In Vitro Inactivation of Antibiotic Resistant Bacteria Using Antibacterial Photodynamic Therapy (aPDT)

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Islamabad

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A thesis submitted in the partial fulfillment of the requirements for the degree of

Master of Philosophy

In

Microbiology

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THESIS & DISSERTATION

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"In the Name of Allah, the Beneficent, the Merciful"

Declaration

The material and information contained in this thesis is my original work that was carried out at the National Institute of Laser and Optronics (NILOP), a college of Pakistan Institute of Engineering and Applied Sciences (PIEAS), Pakistan Atomic Energy Commission (PAEC), Islamabad and Applied, Environmental and Geo-Microbiology Laboratory, Department of Microbiology, Quaid-i- Azam University, Islamabad, Pakistan. I have not previously presented any part of this work elsewhere for any other degree.

Laiq Zada

Dedication

Every challenging work needs self-effort as well as guidance of elders especially those who are very close to our hearts.

My humble effort I dedicate to my sweet and loving.

Father, Mother

And

Sister

Whose affection, love, encouragement, and prayers of day and night make me able to get such success and honor.

Along all the hardworking and respected

Teachers

Certificate

This thesis submitted by **Laiq Zada** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, in partial fulfillment of the requirements for the Degree of Master of Philosophy in Microbiology.

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Dated: 22-05-2023

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List of Abbreviations

$^{1}O2$	Singlet oxygen
AK	Amikacin
ALA	Aminolaevulinic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
APDI	Antimicrobial photodynamic infection
APDT	Antimicrobial photodynamic therapy
ATM	Aztreonam
B.C.	Before Christ
C	Chloramphenicol
CAESAR	Central Asia and Eastern European Surveillance Antimicrobial Resistance
CDC	Center for Disease Control
CFU/ml	Colony forming unit/milliliter
CLSI	Clinical and Laboratory Standard Institute
CLSM	Confocal laser scanning microscope
CN	Gentamicin
COVID-19	Corona virus infectious disease 2019
CRO	Ceftriaxone
CT	Colistin
DNA	Deoxyribonucleic acid
DO	Doxycycline
EARS-Net	European antimicrobial resistance surveillance network
ECDPC	European Centre for Disease Prevention and Control
ESBL	Extended-spectrum beta-lactamases
ESKAPE	*E. Faecium, s. Aureus, k. Pneumonia, a. Baumannii, p. Aeruginosa,* and *enterobacter* species
F	Nitrofurantoin
FDA	Food and Drug Administration

FOT	Fosfomycin
H_2O_2	Hydrogen peroxide
HpD	Hematoporphyrin derivatives
IC	Internal conversion
ISC	Intersystem crossing
KZ	Cefazolin
L	Laser
LEDs	Light Emitting diodes
LMU	Ludwig-maximilians university
LPS	Lipopolysaccharides
LZD	Linezolid
MB	Methylene blue
MDR	Multi-drug resistance
MEM	Meropenem
MHA	Muller Hinton agar
MHT	Magnetic hyperthermia therapy
MM	Millimeter
MRSA	Methicillin resistant *staphylococcus aureus*
MSSA	Methicillin susceptible *staphylococcus aureus*
NA	Nalidixic acid
NAP	National action plan
NILOP	National Institute of laser and Optronics
NIR	Near infrared
OD	Optical density
OX	Oxacillin
PAEC	Pakistan atomic energy commission
PBS	Phosphate buffer saline
PDR	Pan-drug Resistant
PDT	Photodynamic therapy
PIEAS	Pakistan Institute of Engineering and Applied Sciences
PS	Photosensitizer

PTT	Photothermal hyperthermia therapy
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
TB	Toluidine blue
TGC	Tigecycline
UTI	Urinary tract infection
UV	Ultraviolet
WHO	World health organization
XDR	Extensively drug resistant

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Laiq Zada

Abstract

Antimicrobial resistance (AMR) is considered the scourge of this era, and the mortalities associated with infectious diseases induce by multi-drug resistant (MDR) pathogens are high. Keeping AMR in mind, researchers are developing novel antimicrobial photodynamic therapy (aPDT), which has been demonstrated to be efficient against microbes that exhibit resistance to a variety of antimicrobial drugs. This study's objective was to assess methylene blue (MB)-based aPDT's effectiveness in conjugation with a 635nm diode laser (red light), to inactivate MDR- pathogenic *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These MDR strains were exposed to all concentrations of methylene blue without being exposed to light (+MB -L), after that treated with a 635nm diode laser without methylene blue (-MB +L), and then exposed to methylene blue (MB) (15.625, 31.25, 62.5, 125, 250, and 500μg/ml) all concentrations in combination with (18.00, 36.00, 54.00, 72.00, 90.00, 108.00 J/cm^2) 635nm diode laser of 300mW/cm^2 (+MB +L) in sterilized condition (Biosafety cabinet) for 60, 120, 180, 240, 300, and 360 seconds. The inhibition of bacteria was demonstrated by CFU/ml, optical density (OD 600nm), fluorescence spectroscopy, and confocal microscopy (LSM). After irradiation, both bacterial strains were significantly reduced except for MB and diode laser alone. In *P. aeruginosa,* a 39.86% to 100% reduction was reported, and in *S. aureus*, a 49.22% to 100% reduction was reported. The most bactericidal effect was reported at 500, 250, and 125μg/ml for 180, 240, 300, and 360 seconds of exposure with 54.00, 72.00, 90.00, and 108.00 J/cm2 of laser dose at 300 W/cm^2. Antimicrobial photodynamic therapy using methylene blue and 635nm diode laser broadly killed the MDR strains of both grams (positive and negative) bacteria at an equal level (3log and 6log reduction). aPDT could be a promising alternative to conventional antimicrobial therapy, to cope with resistant pathogens in treating bacterial infections.

Chapter 1 Introduction

Antimicrobial or antibiotic resistance (AMR), is globally a public health concern that contributes to the high risk of infectious diseases and death ratio and has a significant economic impact globally, partially caused by the improper use of antimicrobial drugs (Cosgrove, 2006). Antimicrobial resistance presents a new issue for therapeutic medicines or antimicrobial drugs in the period of SARS-CoV-2, especially in the susceptible and frail elderly (Langford et al., 2020). Antibiotics are frequently administered medications in clinical practice, despite the fact that it is believed that approximately 50% of the antibiotics use in hospitals for both indoor and outdoor patients are incorrect, unnecessary, or not as effective as would be ideal for the patients (Pulcini et al., 2007). Every year, a large proportion of people died from treatable and curable diseases prior to the development of antimicrobial resistance as a consequence of inappropriate clinical use of antimicrobial drugs or antibiotics, which promoted the resistance. Antimicrobial-resistant pathogenic bacteria have been on the rise for over a century as a result of inappropriate and excessive antibiotic use. AMR infectious diseases caused by pathogenic bacteria are typically highly challenging to cure as a result of the risk of recurrence and the potential for severe morbidity and death (Anas et al., 2021; Bodie et al., 2019). The World Health Organization (WHO), Geneva, Switzerland, predicted in 2017 that there would be a global antibiotic shortage and the globe would run out of antibiotics since the currently used clinically available antimicrobial drugs were modified to the existing classes of antibiotics, which have demonstrated minimal adverse effects (Aljeldah, 2022). For almost 90 years, antimicrobial drugs have been the major tool used to combat bacterial infections. However, during the past 50 years, the prevalence of AMR has seriously undermined the efficacy of antibiotics, especially the biofilm forming pathogenic bacterial strains, rendering many antibiotics and drugs useless against bacterial infections (Nji et al., 2021).

When bacteria, fungi, viruses, and parasites, among other microbes, are exposed to, survive in, and multiply in antimicrobial drugs that used to affect them, a condition known as "antimicrobial resistance" develops. Antimicrobial resistance is regarded as a

significant public health problem globally, not just in low-income and developing countries (Founou et al., 2017). In 1928, from the byproduct of *Penicillium notatum*, the Alexander Flemings discovered penicillin for the first time, but in the early 1940s, its extensive use was reported. Unfortunately, penicillin resistance was shockingly discovered in 50% of the *Staphylococci* sp. clinical isolates in 1944. Antibiotic resistance (AMR) is becoming a greater concern for humans as a result of widespread antibiotic misuse, which has dramatically increased and propagated the number of resistant microorganisms in the environment. According to the WHO report 2014, antimicrobial resistance (AMR) will be responsible for 10 million deaths per year by 2050. *Staphylococcus aureus* (MRSA) (methicillin-resistant), *Enterococcus faecalis* (vancomycin-resistant), *Mycobacteria* (multidrug-resistant), Gram-negative pathogenic bacteria, and fungi are some of the examples of antibiotic-resistant organisms that are posing an increasing risk to human life. Furthermore, the widespread and excessive use of antibiotics in humans, agriculture, animal husbandry, and industry has been linked to the development of antimicrobial resistance (Harbarth et al., 2015; Maldonado-Carmona et al., 2020; Polat & Kang, 2021). Antimicrobial resistance bacterial pathogens have increased significantly in prevalence during the past ten years, according to the Asian and European epidemiological surveillance networks [the Central Asia and Eastern European Surveillance of Antimicrobial Resistance (CAESAR) and the European Antimicrobial Resistance Surveillance Network (EARS-Net)] ("European Centre for Disease Prevention and Control (2018) Surveillance of Antimicrobial Resistance in Europe Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017," 2018). In April 2014, a study was published by the World Health Organization (WHO) in which they warned the globe about the rapidly coming "post-antibiotic" period, where mortalities will be caused by common diseases and minor injuries. Also in February of 2017, WHO issued a list of deadly pathogenic multi-drug resistance bacteria for which there is an immediate need for novel antimicrobial drug development to emphasize and promote the production of novel antimicrobials and research efforts. This extensive list of superbugs (Gram +ve and -ve) included a group of pathogenic and resistance bacteria, ESKAPE, which contains *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species and is given the status of "high priority" because

they have the ability to show resistance to all kinds of antimicrobials (Asokan et al., 2019; Liu et al., 2015).

According to an estimation, about 25,000 people die in Europe each year from bacterial infections acquired in hospitals. Additionally, around 2 million individuals in the United States get an antimicrobial-resistant bacterial infection every year, and approximately 23,000 deaths occur as the consequences of these bacterial infections, according to the Centers for Disease Control and Prevention (CDC). According to an estimate, antimicrobial resistance is responsible each year for about 700,000 deaths worldwide (Alves et al., 2014; Neill, 2014). Therefore, it has become more crucial than ever to develop new strategies to tackle microorganisms that are resistant to many drugs. So, it is necessary to develop alternate therapies for infectious diseases as a result of the emergence of pathogenic strains that are resistant to antimicrobial treatment. Antimicrobial Photodynamic Therapy (aPDT) is a best approach that could lead to improved antimicrobial therapy (Mahmoudi et al., 2018). Light-based therapeutic alternatives are one of the most promising new antimicrobials (Hutchings et al., 2019).

In ancient times, India, Egypt, and Greece all used light-based therapy as a standard technique for the treatment of skin infections. At the beginning of the 20th century, photodynamic therapy (PDT) was accidently discovered by a germen scientist named "Oscar Raab" in 1900 who documented first the light-based toxicity of acridine red dye on a protist (*Paramecium caudatum*). He also reported that the toxicity was associated with light and later on, the oxygen was also found to be involved in this phenomenon. Moreover, eosin's photodynamic properties, which may be used to treat a variety of skin infections, were also described by Hermann von Tappeiner and A. Jesionek (Youf et al., 2021). Since then, photodynamic therapy has been identified as the application of a photosensitizer (PS) with no toxicity, which is then exposed to the irradiation of light at the correct wavelength for the treatment of the targeted region. The term "photodynamic therapy" was coined by V. Tappeiner, and it was later discovered that oxygen is required for the PDT process in his laboratory (X. Shi et al., 2019). While anti-cancer PDT is a clinical reality for 25 years, The era of antimicrobial photodynamic therapy started in the early 1990s, and the effectiveness of photodynamic therapy as an antimicrobial approach

against antibiotic-resistant diseases and pathogens was first established in healthcare (Mark Wainwright et al., 2017). Antimicrobial photodynamic therapy (aPDT) has been demonstrated to be effective against several common multidrug-resistant bacterial strains, regardless of their antimicrobial resistance rates. Resistance to aPDT has not yet been widely documented, indicating that the risk that bacteria may evolve and evade this therapy is currently low. The development of a more efficient aPDT system is in progress, particularly with the combination of novel chemicals and nanoparticles that make it more efficient and specific. Although APDT shows significant potential for treating skin infections and combating AMR, at this point in its advancement, it is unable to treat systemic infections (Sabino et al., 2020; Youf et al., 2021).

Visible light is used in conjunction with a photosensitizer (PS), oxygen, and other components to perform antimicrobial photodynamic treatment (aPDT). aPDT is mostly performed as a promising substitute approach for local infections, it is safe, easy to use, less expensive, and work in short time (T. G. S. Denis & Hamblin, 2011). The foundation of photodynamic therapy is the interaction of visible light with a photosensitizer chemical that, when exposed to photoactivation energy, is applied to photosensitizers (PSs) that convert the ground-state PSs to an excited singlet state and produce short-lived reactive oxygen species (ROS) on the target site. After the excitation of photosensitizers, through the intersystem crossing (ISC), the PS changes from its singlet state to a triplet state. From there, it either transfers its energy to a nearby molecule to generate singlet oxygen ($^1O2$) or reacts with nearby molecules to generate free radical species ($O2^{\bullet-}$, $OH^{\bullet}$) and hydrogen peroxide (H_2O_2) species of oxygen that can destroy target cells by causing oxidative stress on cell membranes as well as other cellular components (Cieplik et al., 2014). The reactive oxygen species (ROS), superoxide, and other radicals lead to DNA and membrane disruption, and also, in human cells, they induce programmed cell death. The ability of ROS to disrupt cellular components has attracted the attention of scientists to exploit them in diagnostic and treatment approaches. From this point, the concept of antimicrobial PDT evolved (Perni et al., 2021). Antimicrobial photodynamic therapy is specific and is reported to be effective against a variety of microorganisms, including as viral, bacterial, and fungal species. Significant aPDT factors (light, O_2, and PS) must be

adjusted in specific limits to improve activity, including improved the levels of ROS and microbe-killing effects (Polat & Kang, 2021).

In photodynamic therapy (PDT), a specific source of visible light is needed for the excitation of photosensitizers with a specific wavelength of low power. More specifically, red-spectrum light with a wavelength between 600 and 700 nm activates photosensitizers, which allow the light to deeply penetrate from 0.5 to 1.5 cm into tissue (Mahmoudi et al., 2018). For antibacterial photodynamic therapy (aPDT), the following are some light sources that have been applied for the activation of photosensitizers and treatment: UV light, visible light as red, yellow, green, and blue with a wavelength of 600–700, 550–600, 490–550, and 400–490 nm. Also, near-infrared (NIR) with 700–810 nm, light-emitting diodes (LEDs), Xenon lamps, and laser beams have been used (Kashef et al., 2017). For antimicrobial photodynamic therapy (aPDT), a wide range of light sources have been utilized, but due to the deeper penetration into the tissue, light with a longer wavelength is recommended. The surrounded molecular oxygen can be sensitized by various natural or synthetic photosensitizers used in photodynamic therapy (PDT) to produce reactive oxygen species (ROS), which eliminate the microorganisms in their vicinity (Zhuang et al., 2020). In order to have significant penetration into tissue and appropriate triplet state energy for the production of singlet oxygen ($^1O_2$), in the wavelength range of 650–850 nm, an appropriate photosensitizer (PS) is anticipated to have a high absorption. Only a few photosensitizers (PSs) have been clinically approved for human use in conjugation with a specific wavelength of light: for example, methylene blue (MB) and toluidine blue (TB) (Mark Wainwright et al., 2017).

Antibiotics that are commonly used are resistant to *Staphylococcus aureus*, that's why the majority of antimicrobial photodynamic therapy (aPDT) focus on and are primarily directed at gram positive bacteria. According to Paramanantham et al. that approximately 80% biofilm of *Staphylococcus aureus* can reduced by aPDT combined with malachite green photosensitizer (Paramanantham et al., 2019). APDT efficiently inhibits the *Streptococcus mutans* and *Enterococcus faecalis* strains that develop periodontal biofilms and dental infections (Liang et al., 2020). Vancomycin-resistant *E. faecium* infections are common since many strains of *Enterococcus faecalis* are recognized to be ampicillin-

resistant; nevertheless, aPDT may work well to treat these drug-resistant strains of *Enterococcus faecalis* (Woźniak et al., 2021). Gram-negative bacterial strains can also be inhibited by aPDT when used properly. The most frequently researched Gram -ve bacterial target of APDT is *Escherichia coli*. Even in hypoxic conditions, *Pseudomonas aeruginosa* produces severe infections because it is a physiologically adaptable bacterium that can live at both low and normal levels of oxygen. (Fila et al., 2018). According to reports, *Pseudomonas aeruginosa* is significantly killed by curcumin and light. Also, Alam et al. described that the synergism of ampicillin and hypericin with the conjugation of orange light successfully eradicated *Pseudomonas aeruginosa* (Abdulrahman et al., 2020)(S. T. Alam et al., 2019).

Methylene blue is a well-studied broad-spectrum photosensitizer that has been in use against gram positive and negative bacterial species for a long time in photodynamic therapy. MB was the 1st phenothiazinium dye designed in 1870 by Heinrich Caro. MB has best absorption activity between 600-680 nm (red spectrum). The higher wavelength is very useful for penetrating tissues (Felgenträger et al., 2013). MB is a 1st generation photosensitizer used for cancer treatment in the initial time. These PSs are favored in PDT due to their high attachment capacity for a wide range of bacterial isolates, including MSSA, MRSA, *E. coli, pseudomonas aeruginosa* etc. (Vilela et al., 2012). At a high dose, MB has shown antimicrobial activity without exposure to a light source. Moreover, in low concentration, it also has destroyed bacterial and fungal species on exposure to light (Peloi et al., 2008). At normal pH, the positive charge helps the PS locate the membranes. The lipophilic nature of MB supports the dyes distribution in cells and gets into cells easily, also it is less toxic. Based on these properties, these PSs are favored in aPDT in the case of humans (Boltes Cecatto et al., 2020).

