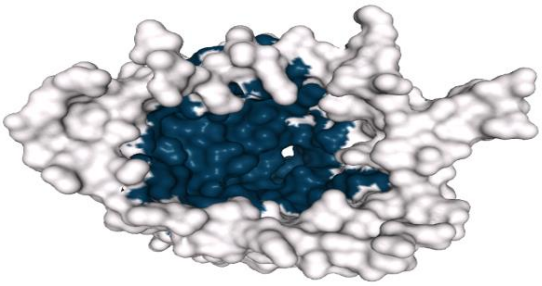
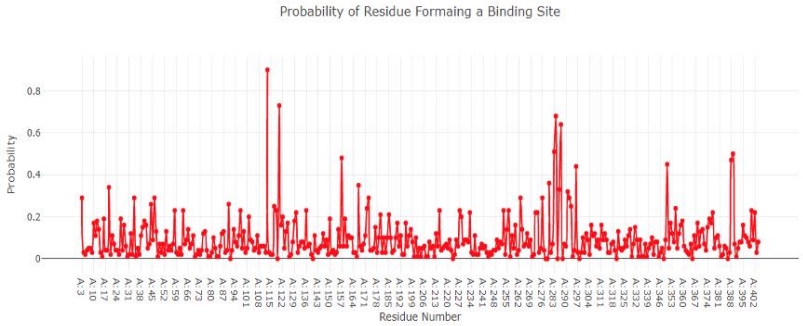
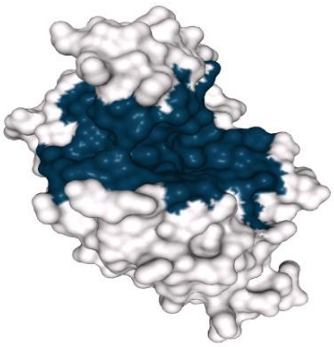


(A)

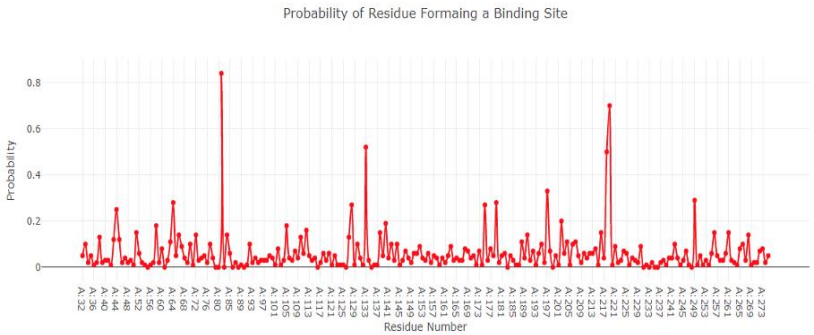


(B)

Figure 4. *(A) Represents the surface of the binding site of OccAB3 obtained from depth residue. (B) Displays the probability if amino acids forming a binding site*



(A)



(B)

Figure 5. *(A) Represents the surface of the binding site of OXA-24 obtained from DEPTH residue. (B) Displays the probability if amino acids forming a binding site*

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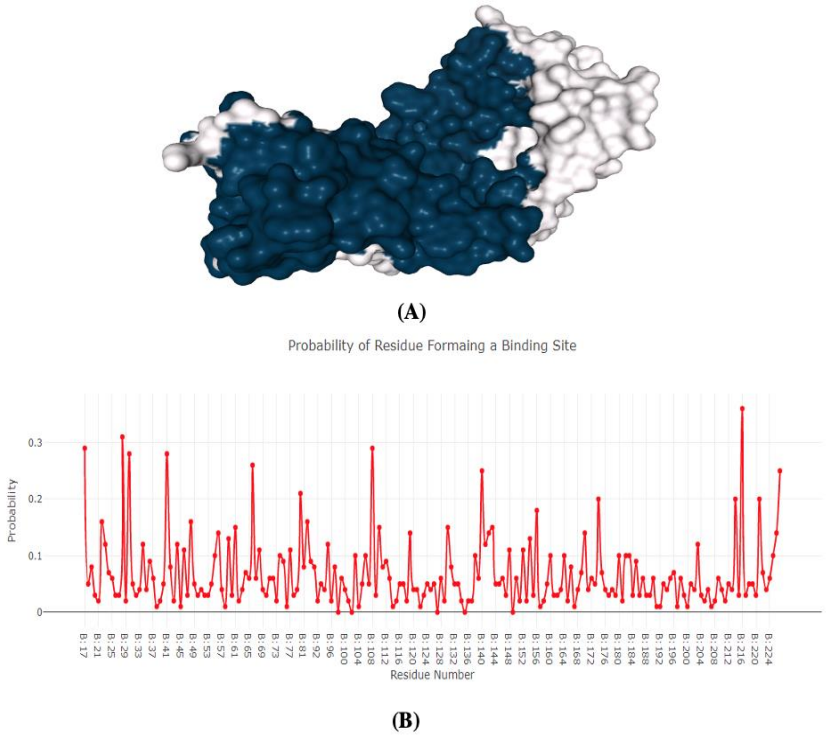

Figure 6. *(A) Represents the surface of the binding site of CarO obtained from depth residue. (B) Displays the probability if amino acids forming a binding site*

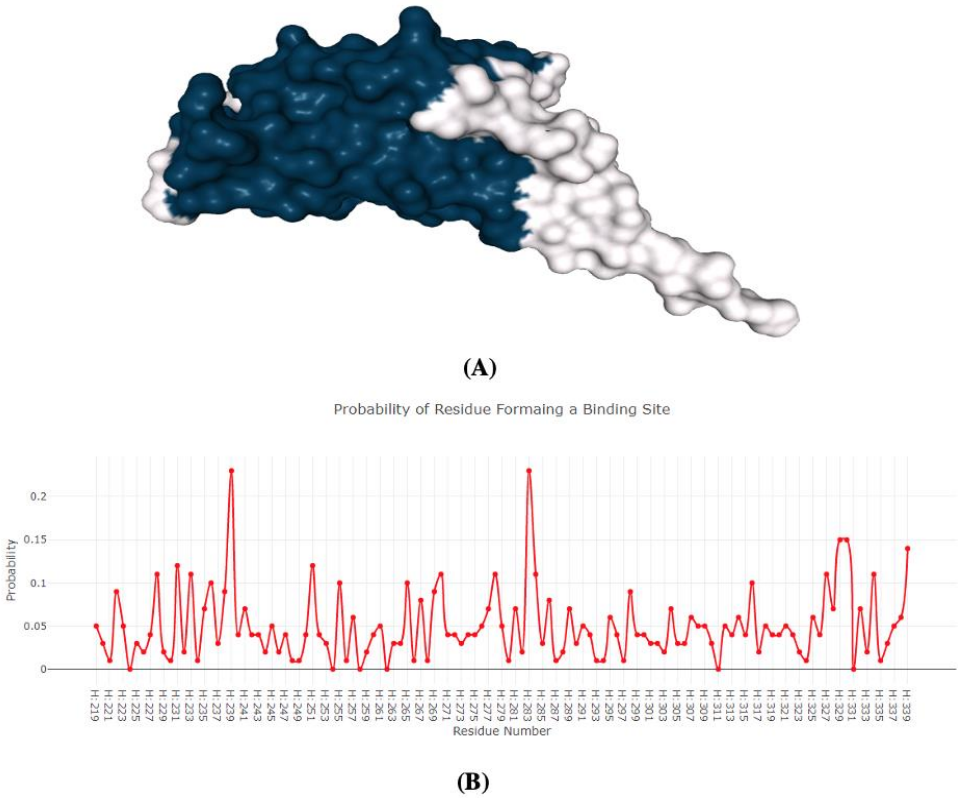

Figure 7. *(A) Represents the surface of the binding site of OmpA obtained from depth residue. (B) Displays the probability if amino acids forming a binding site*

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Binding site and ligands

Examination of active sites shows that the ligand was confined in the binding site of the targeted protein. Docked ligand structure was superimposed to make its binding perfect with the targeted protein. Binding interaction of ligand was shown in the results (*Fig. 8* and *Fig 9; Table 4*).