The benefits of photodynamic therapy over typical chemical antimicrobial agents are as follows: first, it eliminates a broad range of bacteria; second, It quickly kills microorganisms, usually within a few seconds or minutes.; and third, resistance is improbable to the PDT treatment (Michael Wilson, 2004). It has been noticed that photosensitizers do not transfer or attach to the target cell of interest, and consequently, during photodynamic treatment, it is challenging to bind to the affected tissue. As a

result, researchers have focused on improving drug delivery mechanisms to overcome these limitations. In photodynamic therapy the use of nanotechnology in treatment has opened new avenues, and it is expected to add new features to delivery systems and therapeutic approaches in vivo (Allaker & Memarzadeh, 2014). The main aim of this study was to assess the effectiveness of antimicrobial photodynamic therapy (aPDT) by utilizing the photosensitizer methylene blue in conjugation with red diode laser light (630nm) for the deactivation or elimination of drug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro*.

1.1. Aim

In the current study, the main focus was to inactivate antibiotic-resistant bacteria using antibacterial photodynamic therapy (aPDT) *in vitro*.

1.2. Objectives

2. Confirmation of antibiotic resistant activity of clinically isolated and identified *Pseudomonas aeruginosa* and *Staphylococcus aureus*.
3. Preparation the best concentration of methylene blue and their optimization in conjugation with diode laser at specific time duration and intensity.
4. Investigation the efficiency of diode laser on photobleaching of methylene blue and their biodegradation.
5. Evaluation of the antimicrobial activity of methylene blue and diode laser on *Pseudomonas aeruginosa* and *Staphylococcus aureus* in dark state and after photosensitization.
6. Investigation the efficacy of aPDT by using Optical density, Fluorescence Spectroscopy and Confocal Laser Scanning Microscopy (CLSM).

Chapter 2 Literature Review

2.1. Antimicrobial Resistance

The most significant health challenge currently facing the globe is antimicrobial resistance (AMR) (Holmes et al., 2016). Antimicrobial resistance is alarmingly prevalent everywhere in the globe and increasing the rate of morbidity and the ratio of deaths, as reported by the World Health Organization (WHO) in Geneva, (Switzerland) (*WHO. Antibiotic Resistance (Accessed on 23 February 2022).*, n.d.). Thus, in 2019, 3.57 million mortalities were linked to antimicrobial resistance and were caused by six major multi-drug resistant pathogenic bacteria: *E. coli, S. aureus, K. pneumonia, S. pneumonia, A. baumannii,* and *P. aeruginosa* (C. J. Murray et al., 2022). The World Health Organization (WHO) predicted that by 2050, this figure might increase to 10 million (World Health Organization, 2019). Additionally, the COVID-19 pandemic has made the current worldwide situation worse, mostly because of the inappropriate and excessive use of antimicrobial drugs (Mirzaei et al., 2020). Antimicrobial resistance surveillance, containment, and mitigation were further impacted by SARS-CoV-2 by the limited availability of funding, clinical, nursing, and public health workers (Tomczyk et al., 2021).

It is well understood that microbes, especially bacteria, have been recognized to cause major diseases and mortalities in many human communities all over the globe for decades. Penicillin worked well against bacteria as an antibacterial drug in the 1940s, and as a result, it played a critical role in the treatment of several bacterial infections. Therefore, many bacterial species evolve different mechanisms of resistance to penicillin because its efficacy has been reduced due to misuse and overuse. Consequently, this "wonder medication" was no longer effective against 50% of all clinical *Staphylococci* strains by 1944 (Tenover, 2006). The capability of microbes to withstand and replicate when exposed to antibiotics is known as antimicrobial resistance (AMR) (Zhou et al., 2015). In the health system, the main issue is the resistance of pathogenic bacteria and the efficacy of drugs is hampered by antimicrobial resistant bacteria. So, the probability that

bacteria may evolve more complex resistance to drugs increases with the overuse of antibiotics worldwide (M. M. Alam et al., 2019).

Noncontagious infectious diseases kill more people in lower middle-income countries than in high-income countries because infection rates are higher in LMICs worldwide (C. J. L. Murray et al., 2020; WHO, 2020). Because of the COVID-19 pandemic, the distribution and ranking of microbial infections and their burdens around the world may change. Even though the three most common invasive bacterial infections in children are caused by *S. pneumoniae, H. influenzae,* and *N. meningitidis*. However, in adults, the list of pathogenic bacteria may be more varied (Choi, 2001; JIM O'NEILL, 2016; Kayastha et al., 2020). So, leading causes of severe infectious diseases and death in elders are *Staphylococcus, Klebsiella, Acinetobacter* species, *Pseudomonas, Enterobacter species*, and *E. faecalis* (Boucher et al., 2009). Furthermore, the survival of adults who are infected is now in danger from antimicrobial-resistant bacteria, especially *Staphylococcus aureus* (resistant to methicillin), *Klebsiella pneumoniae*, which generates carbapenemase or ESBL enzymes, *Pseudomonas aeruginosa* (resistant to polymyxin or carbapenem), and *Acinetobacter baumannii* species. (Boucher et al., 2009; Laxminarayan et al., 2016; Sanchez et al., 2013).

Presently, infectious diseases caused by bacterial pathogens are the second major cause of mortality worldwide, the third in developed nations, and the fourth in the USA. In Europe, it is the 3rd leading cause of mortality, affecting especially elderly, and frail people (Vicente et al., 2006). In comparison to the previous time, the antibiotic resistance problem has spread quickly from one territory to another. In some areas of the globe, antimicrobial resistance and pathogenic microbes are endemic. During the last eight decades, AMR has undoubtedly increased as a result of the extensive and widespread use of antibiotics (Dcosta et al., 2011). Alternative therapeutic strategies against resistant bacteria are thus urgently needed. Additionally, these techniques have to be created to stop the development of antibiotic resistance after their usage, for example, by working in accordance with a multi-target mode of action rather than the key-lock approach used by traditional antibiotics (Cieplik et al., 2018).

Many educational institutions in the United States of America as shown in Figure 2.1 are implementing antimicrobial stewardship courses for their professional healthcare students on how to accurately recognize the group of patients who will require antimicrobial treatment in order to receive the optimal option of antimicrobial drugs with the optimum dosage duration to reduce the occurrence of antimicrobial resistance.

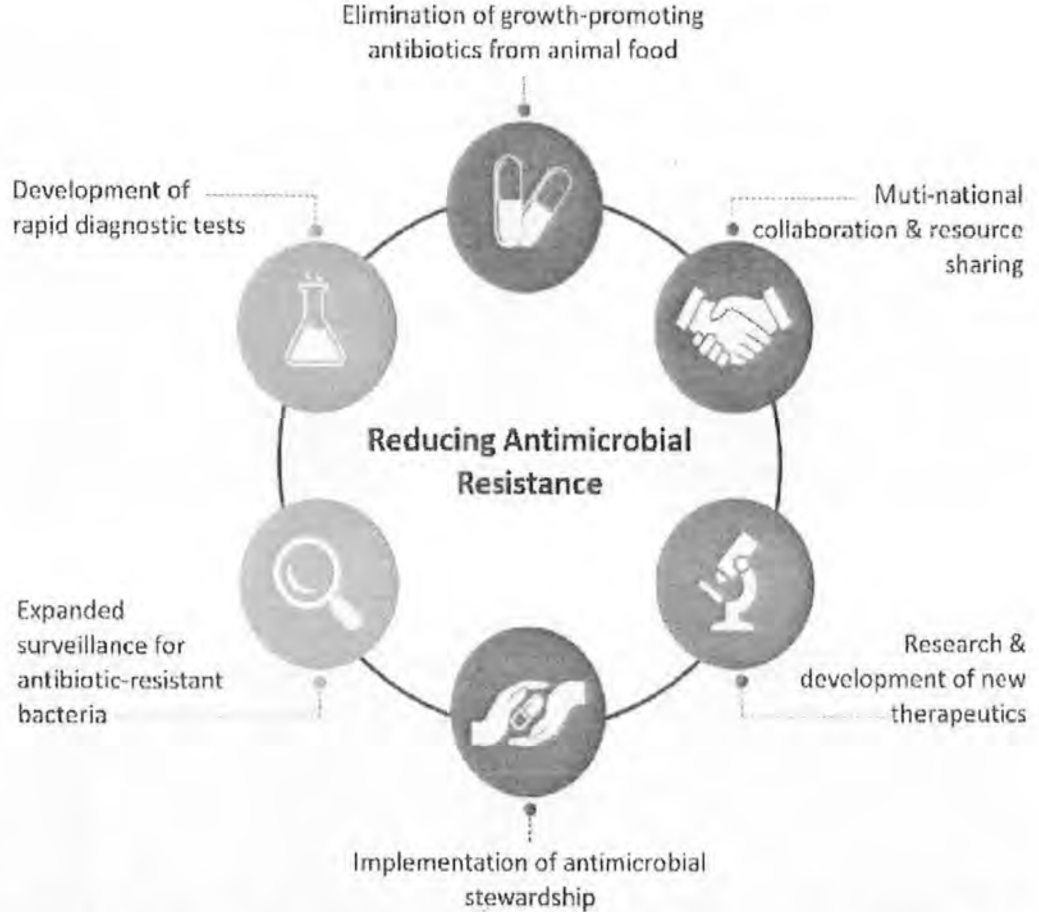

Figure 2-1: Depicts the strategies for reducing antimicrobial resistance (Razzaque, 2021)

2.1.1. History

During World War II, penicillin, an antibiotic initially developed in 1928 by Alexander Fleming, was the first to prove successful in preventing bacterial infection in the military. Unfortunately, the first *S. aureus* strain resistant to penicillin was identified in 1940, prior to the development of penicillin. Methicillin was introduced in 1959 to combat the first penicillinases, but a resistant strain of *Staphylococcus* was discovered to the drug after a

year in 1960 (Sengupta et al., 2013). Vancomycin was first discovered in 1958 to treat methicillin-resistant *Staphylococci*. However, vancomycin-resistant coagulase-negative *Staphylococci* were first identified twenty years later in 1979, and enterococci were discovered to have the same resistance to vancomycin ten years later (Courvalin, 2006), After that, in Japan, the least sensitive *Staphylococcus aureus* strain was identified in 1997 as the vancomycin intermediate *Staphylococcus aureus* (Levine, 2006). Tetracycline was first developed in 1950, and right after nine years, tetracycline resistant *Shigella* strains were first identified in 1959, which is another historical example. Additionally, levofloxacin entered clinical use in 1996, the same year that a *Pneumococcal* strain that was levofloxacin-resistant was first identified (Sengupta et al., 2013). The pharmaceutical industry introduced new antimicrobial drugs in an apparently sufficient amount between 1960 and 1980, a period of twenty years. Unfortunately, the rate of discovering new classes of antimicrobial drugs has declined significantly since the 1980s, until now, when a renewed interest has ignited (Parmar et al., 2018). Bacterial infections caused by antimicrobial resistance or highly antibiotic-resistant pathogenic bacteria are becoming a serious issue in clinical practice around the globe as a result of increasing antimicrobial resistance and the limited availability of novel antimicrobials.

2.2. Types of antimicrobial resistance

Bacterial antimicrobial resistance can be generated in the form of intrinsic, extrinsic, or acquired (Lee, 2019).

2.2.1. Intrinsic Resistance

Intrinsic resistance is the natural or inherent resistance of a bacterial specie to a specific antibiotic or the antibiotic family, because the bacteria have no attachment or target site for specific drugs, and they do not require mutation or the acquisition of genes for resistance. So, it means that antibiotics will never work on that bacterium (Abushaheen et al., 2020; Christaki et al., 2020).

2.2.2. Extrinsic Resistance

Extrinsic resistance is the mechanism in which the bacteria get the resistant gene from other resistant bacteria or the inability of an antimicrobial drug or specific antibiotics to

penetrate or enter because of bacterial outer membrane or cell wall presence or absence. Also, bacteria release enzymes to degrade or inactivate antibiotics (Stefan Schwarz, Axel Cloeckaert, 2006).

2.2.3. Acquired resistance

Acquired resistance is a type of resistance in which the already susceptible bacteria develop resistance due to mutation or by getting the resistant gene from an external source, for example, through horizontal gene transfer. There are three primary methods that can lead to horizontal gene transfer: 1) Transformation 2) Transduction 3) Conjugation as demonstrated in Figure 2.2 (Holmes et al., 2016; Jose M Munita, 2016).

2.2.3.1.Transformation

In the process of transformation, bacteria acquire external naked DNA from the environment or from an already dead bacterium in the environment. Fewer bacteria have the ability to naturally transform (Christaki et al., 2020).

2.2.3.2.Transduction

Transduction is described as a process in which genetic material or external DNA is transferred or introduced from one bacterial cell to another with the help of a vector bacteriophage or virus (Richard P Novick, Gail E Christie, 2010).

2.2.3.3.Conjugation

Conjugation is the most important process of horizontal gene transfer because it involves a donor bacterium physically or directly connecting with a recipient bacterium via the pilus bridge. Through pilus, the donor bacterium transfers DNA or genetic material to the recipient bacterium (Wong et al., 2012).

2.3. Mechanism of Antimicrobial resistance

Replication, survival, and proliferation as quickly as possible are the fundamental goals of microorganisms. Consequentially, bacteria adapt to their environment and perform genetic changes that ensure their survival (MacGowan & Macnaughton, 2017). Furthermore, the genetic changes might occur to make the bacteria resistant to the drugs

and enable them to live if something, like antibacterial drugs, inhibits their capacity to replicate, survive, or proliferate. It's natural for microbes to become resistant to antibiotics (Jose M Munita, 2016).

Furthermore, there are some possible ways that can reduces the potency of antimicrobial drugs as displayed in Figure 2.2 including 1) the production of beta-lactamase enzyme by bacteria for the inactivation and degradation of antimicrobial drugs 2) the modification or alteration in the site for target that have high abhorrence for binding of antimicrobials 3) ejection of antibiotics or efflux pump antibiotics out of the cell 4) reduced or decreased the penetration or uptake of antibiotics to the target (Uddin et al., 2021).

Gram positive bacteria are less likely to employ the mechanisms for regulating the absorption of antimicrobial drugs since there is no outer membrane lipopolysaccharide and efflux pumping process for drug ejection. It is just because of morphological changes in Gram +ve and -ve bacteria, as Gram negative bacteria can use all four antimicrobial resistance mechanisms (Hoffman, 2001).

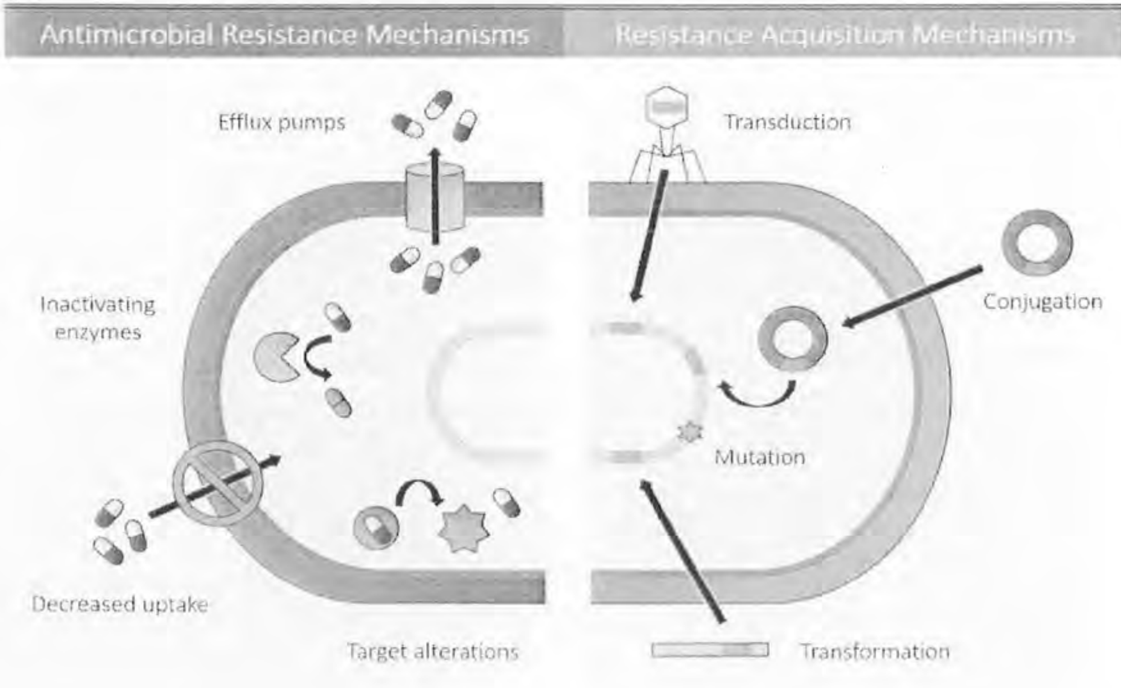

Figure 2-2: Bacterial acquisition and antimicrobial resistance mechanisms (Álvarez-Martínez et al., 2020)

2.4. Antibiotics

Antibiotics actually kill or stop the growth or replication of microbes, enable the immune system of the body to eradicate or eliminate the pathogens. Usually, their mode of action is to stop the synthesis of the bacterial cell wall, block the translation and replication mechanisms to inhibit the protein and DNA synthesis, or disrupt the cell or outer membrane of the bacteria (Levy & Bonnie, 2004). Additionally, antimicrobial drugs attach on outer surface and enter into cell wall of bacteria, and in the ribosomal site, they use energy transport mechanisms that later inhibit the production of protein (Maranan et al., 1997). Antimicrobial drugs have unquestionably saved millions of human and animal lives by combating disease and bacterial infection, and they have been a gift to the human world (Levy, 1992) For the treatment of infectious diseases, different types of antimicrobial drugs have been used over time, In the middle of the twentieth century, antibiotics were considered miracle drugs and there was an optimistic perception at the time that contagious infectious diseases were almost entirely on hold (Aminov, 2010).

Alexander Fleming warned the world about the possibility of resistance to penicillin if used incorrectly (Aminov, 2010). The majority of the new classes of antimicrobial drugs were discovered from the 1950s until the 1970s, and that time was known as the "golden era." As depicted in Figure 2.3 (Davies, 1996). When bacteria become resistant or less susceptible, low or high concentrations of the same drug are recommended for the best efficacy against them. Antibiotic resistance emerged shortly later the implementation of antibiotics into the world (Levy, 1997). Undoubtedly, the discovery of antimicrobials for medicinal use was the most important medical advancement of the 20th century, as illustrated in figure (Katz & Baltz, 2016). Antibiotics make it possible to cure cancer as well as eradicating harmful bacteria and associated infections, perform organ transplantations, and perform open-heart surgery. Nonetheless, some bacterial infections remain incurable due to inappropriate and extensive use of these valuable antimicrobials, which help in resistance establishment tin many bacteria species (Prescott, 2014).

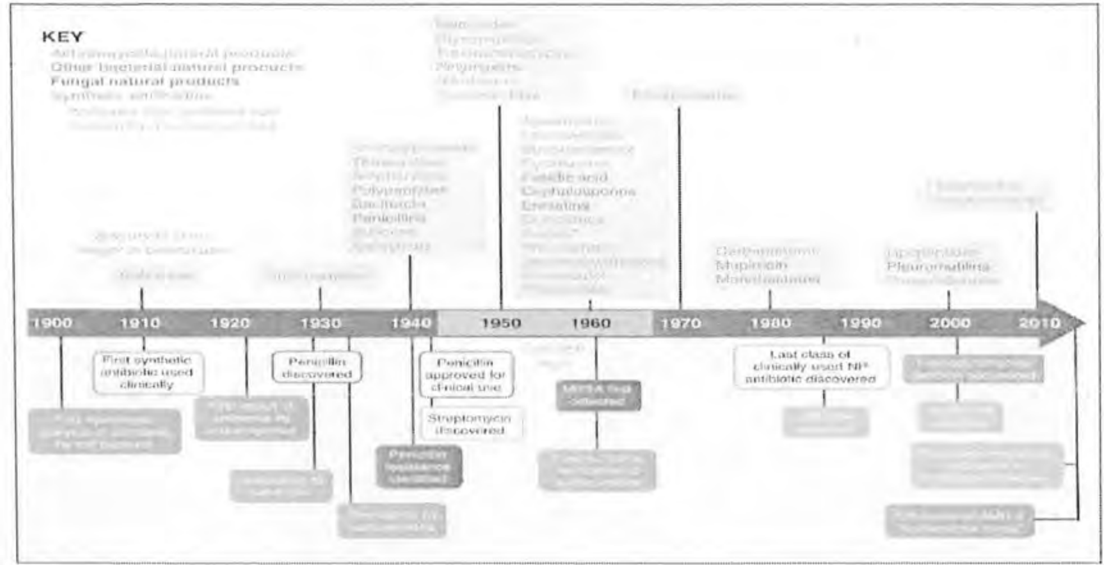

Figure 2-3: Timeline showing the discovery of and resistance to antimicrobial drugs (Hutchings et al., 2019)

2.2.3. Antibiotics' mechanism of action

A typical classification of antibiotics' as shown in Figure 2.4 antibacterial activity includes one of the five mechanisms listed below: 1) Interfering with the synthesis and disrupting the cell wall of bacteria 2) disrupting or blocking the protein synthesis of bacteria by blocking the process of translation 3) inhibiting bacterial nucleic acid synthesis by inhibiting topoisomerase enzymes involved in bacterial DNA replication 4) inhibiting bacterial metabolic pathways by interfering with metabolism or metabolites 5) inhibiting the function of the bacterial membrane by disrupting it (Garima Kapoor, Saurabh Saigal, 2018).

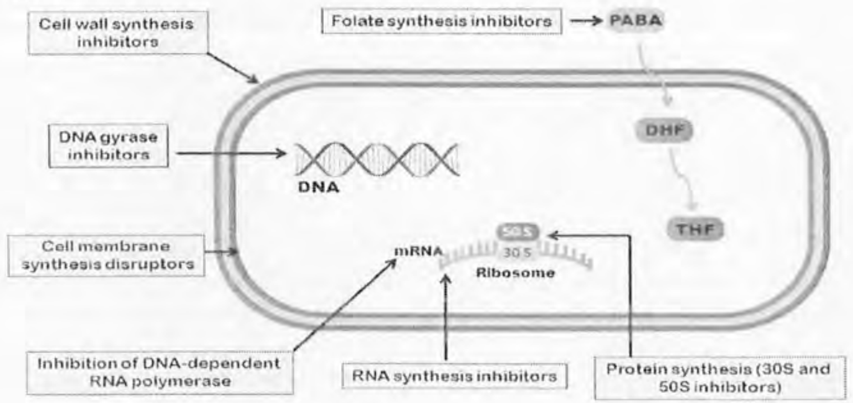

Figure 2-4: Mode of action of antibiotics (Garima Kapoor, Saurabh Saigal, 2018)

2.3. AMR is a matter of concern (AMR issues)

Antimicrobial resistance poses a serious issue to the community. The infections related to AMR cause severe bacterial infections, long-term hospitalization, increase the cost of the healthcare system, and result in failures in the treatment of infections. (Garima Kapoor, Saurabh Saigal, 2018) It is reported that hospital and community-acquired infections are estimated to cause approximately 33,00 mortalities and 874,000 disabilities in patients annually in Europe. The economic cost is approximately 1.5 billion euros per year, which includes increased healthcare system expenses and lost productivity (Antoñanzas & Goossens, 2019). Based on a 2019 study on antibiotic resistance from the reported from CDC, each year In the US, there are over 2.8 million cases of AMR, including more than 35,000 mortalities (Aljeldah, 2022).

According to Klein et al. 65% increase occurred in the consumption of antimicrobial drugs worldwide from 2000 to 2015. Antimicrobial resistance and healthcare costs have been increased as a result of this long-term high consumption and missed use of antibiotics (Klinker et al., 2021). The CDC estimate that antimicrobial resistance in the USA alone may increase hospital costs for treating patients with bacterial infections of any kind, and this might increase dramatically to more than 2 billion dollars annually (van den Bijllaardt et al., 2017) According to the World Bank, antibiotic-resistant bacterial infections may cause a global financial disaster by 2050, causing 28 million people to fall into severe poverty each year at a cost of one trillion US dollars to the worldwide economy (Gajic et al., 2022).

According to a report, in 2019, due to bacterial infection, 7 million deaths occurred, which was about 12% of all mortalities worldwide (Abbafati et al., 2020). Moreover, It is anticipated that antibiotic resistance in bacteria would result in 700,000 mortality annually. If precautionary and control measures are not implemented, then by 2050 this figure might increase to 10 million deaths per year, with a 100 trillion USD loss to the global economy, and this will have a huge impact on lower-middle income countries (JIM O'NEILL, 2016). In order to accomplish the objectives outlined in the National Action Plan (NAP), the CDC received 160 million USD from the Congress of the United

States in the 2016 budget year. Reducing the problems in the healthcare system caused by antibiotic resistance is a government goal, and the budget for combating antibiotic resistance has been raised from 160 million USD to 170 million USD to demonstrate the United States' commitment to the effort (Razzaque, 2021).

2.4. Staphylococcus aureus

The pathogenic strain of *Staphylococcus aureus* is widely distributed, and due to its pathogenicity, persistency, and resistance to antimicrobial drugs, make it a significant pathogenic superbug (Qiu et al., 2010). S. aureus involved in different skin and soft tissue infections because it is a part of the normal flora of the human body (Corrado et al., 2016). Furthermore, *Staph aureus* resistant and sensitive to methicillin strains (MSSA and MRSA) infect 30% of the US population (Gorwitz et al., 2008). Consequently the rapid increase in *Staphylococcus aureus* resistance to many antimicrobial drugs, it is extremely difficult to treat (Jackson et al., 2013).

Since the 1940 discovery of penicillin, it has been possible to treat infections caused by *Staphylococcus aureus*, but the emergence and fast spread of *Staphylococcus aureus* resistant to methicillin ended the use of beta lactams as a therapeutic approach (DeLeo & Chambers, 2009).

2.5. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a common opportunistic (gram-negative) human pathogen that is widely distributed in aquatic habitats and frequently linked to serious infections in immunocompromised patients. whereas 10% of all infections acquired in hospitals are attributed to it, and it is becoming a more widely recognized source of community-acquired infectious diseases. *Pseudomonas aeruginosa* inhabits moist habitats and is thus present in many medical facilities, particularly when it comes to chronic wounds, nebulizers, or UTI devices, where the development of biofilms increases the risk of persistency and resistance to antimicrobial drugs (Høiby et al., 2010; Newman JW, 2017).

For the past ten years, the CDC has classified multidrug-resistant *Pseudomonas aeruginosa* as a major concern due to its contribution of approximately 32,600 infectious cases, 2700 deaths, and 767 million US dollars in annual health expenses (Tabak et al., 2019).

2.6. Photodynamic Therapy (PDT)

In the start of twentieth century the photodynamic therapy (PDT) was found accidently in the laboratory of a Germen scientist name Hermann von Tappeiner. Acridine red dye, according to a student of Tappeiner named Oscar Raab, is toxic to *Paramecium caudatum* (Raab, 1904; von Tappeiner H, 1903). Additionally, he assumed that the light was enough for the toxic effect, but after further investigation, he found that the availability of oxygen is another crucial element in the lethal effect of a compound susceptible to light. When (von Tappeiner H, 1903) used eosin dye to treat patients with basal cell carcinoma of the face skin, they reported its impressive performance. This was the first therapeutic use of photodynamic therapy. Also, to address the phenomenon, (Von Tappeiner & Jodlbauer, 1904) proposed the name "photodynamic therapy.".

Heliotherapy is a method in which sunlight is used to cure many diseases alone or in conjunction with plant chemicals. This method was used and practiced in antiquity (Biju, 2014). Photodynamic therapy has the benefit of being very manageable and of being able to be used in conjunction with various therapeutic strategies, including chemotherapy, immunotherapy, gene therapy, and radiation (Xie et al., 2020). Currently, PDT is the best therapeutic approach to treat variety of diseases because it can eradicate drug-resistant and pathogenic microbes and eliminate cancerous cells and cholesterol from the arteries (Gursoy et al., 2013). For the treatment of pre-cancerous skin diseases, photodynamic therapy (PDT) was given Food and Drug Administration (FDA) approval in 1999 (H. Shi & Sadler, 2020).

2.6.3. History

Light therapy has a long history and was initially used many thousands of years ago. The Egyptians, Indians, and Chinese used it to cure a variety of superficial diseases, including

vitiligo, rickets, psoriasis, skin cancer, and many others (J H Epstein, 1990; John D. Spikes, 1985). Heliotherapy was developed by the Greeks three thousand years ago (3000 B.C.) and was one of the first records of the sun being used as a healing tool. The types of heliotherapies that the Greeks liked were called arenation, which involved people lying naked in specific places while being completely exposed to the sun's light. It wasn't until the latter part of the 18th century that the advantages of sun exposure were once again recognized as a successful treatment for rickets. According to Cauvin, a physician of the 19th century (1815), sunlight is the best therapeutic agent for the treatment of weakness of muscle, rickets, paralysis, swellings, scrofula, and dropsy as displayed in Figure 2.5 (Cauvin, 1815; M. D. DANIELALN, 1991)

Figure 2-5: Heliotherapy treatment of lupus vulgaris for one year (University of Melbourne's Medical History Unit) (M. D. DANIELALN, 1991)

One of the principles for light therapy was discovered in 1877 by Arthur Downes and Thomas Blunt in England, who reported that the sun's UV wavelengths are extremely toxic to bacteria and other microbes (McDonagh, 2001). Niels Ryberg Finse, a Nobel Prize winner in 1903, used light therapy in the current therapeutic approach. Their research demonstrated that a smallpox patient's wound could be treated and pus discharge from the wound eliminated by using light emitted in the red range of the visible spectrum (Niels R. Finsen, 1903). The term "photodynamic therapy" was proposed in 1904 by Von Tappeiner when he was the director in Munich (Ludwig-Maximilians University (LMU)). Oscar Raab had one of his students use light-sensitive Acridine red dye as a photosensitizer for the first time on a protist *Paramecium caudatum*. He demonstrated

that on a sunny day as compared to a cloudy day, the PSs dye has a highly toxic impact on *Paramecium caudatum* (v. Tappeiner, 1909) In 1913, Friedrich Meyer Betz (a German physician) suffered from hyperpigmentation and edoema for more than two months and self-injected hematoporphyrin to treat it. Hematoporphyrin chloride, in combination with sulfuric and hydrochloric acid, was used for the first time for the treatment of cancer, as reported by Lipson in 1960 (Zheng, 2005). In the 1970s, the initial research was immediately performed to check the efficacy of hematoporphyrin derivatives (HpD) in both in vivo and in vitro studies, and first controlled human study was performed (Dennis E.J.G.J. Dolmans, 2003). And finally, after a lot of efforts, in 1993, the Food and Drug Administration (FDA) approved the first photosensitizers for photodynamic therapy as displayed in Figure 2.6 (Ackroyd et al., 2001).

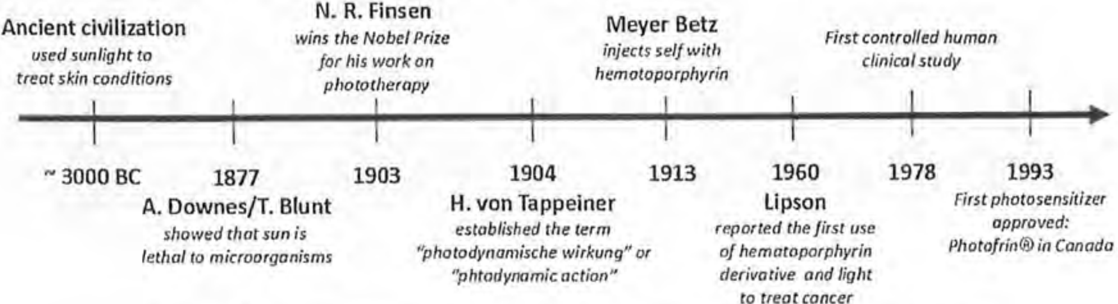

Figure 2-6: Photodynamic therapy (PDT) milestones (Dias et al., 2020)

Although photodynamic therapy (PDT) for the treatment of cancer has been used clinically for at least 25 years, such as in the cure of actinic keratosis or basal cell carcinoma. The antimicrobial effect of photodynamic therapy was introduced into clinical practice and the health care system in 1990 against the first antimicrobial-resistant infectious disease (Mark Wainwright, 2017).

2.7. Antimicrobial Photodynamic therapy

Antimicrobial photodynamic therapy (aPDT) is a potential alternative treatment for infections caused by pathogenic bacteria that are resistant to antimicrobial drugs. The principle of aPDT is the use of a foreign substance or antibiotic alternative known as photosensitizers (PSs) for the photooxidation of bacterial pathogens. The oxidative lethal effect, which is caused by exposing the disease area to light with an excitation wavelength usually in the visible range of light, causes cell death from 400 to 700 nm in

wavelength (Liu et al., 2015). aPDT has evolved as a promising tool and innovation for the elimination of multidrug-resistant bacterial pathogens because it has the ability to inactivate the efflux system, microbial biofilm, spores, and virulence factors. Since the 1990s, numerous pre-clinical experiments have been performed for the investigation of the antibacterial effects of aPDT by using multiple photosensitizers (PSs). As a result, several investigations revealed an optimistic approach of typically having more than $5\log_{10}$ CFU (colonies forming unit) reduction, which is considered to have bactericidal activity in accordance with infectious disease prevention and control measures (Cieplik et al., 2018).

Antimicrobial photodynamic inactivation (aPDI) was used to halt superficial infections and the development of biofilms by several pathogenic microorganisms that are resistant to antibiotics, including *Pseudomonas aeruginosa, Streptococcus, Staphylococcus, Moraxella*, and *Candida* species (Hu et al., 2018). The bacteria that develop biofilm are difficult for antibiotics to eliminate because the complex biofilm acts as a barrier, lowering the efficiency of antimicrobial drugs. aPDT is simple to use for the treatment of dermal infections caused by different microbes by exposing the patients to a specific photosensitizer and source of light. The distribution of antibiotics is known to be lower because of the polymeric matrix that covers the bacteria, acting as the first line of defence for the microbial cells inside the biofilm (Taraszkiewicz et al., 2013). According to Figure 2.7 *Moraxella catarrhalis, Staphylococcus aureus, Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* are biofilm-producing bacteria and have been reported to have a significant decrease in both biofilm quantity and morphological structure after aPDT (Luke-Marshall et al., 2020).

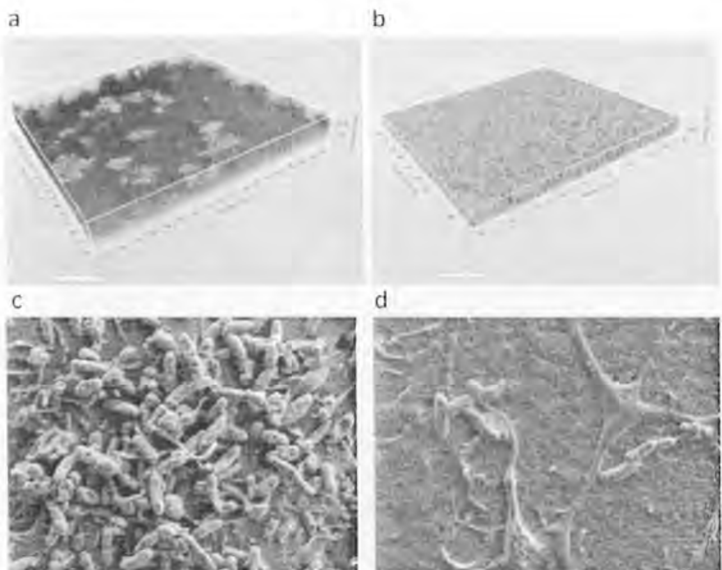

Figure 2-7: Depicting the treatment of biofilm before and after aPDT (a, c, and b, d). The a and b represent confocal microscopy (40μm: scale bar) and the c and d represent scanning electron microscopy (magnification: X5000) (Anas et al., 2021)

With specific concentrations of photosensitizers in combination with light, the PDT plays a vital role in antiviral, antifungal, and antiparasitic activity; also, antibiotic-resistant bacteria are susceptible to photodynamic inactivation (T. G. St. Denis et al., 2011). As compared to antimicrobial drugs, the aPDT targets multiple sites in bacteria, which inhibit and eliminate the chances of resistance development. aPDT is a promising alternative to antibiotics and are active against a wide range of Gram positive and negative bacteria. that can cause severe infection in humans (Cieplik et al., 2017).