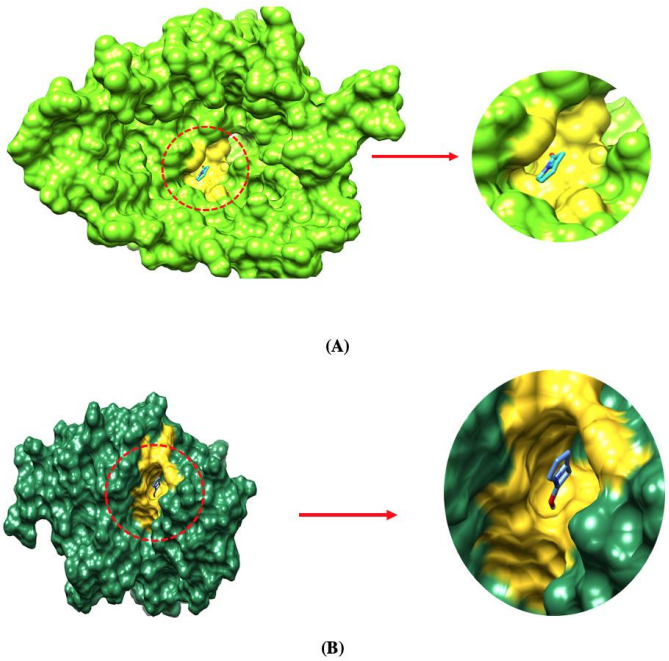

Figure 8. *(A) carbapenem in the binding site of OccAB3. (B) Carbapenem in the binding site of OXA24*

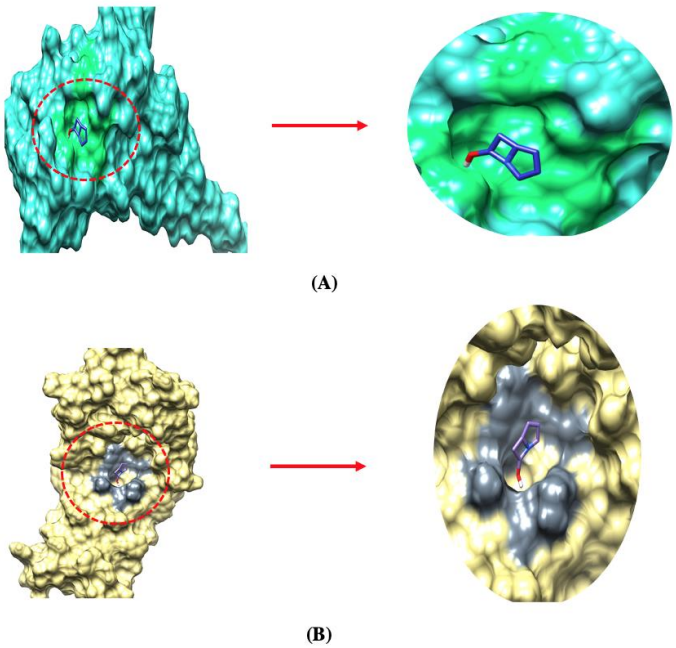

Figure 9. *(A) carbapenem in the binding site of OmpA. (B) Carbapenem in the binding site of Caro*

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Table 4. *Amino acids involved in binding sites*

Ligand	OccAB3	OXA-24	OmpA	Caro
Carbapenem	Phe280, Gly283, Thr284, Ser286, Pro287, Asp291, Phe292, Met293, Asp296	Ser81, Ser128, Tyr133, Thr175, Glu179, Thr197	Leu222, Met228, Arg231, Ser239, Lys251, Thr270, Leu278, Ser283	Lys203, Tyr214, Trp216, Lys221, Tyr227, Phe226

Hydrogen bonding analysis and docking result with OccAB3

Hydrophobic and hydrogen bonds are used to assess the interaction of bonding of docking. OccAB3 interaction with single H-bond at Pro (287). The red dotted line shows the binding distance in Angstrom (A°) as shown in *Figure 10A.*

Docking result with OXA-24

Single hydrogen bond is showed between Carbapenem and OXA-24 at 219 positions. Dotted line shows the distance as shown in *Figure 10B.*