A lot of antimicrobial photodynamic therapy (aPDT) experiments have been carried out on pathogenic fungi and bacteria in their biofilm and planktonic forms; for instance, multi-drug-resistant *P. aeruginosa, S. aureus* resistant to methicillin (MRSA), and other fungal growth were eliminated to 99.99% by using a photosensitizer (Methylene blue) in conjugation with laser light (670 nm) (Biel, Sievert, Usacheva, Teichert, & Balcom, 2011). Also, aPDT using two photosensitizers (MB + protoporphyrin IX) was excited by 652nm laser light for the elimination of rod-shaped, gram-negative *Acinetobacter baumannii* (biofilm-forming) in biofilm and planktonic form (Anane et al., 2020). As a result, irreparable damage occurred in molecular components of the cell, including deoxyribonucleic acid (DNA), enzymes, lipids, and proteins, by the production of singlet

oxygen ($^1O2$) and reactive-oxygen-species (ROS), during photosensitization of specific photosensitizer by visible laser light (Girotti, 2001).

2.7.3. Photosensitizers

Photosensitizers (PSs) are composed of inorganic substances or of plant chemicals that are excited by a specific wavelength of light and produce $^1O2$ or ROS, which induce the elimination of microorganisms in their locality (Castano et al., 2004). In order to achieve significant penetration into tissue and adequate triplet state energy for singlet oxygen (1O2) production, an optimal photosensitizer is anticipated to have strong absorption between wavelengths of 650–850 nm (Plaetzer et al., 2009).

Photosensitizers (PSs) are characterized on the basis of precursors, chemical structure, and mechanisms of action (Lan et al., 2019).

Photosensitizers are classified into three subcategories: 1st generation, 2nd generation, and 3rd generation photosensitizers (PSs): The 1st generation photosensitizers includes hematoporphyrin's, also known as porphyrins, which are soluble in water; the second generation includes methylene blue (MB), toluidine blue (TB), and aminolaevulinic acid (ALA), which produce more $^1O2$ and are highly selective; and recent studies on third-generation photosensitizers have focused on decreasing toxicity to normal tissues and improving solubility (Babu et al., 2020; Sowa & Voskuhl, 2020).

E. coli and *P. aeruginosa*, are the pathogens that cause infectious diseases in humans and animals, are efficiently eliminated by antimicrobial photodynamic therapy (aPDT). The disruption of cell walls and DNA breakdown in pathogenic microbes targeted by methylene blue (MB), a 2nd generation photosensitizer, and also photooxidation have been demonstrated to reduce the efficiency of various virulence components, including lipopolysaccharides (LPS) and proteases (Kömerik et al., 2003). Moreover, the bactericidal efficacy is attributed to $^1O2$ and ROS species such as free radicals, which influence a variety of tissues and cell targets, as the resistance to aPDT is improbable.

The majority of photosensitizers used in photodynamic therapy are susceptible to wavelengths of light from 630 to 700 nm. Table 2.1 gives a summary types and excitation wavelength of photosensitizers (Fekrazad et al., 2015).

Table 2-1: Photosensitizer Types and Their Wavelength of Excitation (Fekrazad et al., 2017)

Photosensitizer Types	Excitation Wavelength
Plant based therapeutic agents	550-700 nm
Phthalocyanine	660-700 nm
Hematoporphyrin derivatives	620-650 nm
Cyanine	600-805 nm
Phenothiazine (toluidine blue + methylene blue)	620-700 nm

Since 1990, the discovery of photosensitizers for PDT has been more widespread than the conventional antimicrobial drugs, with a significant rise after the start of the resistant era, as shown in Figure 2.8 (Mark Wainwright et al., 2017).

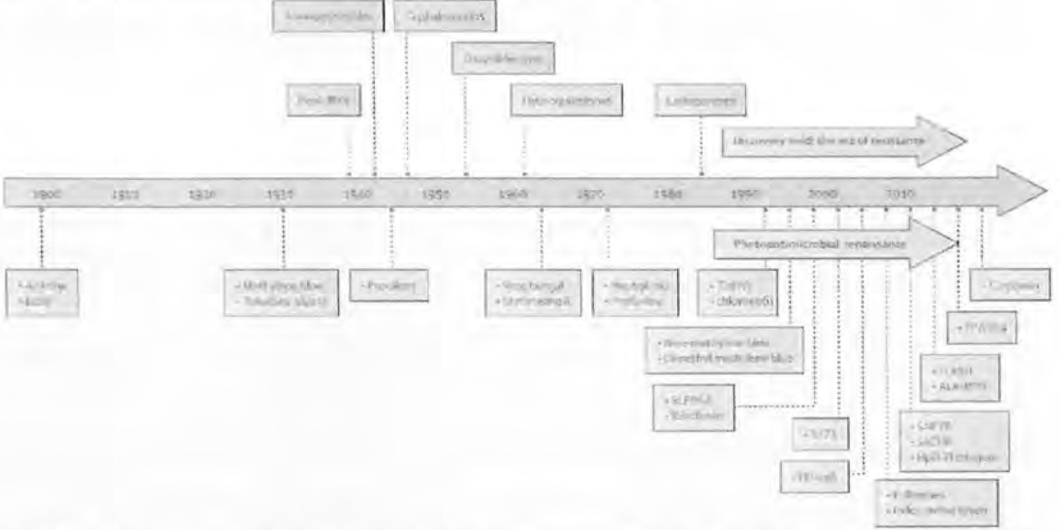

Figure 2-8: Discovery of antimicrobial photosensitizers before and after the resistant era (Mark Wainwright et al., 2017)

The penetration and attachment of photosensitizers into the microbial outer membrane depend on the species of bacteria. Gram positive bacteria have porous and permeable membrane while gram negative have a complex peptidoglycan and LPS which make it less permeable, that's why gram positive are more susceptible to aPDT. Therefore, cationic photosensitizers (PSs) should be used against both bacterial species for

antimicrobial photodynamic therapy (aPDT). It is demonstrated that MB, TB, and hematoporphyrin photosensitizers have a considerably more lethal and toxic impact on microorganisms due to their cationic state (Merchat et al., 1996; Minnock et al., 1996; M. Wilson et al., 1995).

2.4.1.1.Methylene blue

Heinrich Caro, a German chemist, discovered the first phenothiazinium, Methylene Blue dye, in the 1870s, which is included in the second generation of photosensitizers (Caro, 1878). Figure 2.9 demonstrated that MB dye has a 3-ring p-system structure with a single +ve charge on it and a $^{1}O2$ quantum yield less than 0.5; it also has an auxochromic side group (Wilkinson et al., 1993).

Figure 2-9: Methylene blue's chemical composition (Ghosh et al., 2019)

The entire absorption spectrum of methylene blue in liquid ranges from 600 nm to 680 nm, with the upward part of the absorption curve having the lowest slope while the downward part suddenly drops. So, MB at wavelengths of 635 nm and 670 nm has two absorption peaks as depicted in Figure 2.10 (Tardivo et al., 2005). Since longer wavelengths of laser light penetrate into tissue more efficiently than shorter ones, MB exhibit a significant absorption peak in the red spectrum at 600nm to 680nm, which is ideal for their use as photosensitizers in PDT (Felgenträger et al., 2013).

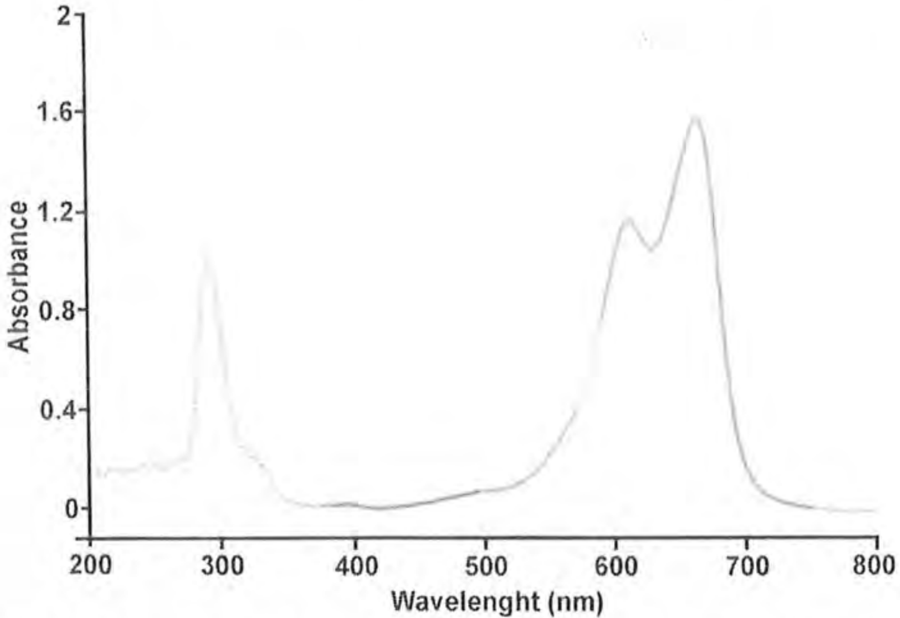

Figure 2-10: Methylene blue's absorption spectra (Giannelli & Bani, 2018; Tardivo et al., 2005)

A 1) visible light 2) photosensitizer and 3) molecular oxygen are the three most important requirements for photodynamic therapy. As demonstrated in Figure 2.11, the photosensitizer is safe until it is not exposed or photosensitized by specific range of laser light (Fekrazad et al., 2014).

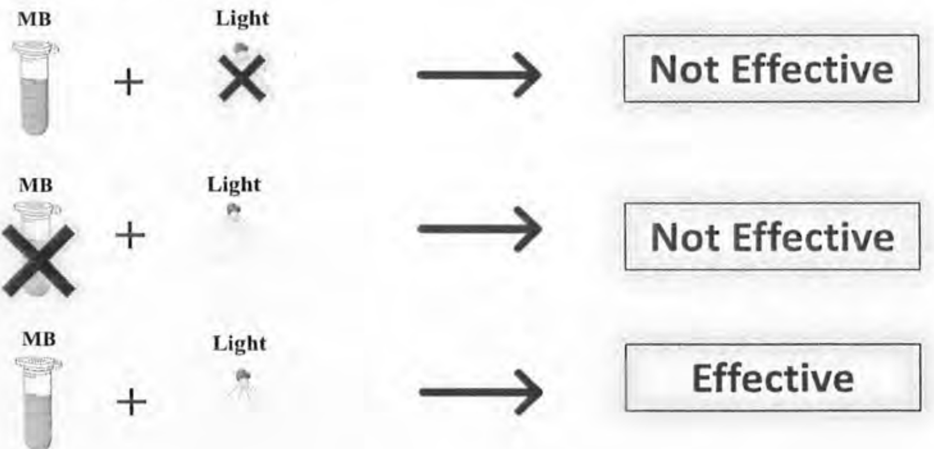

Figure 2-11: Demonstrate the efficiency of methylene blue with and without light

Even antimicrobial-resistant bacteria are effectively eradicated by methylene blue, as it is very commonly used in microbiology research. It also exhibited antimicrobial activity at high doses without exposure to laser light. As a photosensitizer, a low dose in

combination with light can effectively eliminate both gram +ve and -ve bacterial and fungal species (Vilela et al., 2012; M. Wainwright & Crossley, 2002). The efficacy of aPDT-methylene blue (MB) has also been studied in a maxillary sinus model on biofilms of multi-drug-resistant *S. aureus* resistant to methicillin (MRSA) and *P. aeruginosa*. According to this pre-clinical study, after a single therapy, the biofilm of severe rhinosinusitis was eliminated by more than 99.99% (Kofler et al., 2018).

2.4.2. Source of light

For antimicrobial photodynamic therapy (aPDT) there are three major sources of light for the excitation of photosensitizers (PSs) have been reported: for example, 1) Gas-discharge lamps such as Xenon or quartz tungsten halogen lamps, 2) LEDs (light emitting diodes) and 3) Lasers: including aluminum, argon, neodymium doped, yttrium, garnet and diode lasers as shown in Figure 2.12 (Nagata et al., 2012).

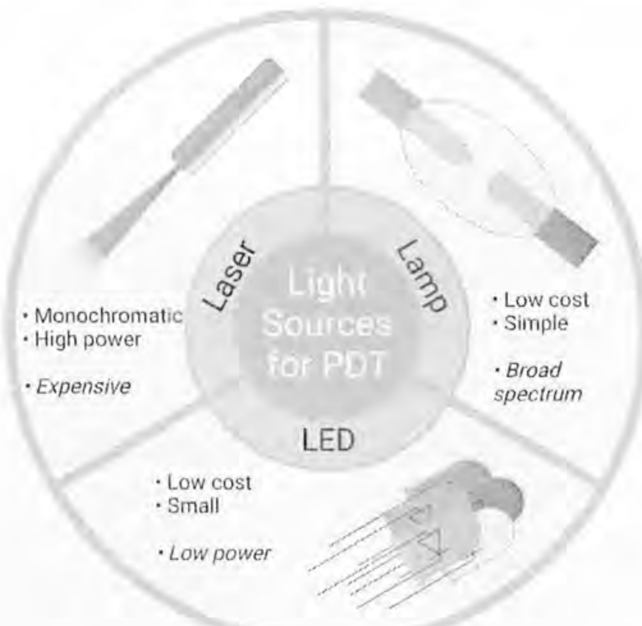

Figure 2-12: Sources of light used in photodynamic therapy (PDT): 1) Lasers 2) LED (light emitting diodes), 3) lamps (Gunaydin et al., 2021)

Generally, rather than the source of light for example (LEDs, lasers or lamps), the wavelength and intensity of light sources, also the mode of action are very essential factors for the photosensor excitation (Cieplik et al., 2018). A source of light is needed in

PDT to expose the photosensitizers for excitation to visible light at a certain wavelength. Mostly red laser-lights are used for the excitation of photosensitizers with a wavelength from 600 to 700 nm, which have the ability to penetrate in tissue from 0.5 to 1.5 cm absorption, , which can cause the cell death, necrosis or apoptosis in tissue (Salva, 2002). For the treatment of tissues and use of photosensitizers (PSs), the wavelength, intensity, dose of light and penetration of depth for damage varies (Rajesh et al., 2011).

Presently, for photodynamic therapy (PDT) different sources of light are used as substitutes for sunlight therapy such as LEDs, discharge lamps, lasers, and other optical fiber systems. For antibacterial photodynamic therapy (aPDT), the following are some light sources that have been applied for the activation of photosensitizers and treatment: UV light (300-400nm), visible light as red, yellow, green, and blue with a wavelength of 600–700, 550–600, 490–550, and 400–490nm. Also, near-infrared (NIR) with 700–810 nm, LEDs, Xe lamps, and laser beams are used (Kashef et al., 2017). For antimicrobial photodynamic therapy (aPDT), many different types of visible light have been employed, but due to the deeper penetration into the tissue, light with a longer wavelength is recommended (Babu et al., 2020). Since the greater light intensity may cause heating concerns, while the appropriate range of light for antimicrobial photoexcitation is between 5 to 1000 W/m^2, as the exposure time duration depends on the intensity of light (Luksiene & Brovko, 2013).

2.5. Mechanism and efficiency of aPDT

Antimicrobial photodynamic therapy (aPDT) addresses a variety of bacterial infections, for example, it can treat the life-threatening MDR-*S. aureus* causing cutaneous, bloodstream and soft tissue chronic microbial infections (Paramanantham et al., 2019). Previously numerous pre-clinical investigations demonstrated that aPDT effectively eliminates a wide range of harmful microbes. The activity of aPDT is influenced by the morphological and molecular composition of Gram positive and Gram-negative bacterial microbes (Polat & Kang, 2021). Under certain circumstances, a PS has the capability to generate ROS. Normally, when the ground state photosensitizer (PS^0) irradiated by an appropriate range of irradiation, after exposure to light and photon (hv) absorption the

photosensitizer transformed from the normal condition to photosensitized condition photosensitizer (1PS)* with a short lifetime and high reactivity (Tim Maisch, Jürgen Baier, Barbara Franz, Max Maier, Michael Landthaler, Rolf-Markus Szeimies, 2007). The photosensitizer then initiates internal conversion (IC), in that case it loses the energy by releasing fluorescence and returns back to its initial ground state (PS^0), or in that time it performs inter-system conversion (ISC), in which it transfers to the more stable long life excited triplet state PS (3PS) *. During this conversion Type I (e-transfer) and Type II (E transfer) are two different forms of chemical reaction pathways that take place (Foot, 1991). Free radicals' species (O2•−, OH• and hydrogen peroxide H_2O_2) generated during Type I pathway which cause proteins and lipid peroxidation in cells after interaction, (Athar et al., 1988) while in Type II pathways ($^1O2$) singlet oxygen is produced as a result of energy transfer from the highly reactive triplet state PS as displayed in Figure 2.13 . In addition to producing more oxygen radicals, the singlet oxygen may interact directly with nearby biological molecules in their locality (Redmond & Gamlin, 1999). In aPDT for microbial infectious diseases and other disorders, it is believed that singlet oxygen generation is essential (Yin et al., 2015). Bacterial inhibition induced by the reaction of ROS from both pathways, inside the bacterial cell or in their locality. It should be noticed that the lifespan of ROS and $^1O2$ is very short (Fu et al., 2013).

Because of its unstable electrical composition, $^1O2$ is very reactive and has a very limited lifespan. Depending on the surrounding environment, it has a limited lifespan in water (about 3-4μs) with a small diffusion range, while in pure water it is roughly 1μm, and it is less than 50 nm in lipid layers that are high in protein (Alves et al., 2014).