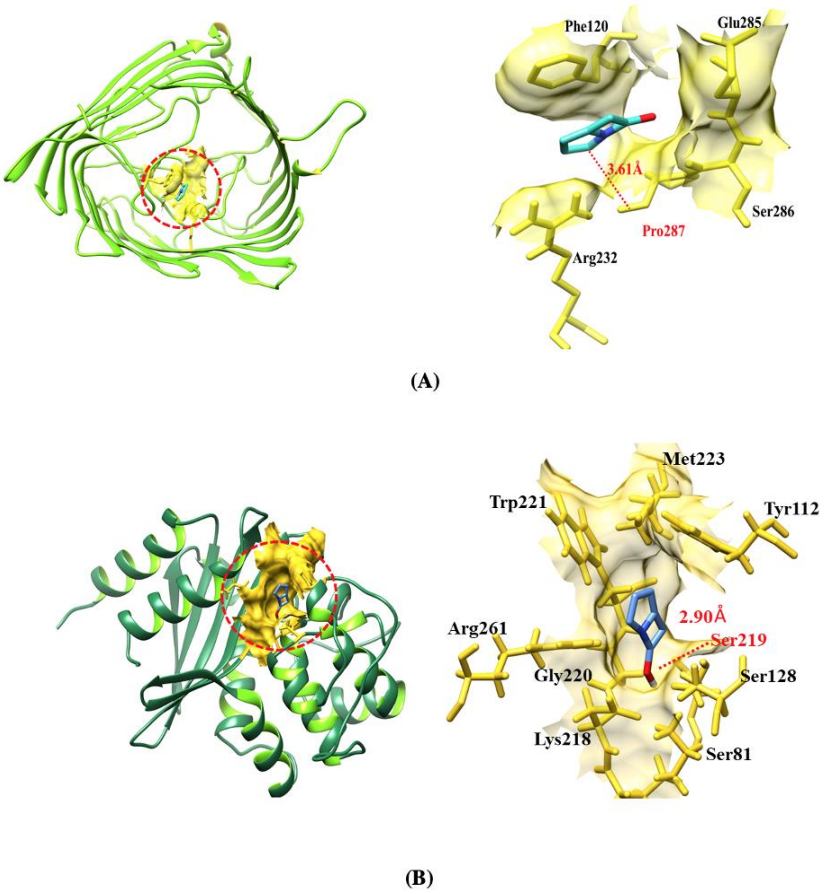

Figure 10. *(A) Docking complex of OccAB3. (B) Docking complex of OXA-24*

Docking result with OmpA

Carbapenem showed two Hydrogen bonds with Arg (329) and Ala (320). Bond distance shows in red dotted line as shown in *Figure 11A.*

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Docking result with Caro

Single hydrogen bond formation between carbaenem and proline at the position 218. The binding distance is in angstrom 1.87A° as shown in *Figure 11B.*