The effectiveness of aPDT can be increased by focusing on essential components of microbes. In targeted pathogens it is not the only cause of cell death to disrupt their DNA by breakdown its bonding and supercoiling by using photosensitizers and specific light in aPDT [28]. According to Figure 2.14 the other proposed reasons for cell death including disintegrate synthesis of cell wall, loss of potassium ions, disorganized proteins in cytoplasmic membrane, and disrupt outer membrane and increased their porosity (Hamblin & Hasan, 2004). By employing specific photosensitizers (e.g., Methylene blue)

in antimicrobial photodynamic therapy (aPDT) can potentially cause damage in DNA and RNA, by inhibiting their multiplication and synthesis of DNA (Hamblin & Abrahamse, 2020).

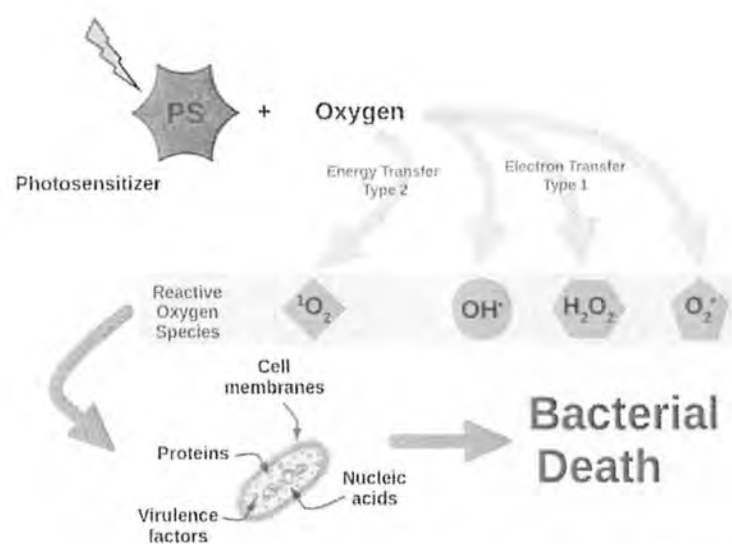

Figure 2-13: The aPDT mechanisms' schematic depiction (Maldonado-Carmona et al., 2020)

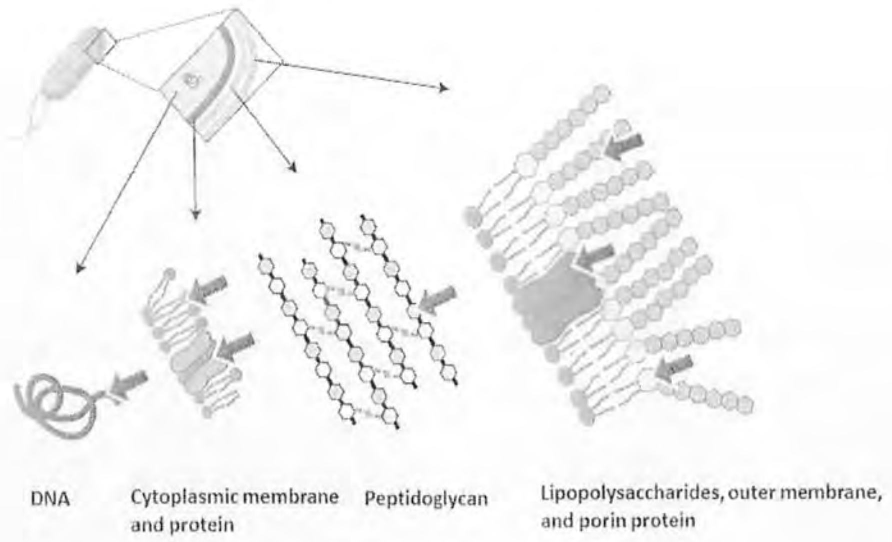

Figure 2-14: Target sites for aPDT treatment in bacteria cell (Liu et al., 2015)

2.6. Application of PDT

Photodynamic therapy (PDT) was initially identified as a comparatively novel anticancer treatment method (Diamond et al., 1972). Since then, it has developed and is currently

used in a wide range of therapeutic approaches. For instance, it is used to treat neurovascular diseases, disinfect environmental contaminants, control long-term insects and pests, and also have antiviral activity which better showed in recent SARS-CoV-2, (Svyatchenko et al., 2021) bacteria, fungi, and parasites, among other things. Despite the cancer therapy, the application of photodynamic therapy has revealed a magnificent approach against bacteria and established the domain of aPDT. In both free living bacteria and biofilm, PDT has been proven to eliminate a broad range of microbial pathogens (Songca & Adjei, 2022).

Photodynamic therapy is used in conjugation with many therapeutic drugs to improve the efficacy of aPDT, and it has also been demonstrated in conjunction with various chemotherapeutic chemicals against various infections caused by pathogenic bacteria (Pérez-Laguna et al., 2019). Photodynamic therapy with chemotherapeutics and photothermal hyperthermia therapy (PTT) is another synergistic therapy for the treatment of cancer that has been studied, as well as against different pathogenic fungi and bacteria (Rodríguez-Cerdeira et al., 2021). Magnetic hyperthermia therapy (MHT) has also been investigated in conjugation with photodynamic therapy for cancers that are difficult to treat, like brain and bone tissue cancer, and for many other applications (Matsubara et al., 2013). The efficiency of aPDT is investigated through the elimination of cutaneous infectious diseases produced by different pathogenic bacterial, fungal, protozoan, and viral strains. In this case, PDT is used to irradiate the skin lesion, preventing pathogens from entering the blood circulation system. Additionally, aPDT has been extensively used as a disinfectant technique, both for surfaces and food as depicted in Figure 2.15 (Seidi Damyeh et al., 2020) (Fig. 4).

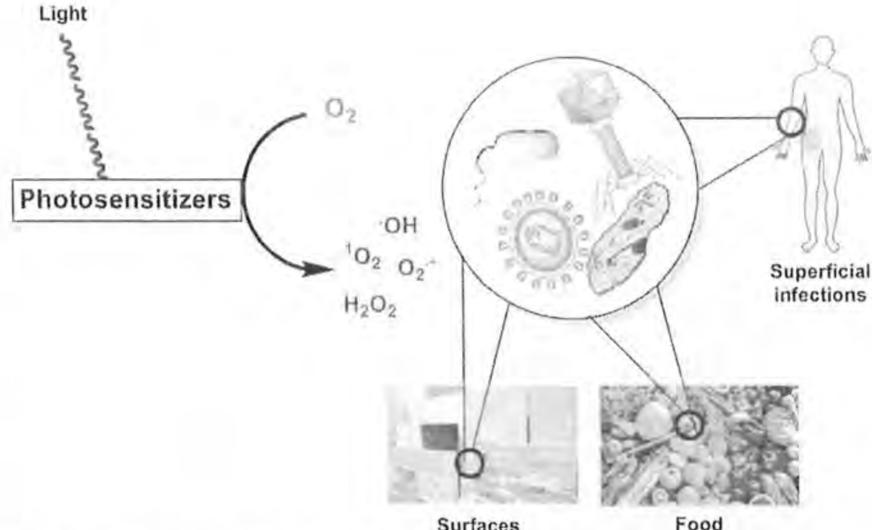

Figure 2-15: Antimicrobial photodynamic therapy for the treatment of skin infections and for the decontamination of food and surfaces (Dias et al., 2020)

2.7. Benefits and drawbacks of photodynamic therapy

Antimicrobial photodynamic therapy (aPDT) has a number of important benefits, including being less expensive than conventional chemical therapy, friendly to the environment, and having high safety in a variety of applications (Cantelli et al., 2020). The following are the key benefits of antimicrobial photodynamic therapy: As compared to chemical therapy, the aPDT effectively eliminates a broad range of microbes. They have effective phototoxicity to kill both antimicrobial-resistant and wild bacterial and other microbial strains as shown in Figure 2.16. In addition, they have a low mutational potential and are highly selective in eliminating pathogenic strains when compared to the host. very specific and efficient in terms of time and space selectivity, and produce highly toxic ROS and oxygen for microbe eradication. and other beneficial properties like being ecologically friendly and being less expensive (Anas et al., 2021).

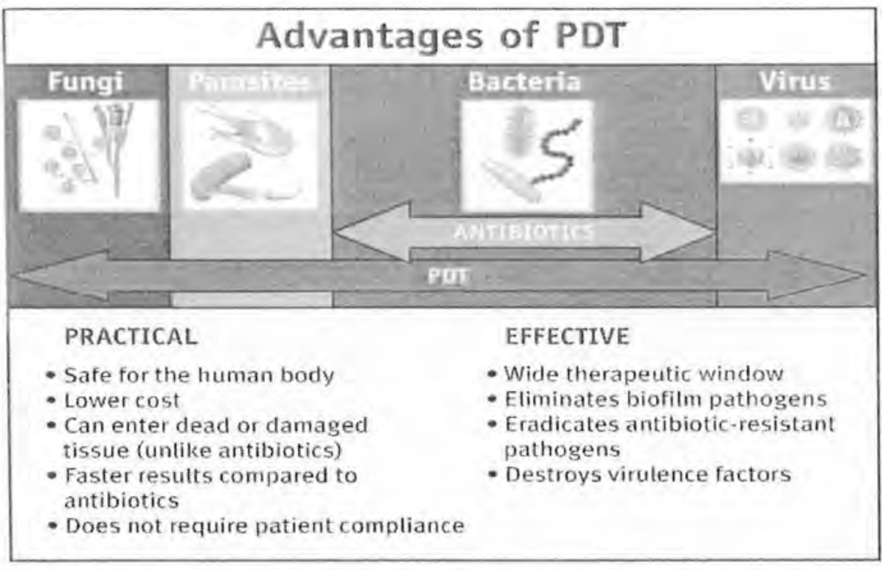

Figure 2-16: Advantages of PDT for localized infection (Dai et al., 2009)

There are some reasons why resistance to antimicrobial photodynamic therapy (aPDT) is improbable, even after repeated use (Lauro et al., 2002). 1) Bacteria cannot develop resistance to therapy due to the insufficient time interval between photosensitizer excitation and PDT. 2) Photosensitizers have no toxicity in the dark; that is why there is no need for bacteria to develop resistant mechanisms to combat PSs. 3) After photodynamic treatment, the cells are too disrupted, making it impossible for them to impart cross-generational adaptivity. 4) As compared to antibiotics, the aPDT does not focus on only one target site in bacteria (Schastak et al., 2010).

If PDT has a lot of benefits, there are some drawbacks as well. Because of the broad spectrum of effects of ROS (reactive oxygen species) produced during PDT treatment, it has the potential to eliminate both harmful (pathogenic) and beneficial microorganisms. For instance, if ROS concentrations are higher than what the host can tolerate, in that situation, the host cells might be inactivated due to the unintended effects. The reaction may be managed using the most recent technological advancements by regulating the concentrations of photosensitizers, exposure times of light and chemicals, and intensity of light (Montanha et al., 2017)

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Chapter 3 Material and Methods

3.1. Research location

The current research work was carried out at the National Institute of Laser and Optronics (NILOP), a college of the Pakistan Institute of Engineering and Applied Sciences (PIEAS), Pakistan Atomic Energy Commission (PAEC), Islamabad, in collaboration with the Department of Microbiology, Quaid-i-Azam University, Islamabad. The entire research project was done using standard microbiological practices.

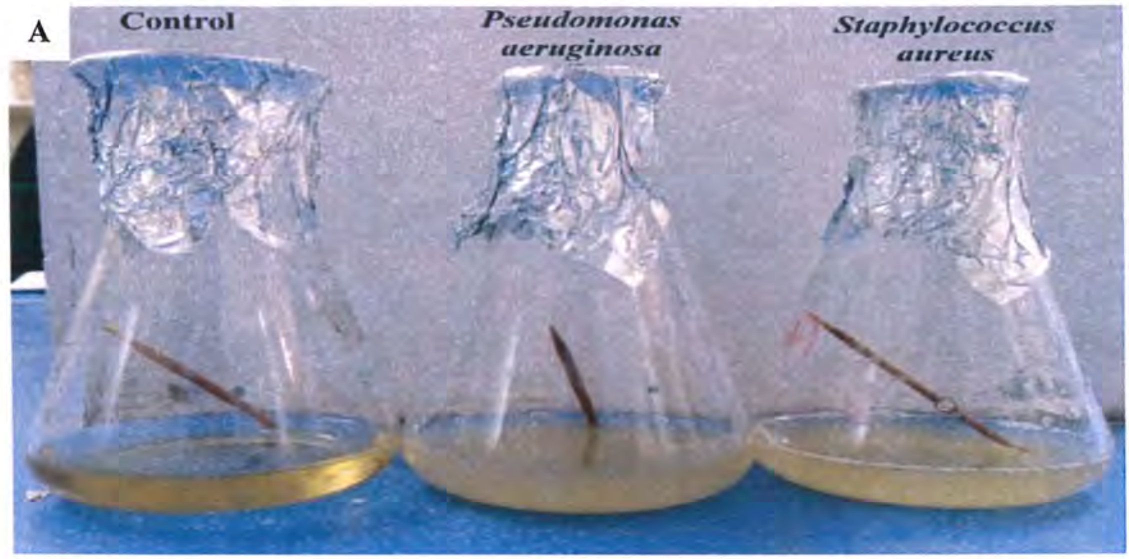
3.2. Materials and chemicals

In this research, different chemicals and materials were used, including Muller-Hinton agar (MHA), nutrient broth, and agar, which were obtained from Oxoid (UK). The following classes of Meropenem, Gentamicin, Fosfomycin, Colistin, Chloramphenicol, Doxycycline, Cefazolin, Nitrofurantoin, Tigecycline, Nalidixic Acid, Ceftriaxone, Amikacin, Aztreonam, Oxacillin, Ampicillin, and Linezolid antibiotic discs were purchased from Sigma-Aldrich. Photosensitizer methylene blue (MB) (VWR, PROLABO, Belgium) was used as an alternative to antibiotics. Barium chloride ($BaCL_2$), sulfuric acid (H_2SO_4), and phosphate buffer saline (PBS) (Thermo Fisher Scientific) were used for the McFarland standard and other solutions. FluoroMax-4 spectrofluorometer (HORIBA Scientific, Jobin Yvon), UV-Vis spectrophotometer (Thermo Scientific, San Jose, CA), and confocal laser scanning microscope (Zeiss LSM 510-META, Germany) were also used.

3.3. Bacterial strains and their culture

Clinically isolated and 16S rRNA sequence identified Gram negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* strains were studied in this research, which was provided by the Applied, Environmental, and Geo Microbiology Lab, Quaid-i-Azam University Islamabad. The bacterial strains were grown in Nutrient Broth Oxoid (UK) and overnight incubated at 37°C in a New Brunswick INNOVA 43 (USA) constant shaker at 144 rpm. The overnight incubated culture was streaked on

Nutrient Agar containing petri dishes Oxoid (UK) and for 24 hours incubated at 37°C to revive both strains as shown in Figure 3.1. Both pure cultured strains were preserved in a 70% glycerol stock solution (Thermo Fisher Scientific) at -80°C until use.



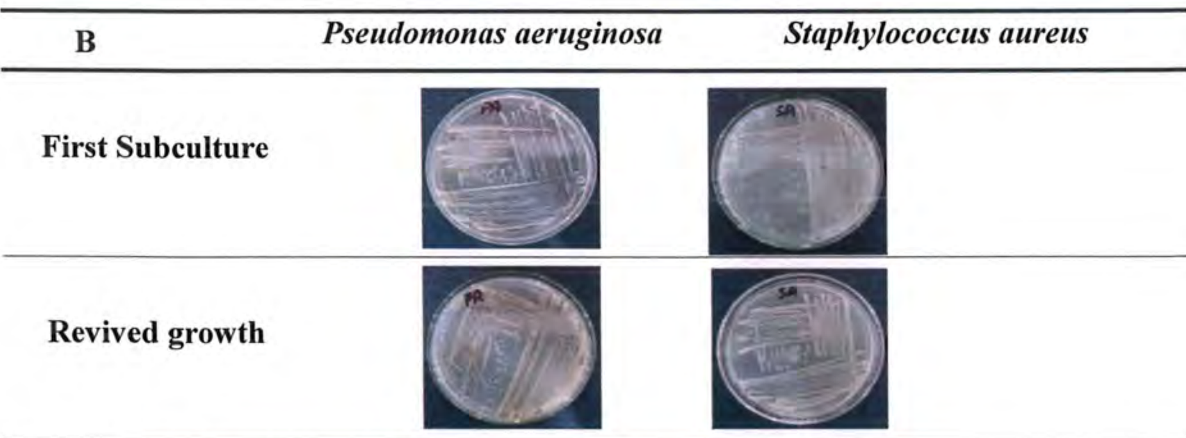


Figure 3-1: a) Inoculation; b) Subculturing of *P. aeruginosa* and *S. aureus*

3.4. Antibiotic sensitivity testing

The Kirby-Baur agar disc diffusion technique was employed on the identified and revived bacterial strains to identify the level of antibiotic resistance. In this method, using a sterile toothpick, a single colony of selected and cultured bacterial strains was mixed with 0.9% autoclaved normal saline. Further, the suspension's turbidity and the 0.5 McFarland solution's turbidity were compared. Then, a pipet was used to pour 100µl of bacterial

suspension onto the Muller-Hinton agar plates (Oxoid, UK). By repeatedly and thoroughly swabbing with sterile swabs, this suspension was applied to the MHA agar. With a sterile syringe, the above-mentioned antibiotic discs were then applied on media containing plates (Hudzicki, 2012). Following a 24-hour incubation period at 37 °C in these petri dishes, the zone of inhibition was measured and recorded in accordance with the CLSI 2020 recommendations.

3.5. McFarland standard solution

For this standard, H_2SO_4 (99.5 ml) and barium chloride (BaCL2: 0.5 ml) (Sigma-Aldrich) were mixed for the preparation of 0.5 McFarland solution and stored at 4°C for further use. This standard is used to accurately measure turbidity in normal saline using freshly inoculated bacterial cultures. The optical density of the 0.5 McFarland turbidity standard is equivalent to 1.5×10^8 CFU/ml of bacterial suspension (Kralik et al., 2012).

3.6. Photosensitizer preparation

Methylene blue (MB) (VWR, PROLABO, Belgium) was employed as a photosensitizer against both bacterial strains as an alternative to antibiotics. Its 10mg/ml stock solution was prepared in autoclaved deionized water, and further diluted into concentrations in µg/ml (500, 250, 125, 62.5, 31.25, and 15.625) (Figure 3.2). All methylene blue concentration tubes were wrapped in aluminum foil and stored in the dark at 4°C until use.

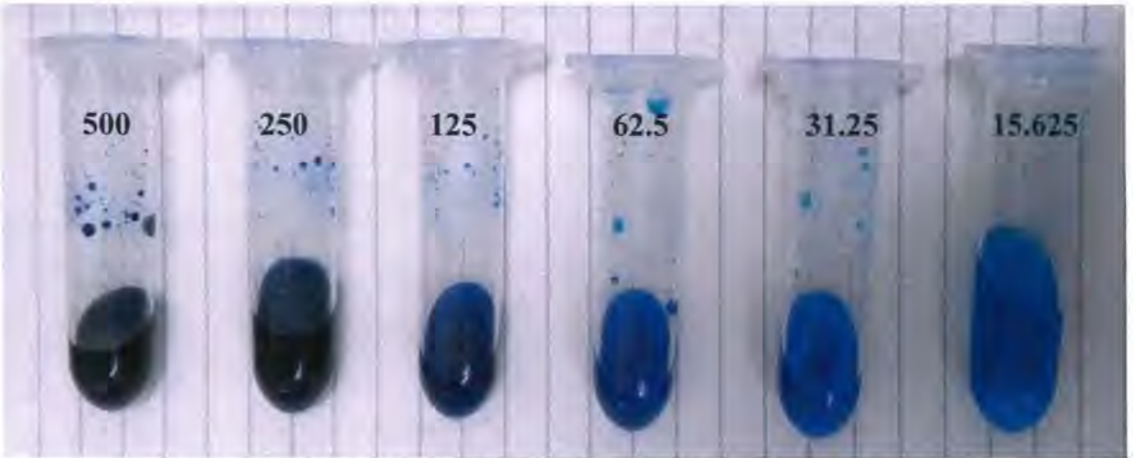


Figure 3-2: Concentration of methylene blue in µg/ml for use in aPDT

3.7. Source of light

A red laser light source with a power of 300mW/cm^2 and 635 nm wavelength was employed to irradiate the bacterial strains used for the aPDT experiment at a distance of 5 cm (developed by the National Institute of Laser and Optronics, Islamabad) illustrated in Figure 3.3. The time and dosage for excitation in an aPDT are shown in Table 3.1.

Table 3-1: Time and dosage for photosensitization at 300mW/cm^2

Time (Sec)	60	120	180	240	300	360
Dose (J/cm^2)	18	36	54	72	90	108

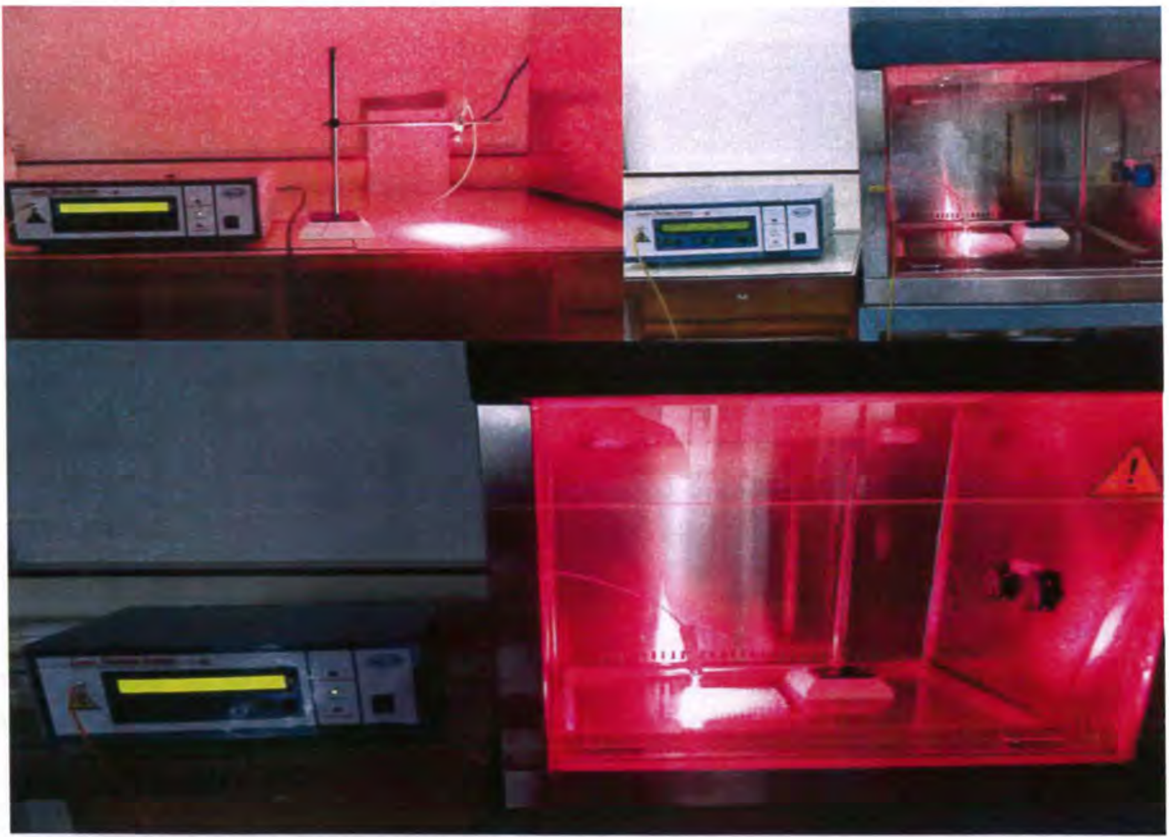


Figure 3-3: A 635nm diode laser developed by NILOP, Islamabad, is used as a light source

3.8. Methylene blue photobleaching

In order to check the efficiency of diode laser light on dye degradation, a photobleaching experiment was performed using methylene blue. In this experiment, 200µl aliquot of

methylene blue from each concentration, from 500μg/ml to 15.625μg/ml was poured into each well of a 96-well plate and exposed to diode laser for a specific time and dose at 300mW/cm^2 as shown in Table 3.1. The optical density (OD_{600nm}) was recorded using a UV-Vis spectrophotometer (Thermo Scientific, San Jose, CA), and decrease in the optical density/absorbance was determined as the process of photodegradation.

3.9. Biodegradation of methylene blue

For the biodegradation of methylene blue, both bacterial strains, *Pseudomonas aeruginosa* and *Staphylococcus aureus,* were used. A fresh bacterial culture was inoculated into nutrient broth and incubated overnight at 37°C. After overnight incubation 5ml of cultured broth were poured in each test tubes and another single set of test tubes contains only nutrient broth with no bacteria, also 1ml of methylene blue from each concentration were mixed in all test tubes (a total of 7 sets of test tube were used) and overnight incubated at 37°C. after incubation all test tube were compared with each other.

3.10. Cytotoxic activity of methylene blue in dark

For the evaluation of the efficiency of selected serially diluted concentrations of methylene blue, a dark experiment without exposure to laser (+MB -L) was performed. Each bacterial inoculum was incubated for 20 minutes in dark with 100μl of methylene blue from each concentration. Following incubation, 10μl of bacteria with MB from each group were seeded on nutrient agar-containing petri dishes and for 24 hrs. incubated at 37°C. After incubation, the colonies were counted to investigate the efficiency of the methylene blue concentration used in aPDT.

Also, bacterial colonies were mixed in 0.9% normal saline, and the suspension's turbidity and the 0.5 McFarland solution's turbidity were compared. Then, a pipet was used to pour 100μl of bacterial suspension onto the Muller-Hinton agar plates. By repeatedly and thoroughly swabbing with sterile swabs, this suspension was applied to the MHA agar. After swabbing for the well diffusion method, wells were made through a cork borer in MHA, and 10μl from each methylene blue concentration were poured. At 37°C for 24 hrs. all petri dishes were incubated. After incubation, zone of inhibitions was analyzed.

3.11. Antimicrobial photodynamic therapy

In this study, two *P. aeruginosa* and *S. aureus* bacterial strains were studied as pathogenic models. The bacterial strain was cultured overnight in a shaker incubator at 37°C after being introduced in nutrient broth. After incubation, 5 ml of bacterial culture were centrifuged in 15-ml Falcon tubes at 5000 rpm for 10 minutes at 15°C. After centrifugation, the supernatant was removed, and autoclaved phosphate buffered saline (PBS) was used to rinse both bacterial strains' pellets three times. Both bacterial strains were resuspended in PBS to achieve an optical density comparable to 10^8 CFU/ml. A UV-Vis spectrophotometer (Thermo Scientific, San Jose, California) was utilized to measure the OD value of 0.8 at 600 nm. The measurement was adjusted to get 10^8 CFU/ml.

Further, 200μl of bacterial samples were transported to a 96-well plate without methylene blue (MB) (-MB +L) and irradiated with 300mW/cm^2 with different dosages to know the efficiency of red-light diode laser without MB, and 10μl of aliquots were seeded from the irradiated samples on petri dishes containing nutrient agar and for 24hrs. incubated at 37°C. After the incubation, bacterial growth was observed by measuring CFU/ml. Additionally, in second experiment both bacterial inoculums were incubated for 20 minutes in dark with 100μl/ml of methylene blue from each concentration in μg/ml (500, 250, 125, 62.5, 31.25, 15.625). After incubation, 200μl of bacterial samples were transported to a 96-well plate and in sterile condition (biosafety cabinet) exposed to a 635 nm (300mW/cm^2) blue diode laser at a distance of 5 cm for specific time durations (60, 120, 180, 240, 300, and 360 seconds) and dosages (18, 36, 54, 72, 90, and 109 J/cm^2) as depicted in Table 3.1; the control was left untreated. Following treatment of both bacterial strains 10μl from each well of 96-well plate were seeded on nutrient agar containing plates and incubated for 24 hours at 37°C. Both bacterial growths were measured by colony forming units (CFU) after incubation. The rate of survival of both bacterial strains after irradiation were compared with (-MB -L, -MB +L, +MB -L, +MB +L) treated plates in dark and also compared with controls.

3.12. Optical density

In order to measure the optical density, microplate spectrophotometer (INNO^{TM} & INNO-M^{TM}) were used after photosensitization of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* with a 635 nm red light diode laser at 300mW/cm^2. The optical densities were performed in a 96-well plate with all concentrations of methylene blue from 500 to 15.625μg/ml at 600 nm. The decrease in optical density determined the efficiency of antimicrobial photodynamic therapy (aPDT) on both multi-drug resistant strains.

3.13. Fluorescence spectroscopy of bacterial strains

For fluorescence spectroscopy, fresh cultures of both bacteria were inoculated in nutrient broth and incubated overnight at 37°C in a shaker incubator. After incubation, the 5ml bacterial cultured broth were transferred to 15-ml Falcon tubes and centrifuged at 5000 rpm and 15°C for 10 minutes. After centrifugation, the supernatant was removed, and autoclaved phosphate buffered saline (PBS) was used to rinse both bacterial strains' pellets three times and again resuspended in PBS. Furthermore, 100μl of methylene blue from each concentration was mixed with each bacterial PBS solution and incubated for 20 minutes in quartz cuvettes in the dark. After incubation, the samples were immediately irradiated in sterile conditions (biosafety cabinet) with a 635nm diode laser (300mW/cm2) at a specific time and dose as mentioned in Table 3.1. After photosensitization, the emission spectra of treated and untreated samples were analyzed by using a FluoroMax-4 spectrofluorometer (HORIBA Scientific, Jobin Yvon) from 285nm to 525nm, with 270nm as the excitation Soret bands. The bandpass settings for both the emission and excitation slits were set at 8 nm.

3.14. Confocal microscopy cell viability

Confocal laser scanning microscopy was used to visualize the impact of methylene blue-associated aPDT on MDR *P. aeruginosa* and *S. aureus* after and before photosensitization. For high-resolution confocal microscopy images, some important steps were followed: both bacterial strains were inoculated, incubated, centrifuged, and

resuspended in PBS. The bacterial solution in PBS was incubated with 100μl/ml of all concentrations of methylene blue for 20 minutes at room temperature in the dark. Both bacteria strains were photosensitized after incubation with 18, 36, 54, 72, 90, and 109 (J/cm^2) of diode laser dose for 60, 120, 180, 240, 300, and 360 seconds at a power of $300mW/cm^2$. After irradiation, each concentration's 10μl bacterial sample was deposited on a glass slide for 20 minutes at room temperature and allowed to dry. The glass slides were rinsed for three times with and autoclaved PBS to remove adherent bacterial cells and covered with a cover slip.

For fluorescence high-resolution images, the confocal laser scanning microscope (Zeiss LSM 510-META, Germany) was used before and after irradiation. Immersion oil was used to fix the glass slides beneath a plan-apochromat 100x/1.40 Oil DIC M27 objective lens. Argon (488nm, 30mW) and HeNe (543nm, 1mW) lasers, both installed in the confocal laser scanning microscope, were used for excitation.

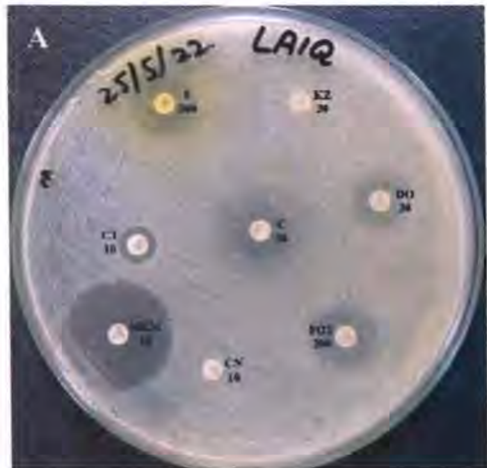
3.15. Statistical analysis

To ensure the accuracy of the results and the standard deviation of each experimental phase, each experiment was carried out three times. Microsoft Excel and OriginPro 2017 were utilized for data analysis, and one-way ANOVA software was employed for all statistical analyses.

Chapter 4 Results

4.1. *P. aeruginosa* and *S. aureus* antimicrobial susceptibility

The multi-drug-resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacterial strains were clinically isolated and identified by 16S rRNA sequencing. For the confirmation of their antibiotic resistance activity, antimicrobial susceptibility testing was performed, in which different classes of antibiotic discs were employed on Muller-Hinton agar plates for these bacterial strains, including: Meropenem (MEM), Gentamicin (CN), Fosfomycin (FOT), Colistin (CT), Chloramphenicol (C), Doxycycline (DO), Cefazolin (KZ), Nitrofurantoin (F), Tigecycline (TGC), Nalidixic Acid (NA), Ceftriaxone (CRO), Amikacin (AK), Aztreonam (ATM), Oxacillin (OX), Ampicillin (AMP), and Linezolid (LZD) The zone of inhibition was measured in millimeters (mm) as a result of their antibiogram activity, according to the CLSI guidelines 2020. Figure 4.1 shows the zones of inhibition around antibiotic discs, which depict the activity of antibiotics on bacterial strains.



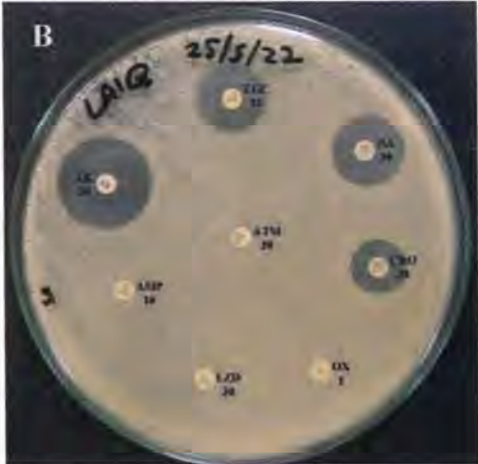


Figure 4-1: Display the antimicrobial activity of a) *P. aeruginosa* b) *S. aureus.*

Consequently, after measuring the zone around antibiotics, the bacterial strains were sensitive to very few antibiotics. The *Pseudomonas aeruginosa* was resistant to four, susceptible to three, and intermediate to a single class of antibiotics, which is mentioned in Figure 4.2 and Table 4.1. Also, Figure 4.3 and Table 4.2 depict the response of *Staphylococcus aureus* to antibiotic discs, which show a higher resistance of *S. aureus* to

antibiotics than *P. aeruginosa*. This gram-positive strain was resistant to five antibiotic discs and susceptible to three class of antibiotics.