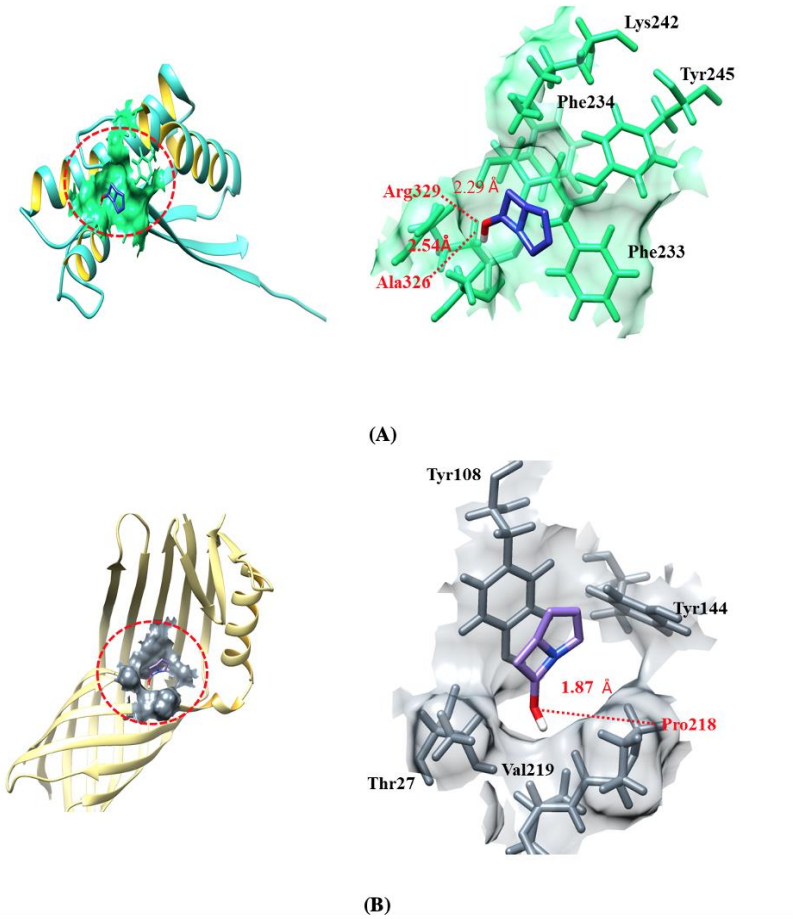

Figure 11. *(A) Docking complex of OmpA. (B) Docking complex of Caro*

Ligands binding energies

Carbapenem was docked to OccAB3 by using PyRx. The docking result of OccAB3 and binding energy was 4.9 Kcal/mol with standard drug carbapenem. The binding score which shows in the graph is -4.3 Kcal/mol. The docking result of carbapenem with OXA-24 shows their binding energy. The standard drug carbapenem was docked to Caro by using PyRx. The docking energy was -3.9 Kcal/mol showed in the binding score. The binding energy results shows the binding score -4.1 Kcal/mol. Carbapenem with OmpA demonstrated their binding energies as shown in *Table 5*.

Table 5. *Ligands binding energies*

Protein	Carbapenem
OccAB3	4.9 Kcal/mol
OXA-24	-4.3 Kcal/mol
CarO	-3.9 Kcal/mol
OmpA	-4.1 Kcal/mol

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Discussion

Methylene blue (MB) is an attractive molecule with exceptional properties mainly used for biomedical applications and used as an effective therapeutic agent to treat anemia, malaria and Barrett's esophagus (Khan et al., 2022; Dao et al., 2020). MB is used in human and veterinary medicine for various diagnostic and therapeutic procedures (Hou et al., 2018). MB was used as synthetic antimalarial for the first time in the late 19th and the early 20th centuries against all types of malaria and it can also act as a chloroquine sensitizer (Lu et al., 2018; Schirmer et al., 2003). Moreover, it is also used for the photodynamic treatment of cancer and alsoas a dye in microbiology labs but in this study first time it is used as a resisting agent against *Acinetobacter baumannii.* MB inhibits the growth of AB strains and found lower MICs results in Carbapenem. The main reason to use MB is to check the ability to be used against carbapenem resistance AB strains. EMB is one of the most used media in microbiology labs. EMB media inhibited the growth and showed no growth on media plates (Uddin et al., 2020).

This study has the main objective which is to find the methylene blue potential used against AB strains as a drug. In comparison with previous study (Lim et al.,2021; Gazel et al., 2019) this article shows that methylene blue has some ability to use against AB. This research was an in-vitro study to confirm the drug ability of MB by using AB strains. And the second point is to check the combinational drugs susceptibility. Both additive and synergistic drug combinations were visualized. In this study, I could not perform the molecular level of the MB effect. Further in the future may be the molecular and genetic examination are required that how much MB susceptibility evolve in AB strains.

Conclusions

In this research, twenty strains of *Acinetobacter baumannii* were demonstrated their results in antimicrobial activity. The results infer that *A. baumannii* shows sensitivity against Meropenem. In-vitro studied effect of methylene blue showed low MIC results against carbapenem resistance *A. baumannii.* The growth of AB was inhibited by EMB agar. On the other hand, AB strains showed sensitivity against synergistic combinational drugs (Meropenem + Amikacin), (Meropenem + Ciprofloxacin), and these same strains gives resistance against additive combinational drugs (Meropenem + Cefepime) and (Imipenem + Tobramycin), (Ciprofloxacin), (Cefepime) as compared with individually.

Conflict of Interest. The authors declare no conflict of interest.

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