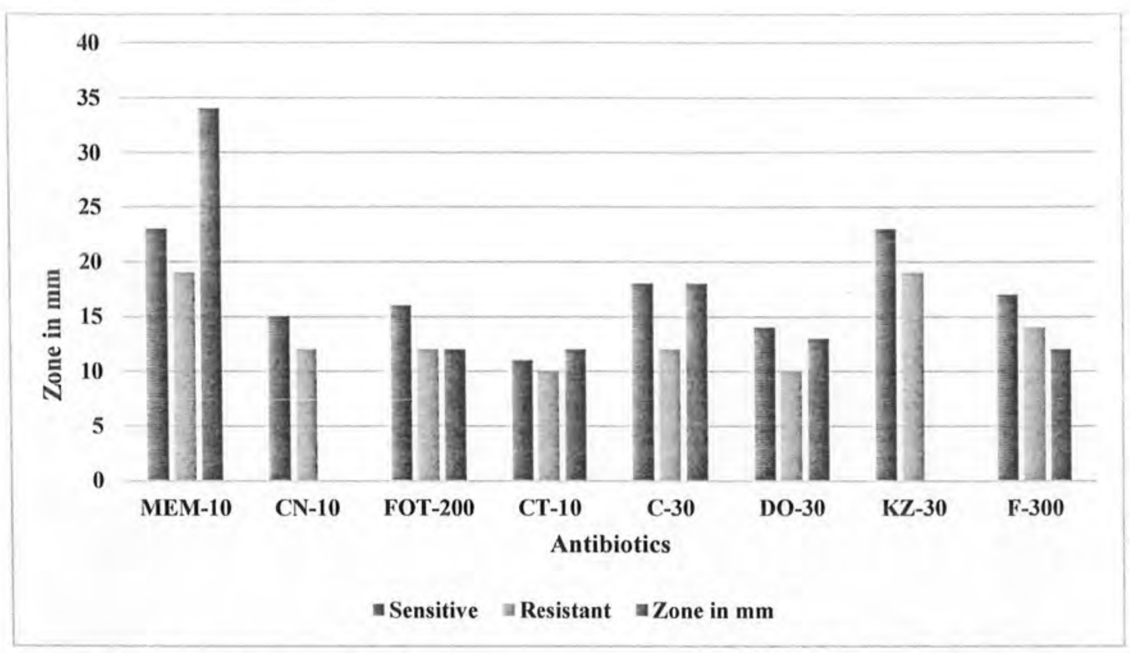


Figure 4-2: Antibiotic susceptibility of *Pseudomonas aeruginosa* according to CLSI 2020

Table 4-1: Antibiogram of *Pseudomonas aeruginosa*

Antibiotics	Concentrations	S/R/I
Meropenem (MEM)	10 µg	S
Gentamicin (CN)	10 µg	R
Fosfomycin (FOT)	200 µg	R
Colistin (CT)	10 µg	S
Chloramphenicol (C)	30 µg	S
Doxycycline (DO)	30 µg	I
Cefazolin (KZ)	30 µg	R
Nitrofurantoin (F)	300 µg	R
MM: Millimeter, R: Resistant, S: Sensitive, I: Intermediate		

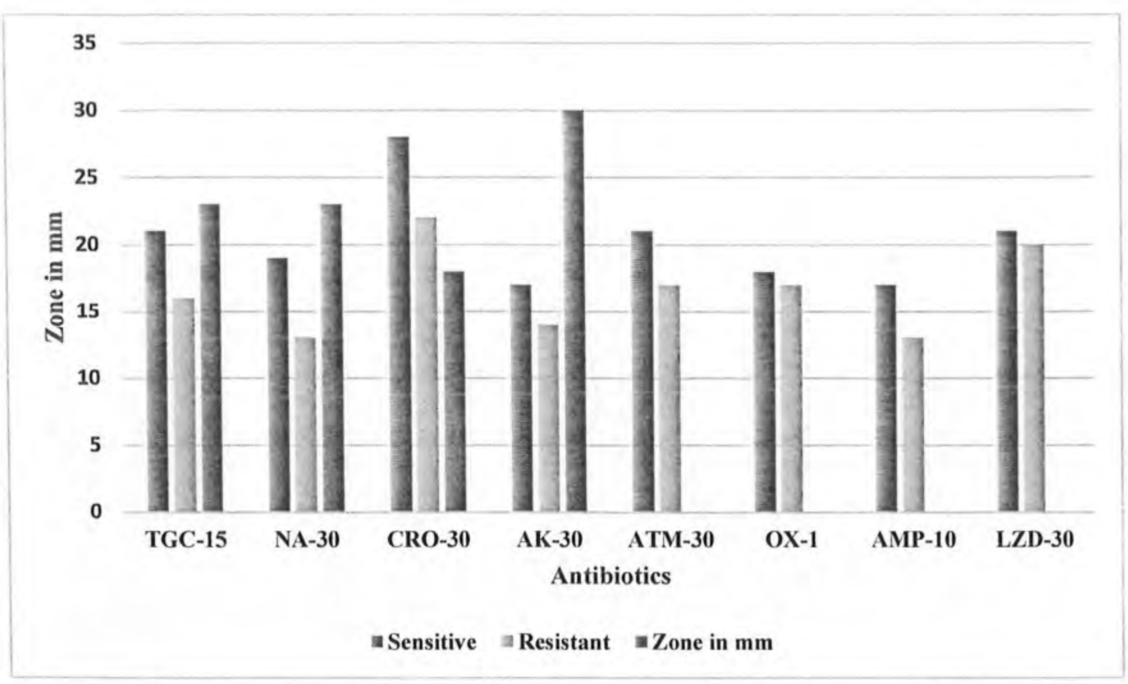


Figure 4-3: Antibiotic susceptibility of *Staphylococcus aureus* according to CLSI 2020

Table 4-2: Antibiogram of *Staphylococcus aureus*

Antibiotics	Concentrations	S/R/I
Tigecycline (TGC)	15 µg	S
Nalidixic acid (NA)	30 µg	S
Ceftriaxone (CRO)	30 µg	R
Amikacin (AK)	30 µg	S
Aztreonam (ATM)	30 µg	R
Oxacillin (OX)	1 µg	R
Ampicillin (AMP)	10 µg	R
Linezolid (LZD)	30 µg	R

MM: Millimeter, R: Resistant, S: Sensitive, I: Intermediate

4.2. Light induced degradation of methylene blue

Photodegradation is a normal phenomenon that occurs in normal compounds when they are exposed to light or irradiated by sunlight, as a result of light irradiation, they produce ROS by themselves. For the investigation of the dye degradation efficiency of the red light (635 nm) diode laser, photobleaching was performed in the lab. Consequently, this technique shows high activity of photodegradation of all methylene blue concentrations after exposure to different doses of light at specific time durations of 300mW/cm^2, as shown in Table 3.1. The photodegraded samples were compared with the untreated ones.

At 500 µg/ml the laser shows high photostability at 60, 120, and 180 sec with 18, 36, and 54 J/cm^2, but at 240, 300, and 360 sec with 72, 90, and 108 J/cm^2, it shows the best activity and changes the color from dark blue to transparent. Furthermore, 250 g/ml showed less activity on 60 and 120 seconds with 18 and 36 J/cm^2 and highest activity at 240, 300, and 360 seconds with 54, 72, 90, and 108 J/cm^2. Other all the concentrations were completely degraded after light exposure and changed the colour of methylene blue, as shown in Figure 4.4. Microplate spectrophotometer (INNO^{TM} & INNO-M^{TM}) were used to determine the optical density of methylene blue degradation before and after exposure to a diode laser, as mentioned in Figure 4.5. The higher the concentration of dye, the more exposure and time are required.

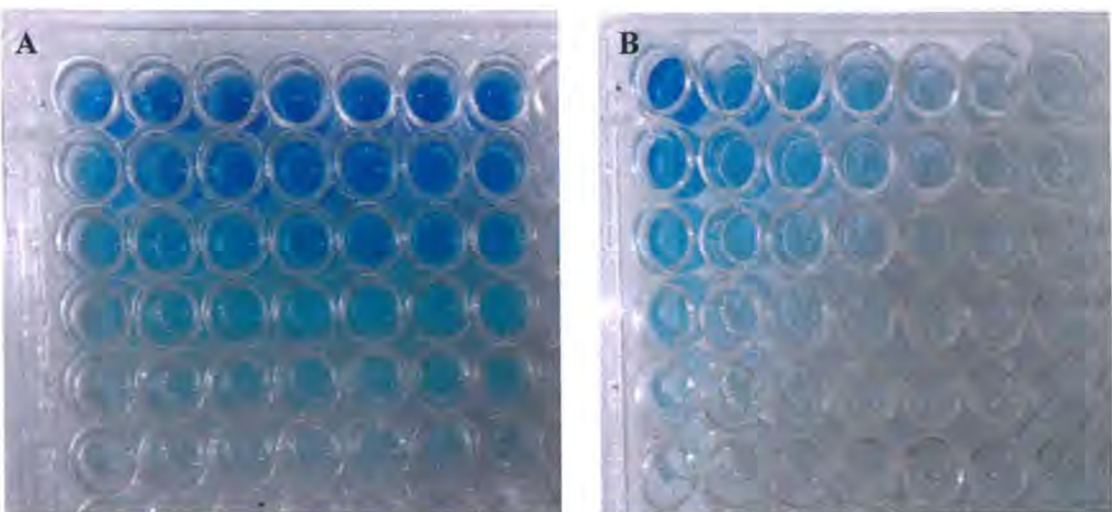
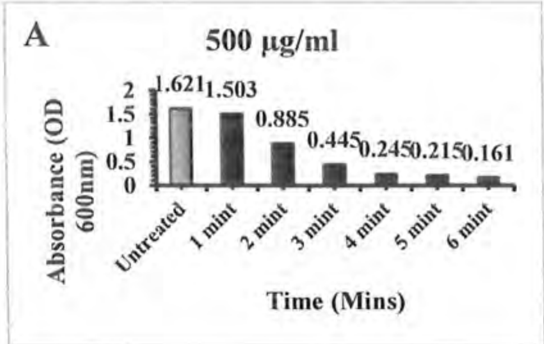
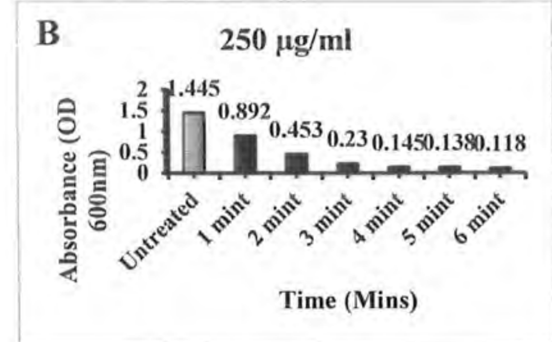
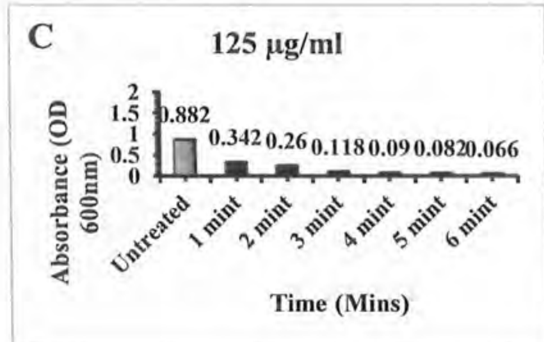
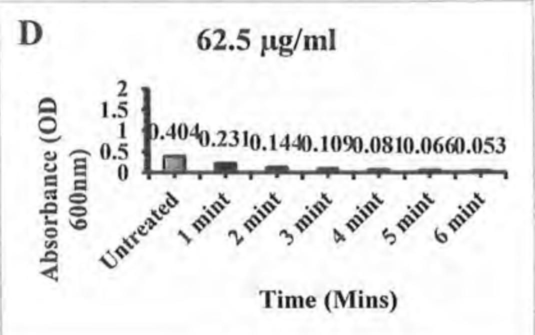


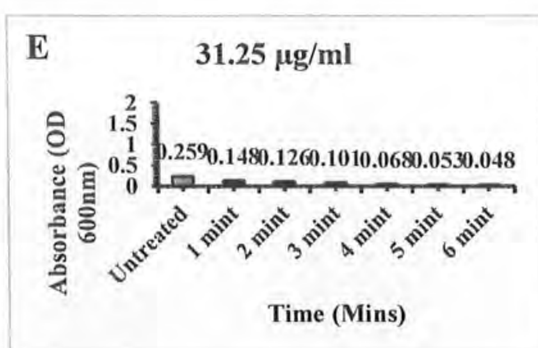
Figure 4-4: Photobleaching of methylene blue a) before and b) after diode laser exposure











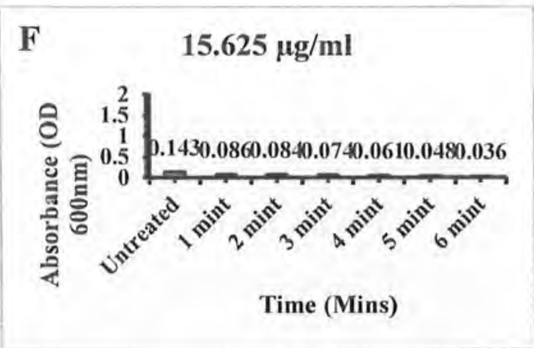


Figure 4-5: Optical density (OD 600nm) of methylene blue before and after photodegradation

4.3. Microbial induced degradation of methylene blue

In this experiment, methylene blue degradation was visually observed by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. After overnight incubation of bacteria with methylene blue in nutrient broth, a clear change was observed in the methylene blue color, which was degraded by bacterial strains, as illustrated in Figure 4.6. Several studies have shown that *Pseudomonas aeruginosa* and *Staphylococcus aureus* can degrade and decolorize various dyes, including methylene blue. Many bacterial strains

have enzymes that decolorize methylene blue in aqueous solutions, for example, peroxidase, laccase, reductase, and oxidase (Ikram et al., 2022). This study demonstrated that both the bacterial strains *P. aeruginosa* and *S. aureus* are resistant pathogenic strains that can efficiently decolorize methylene blue dye in aqueous solutions.

Figure 4-6: Illustrated the methylene blue decolorization by bacteria strains a) before and b) after incubation.

4.4. Evaluation of cytotoxicity of methylene blue in dark

Before aPDT the cytotoxicity of methylene blue was analyzed without irradiation at concentrations ranging from 15.625μg/ml to 500μg/ml. After 24 hours of incubation, the control and methylene blue-treated bacterial growth were compared, and it was known that there was no inhibition in both bacterial strains reported, as shown in Figure 4.7. Also, as Figure 4.8 depicts, the Muller-Hinton agar-containing plates were observed; the methylene blue was diffused in the MHA, but no zone of inhibition was created. It means that methylene has no activity without irradiation against pathogenic strain.

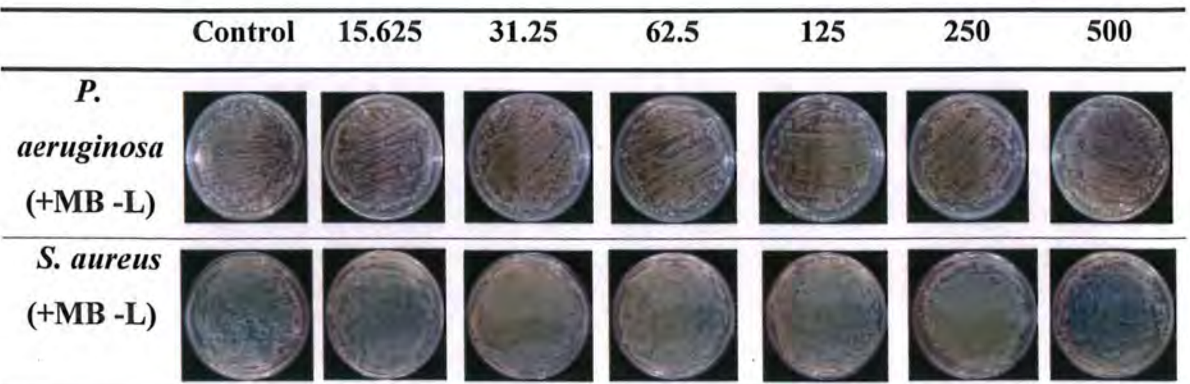


Figure 4-7: Depicts methylene blue's concentration (μg/ml) cytotoxicity in the absence of irradiation.

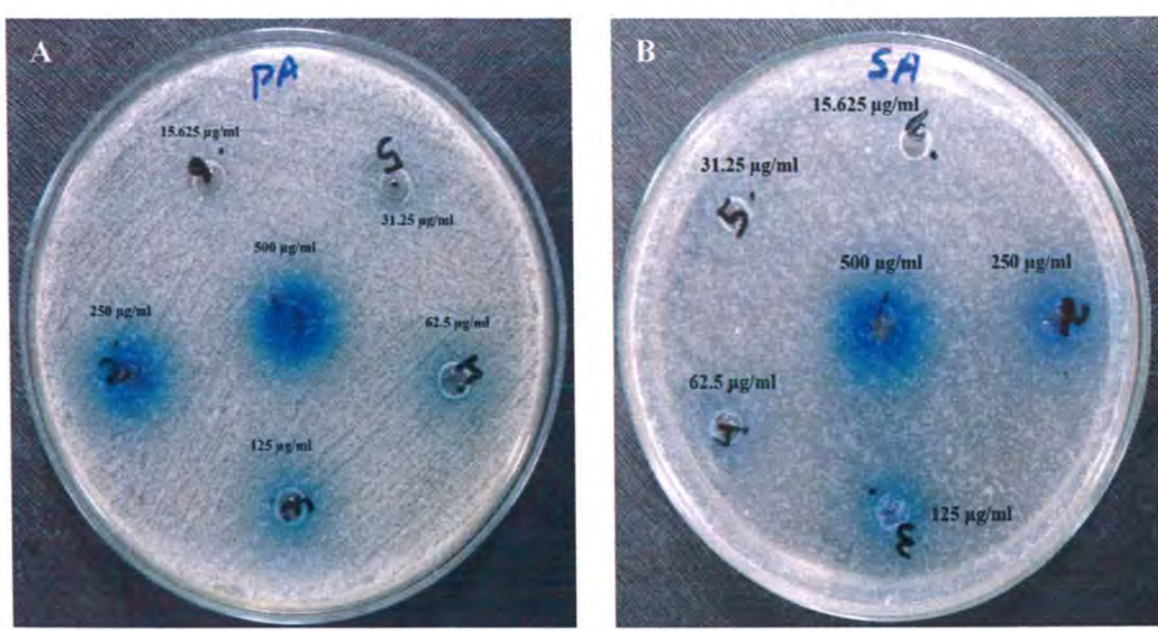


Figure 4-8: Demonstrate methylene blue's antimicrobial activity in the dark.

4.5. Methylene blue effect on bacterial growth in aPDT

Prior to looking into methylene blue effect in aPDT, the efficiency of diode lasers in the absence of methylene was reported on both bacterial strains. After exposure of bacterial strains to a diode laser in 96-well plates for a specific time and dose exposure, it was observed that there was no inhibition reported after treatment with the laser without methylene blue, as shown in Figure 4.9. The diode laser remained ineffective without the addition of methylene blue.

	Control	**60s**	**120s**	**180s**	**240s**	**300s**	**360s**
		18 J/cm^2	**36 J/cm^2**	**54 J/cm^2**	**72 J/cm^2**	**90 J/cm^2**	**108 J/cm^2**
***P. aeruginosa* (-MB +L)**							
***S. aureus* (-MB +L)**							

Figure 4-9: Show the efficiency of 635nm diode laser at 300mW/cm2 without methylene blue. (S: seconds)

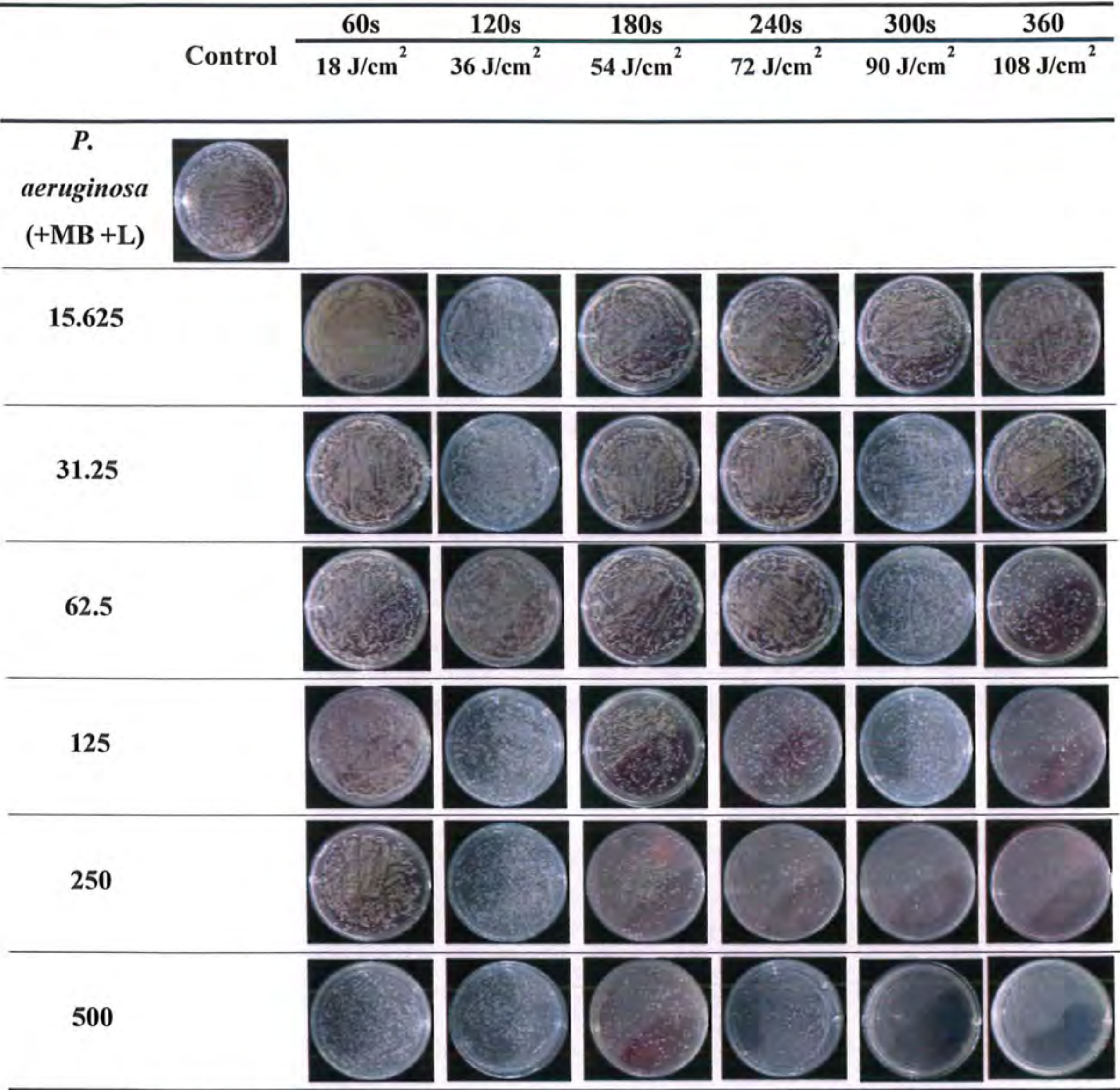


Figure 4-10: Effect of aPDT on Gram-negative *P. aeruginosa* at 300mW/cm2 (+MB +L). MB concentration (µg/ml) Energy (J/cm2) Time (Seconds)

Colony forming units (CFU/ml) were used to evaluate and quantify the antibacterial activity of methylene blue combined with 635nm diode laser light against *P. aeruginosa* and *S. aureus*.

Colony forming unit (CFU/ml) = number of colonies × dilution factor / volume of culture plated (Manzoor et al., 2022) as shown in Figure 4.10 and 4.11.

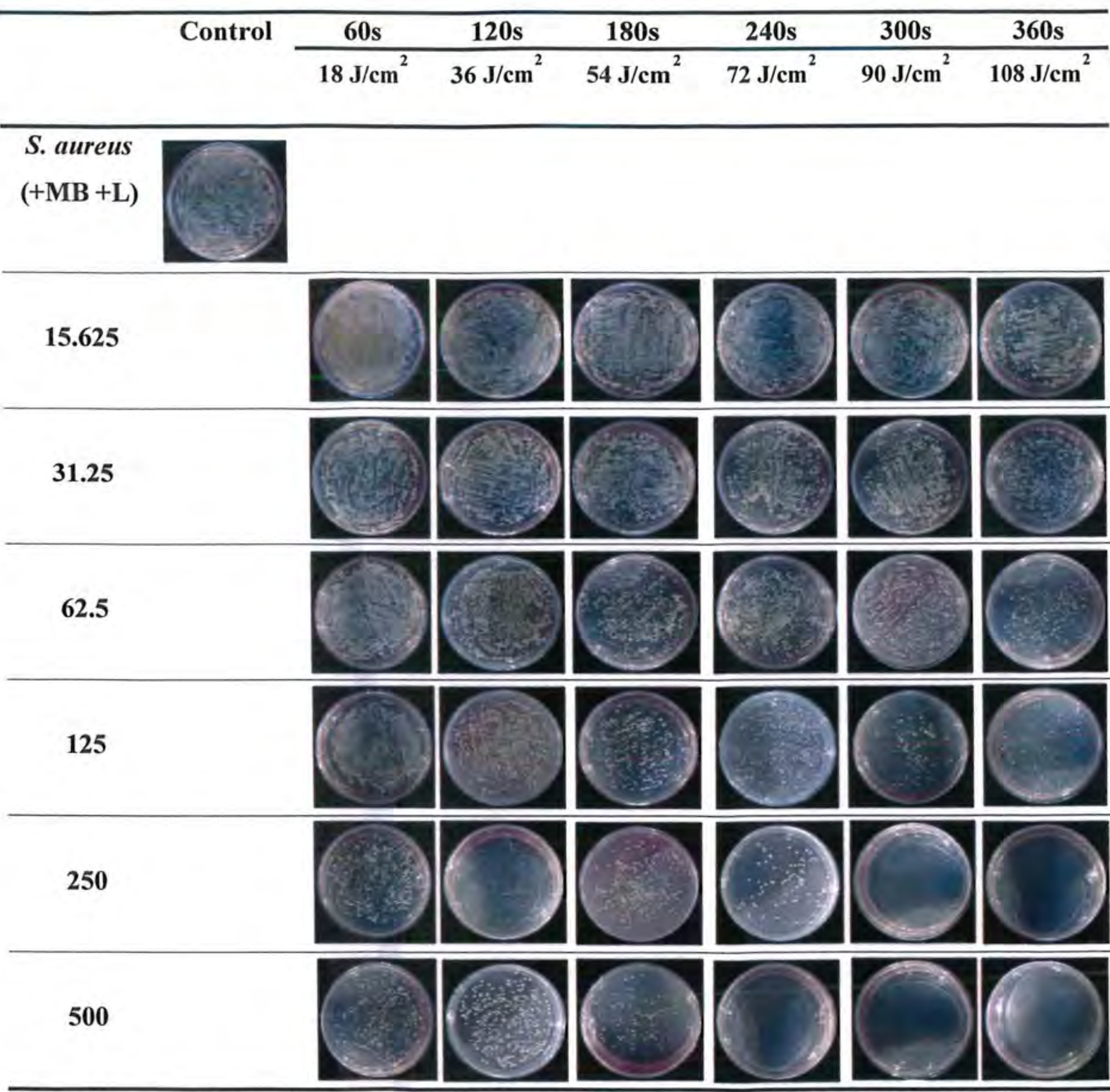


Figure 4-11: Effect of aPDT on Gram-positive *S. aureus* at 300mW/cm2 (+MB +L). MB concentration (μg/ml) Energy (J/cm2) Time (Seconds)

Methylene blue in photoinactivation shows great potency against both gram +ve and -ve strains. In the case of *P. aeruginosa,* a 100% reduction was observed at 300mW/cm^2 by 500 μg/ml after irradiation for 300 and 360 seconds (90 and 108 J/cm^2), and 94 to 99.72% was reported after irradiation for 60, 120, 180, and 240 seconds (18, 36, 54, and 72 J/cm^2). 100% reduction was also observed at 250 μg/ml after 360 seconds, 95–99.79% after 120, 180, 240, and 300 seconds, and 89% after 60 seconds of irradiation. After photosensitization for 60 to 360 seconds, 78.47 to 99.73% reduction by 125 g/ml, 66.44 to 97.89% reduction by 62.5 μg/ml, 54.89 to 94.48% reduction by 31.25 μg/ml, and

39.89 to 83.57% reduction by 15.625 µg/ml were reported, as shown in Figure 4.12 and Table 4.3.

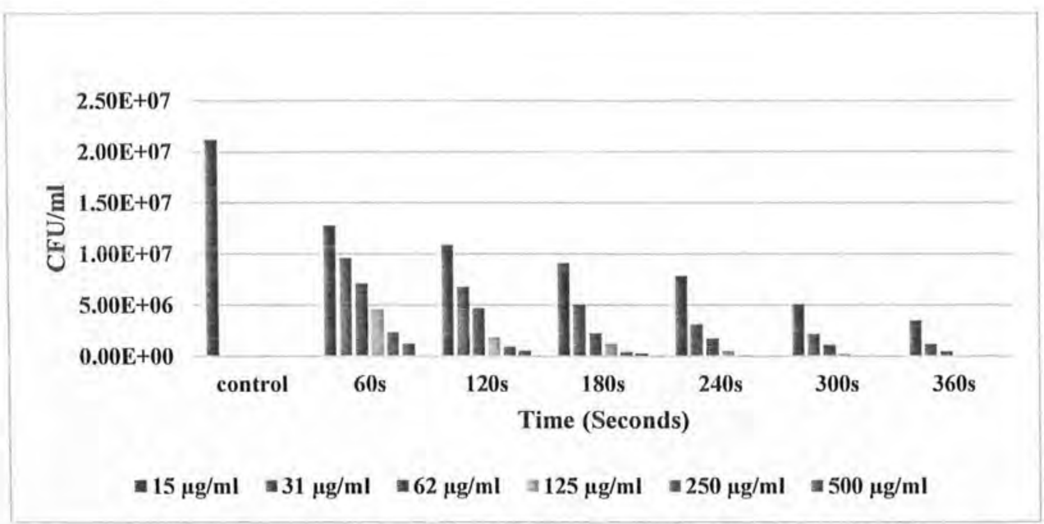


Figure 4-12: Colony Forming Unit (CFU/ml) *Pseudomonas aeruginosa* after aPDT (300mW/cm²)

Table 4-3: Growth reduction of *P. aeruginosa* after aPDT (300mW/cm²)

MB Concentration	60s 18 J/cm²	120s 36 J/cm²	180s 54 J/cm²	240s 72 J/cm²	300s 90 J/cm²	360s 108 J/cm²
15.625 µg/ml	39.86%	48.70%	56.93%	63.05%	76.11%	83.57%
31.25 µg/ml	54.89%	68.19%	76.12%	85.27%	89.80%	94.48%
62.5 µg/ml	66.44%	78.01%	89.35%	91.99%	94.86%	97.89%
125 µg/ml	78.47%	91.31%	94.41%	97.53%	99.01%	99.73%
250 µg/ml	89.04%	95.47%	98.04%	99.50%	99.79%	100.00%
500 µg/ml	94.26%	97.54%	98.86%	99.72%	100.00%	100.00%

The same experiment was performed for gram-positive Staphylococcus aureus, and a 100% reduction was observed at 500 µg/ml after exposure for 300 and 360 seconds, and a 94.81% to 99.95% reduction after 60, 120, 180, and 240 seconds. After 360 seconds of

exposure at 250 μg/ml, a 100% reduction was reported, and a 92.61–99.98% reduction was observed after irradiation for 60, 120, 180, 240, and 300 seconds. Furthermore, after irradiation for 60 to 360 seconds of S. aureus, 83.95 to 99.83% reduction by 125 μg/ml, 72.05 to 98.50% reduction by 62.50 μg/ml, 59.08 to 96.29% reduction by 32.25 μg/ml, and 49.22 to 87.19% reduction by 15.625 μg/ml were reported, as demonstrated by Figure 4.13 and Table 4.4.

500 μg/ml, 250 μg/ml, and 125 μg/ml show great potency, and 62.5 μg/ml show less activity than 500, 250, and 125 μg/ml but more than 31.25 and 15.625 μg/ml of methylene blue concentration against both multi-drug resistant strains. In comparison, *Pseudomonas aeruginosa* was a little bit less sensitive than *Staphylococcus aureus* to aPDT. The reduction in both bacterial strains after aPDT was reported from 3log to 6log reduction.

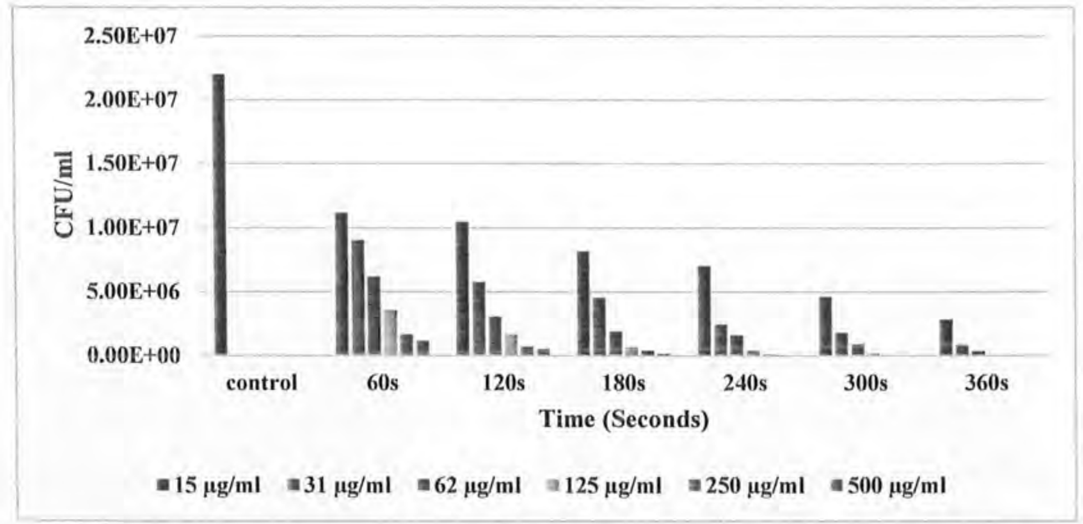


Figure 4-13: Colony Forming Unit (CFU/ml) *Staphylococcus aureus* after aPDT (300mW/cm²)

Table 4-4: Growth reduction of *S. aureus* after aPDT (300mW/cm²)

MB Concentration	60s	120s	180s	240s	300s	360s
	18 J/cm²	36 J/cm²	54 J/cm²	72 J/cm²	90 J/cm²	108 J/cm²
15.625 μg/ml	49.22%	52.42%	62.92%	68.18%	79.22%	87.19%
31.25 μg/ml	59.08%	74.01%	79.48%	89.01%	91.96%	96.29%

62.5 μg/ml	72.05%	86.27%	91.43%	92.90%	96.04%	98.50%
125 μg/ml	83.95%	92.70%	97.13%	98.23%	99.54%	99.83%
250 μg/ml	92.61%	96.93%	98.33%	99.67%	99.98%	100.00%
500 μg/ml	94.81%	97.74%	99.44%	99.95%	100.00%	100.00%

4.6. aPDT effect on optical density of bacterial strains

An optical density 0.8 was measured before antimicrobial photodynamic therapy by using microplate spectrophotometer (INNO™ & INNO-M™), at 600nm. A large fold of reduction in bacterial growth optical density was reported after photosensitization with methylene blue in conjugation with 635nm diode laser. The treated sample OD were compared with the control OD which show that *S. aureus* have higher reduction that *P. aeruginosa* as depicted in Figure 4.14 and 4.15.

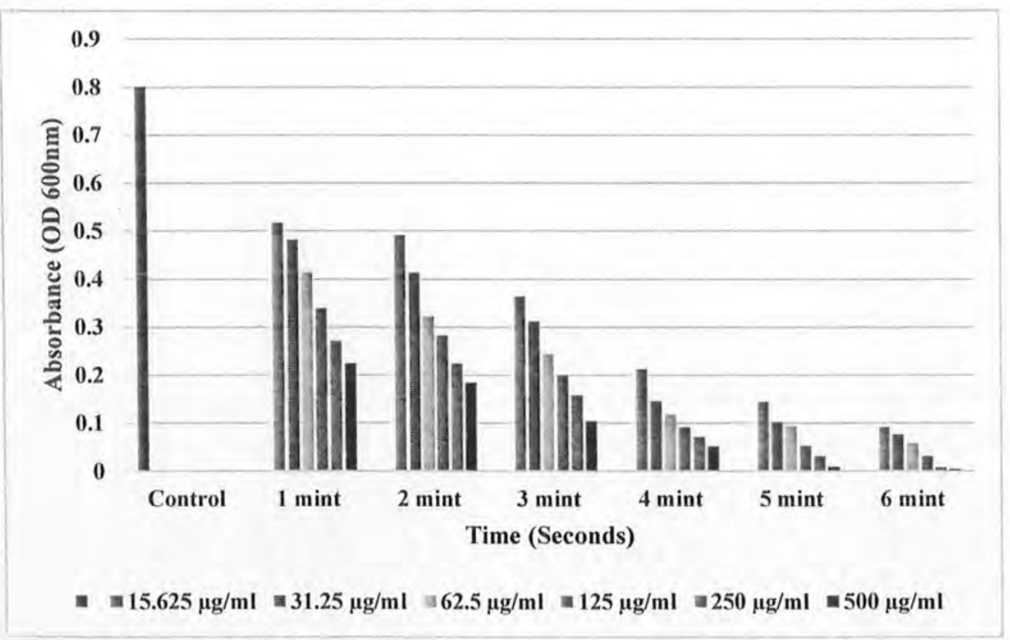


Figure 4-14: Demonstrate *P. aeruginosa* optical density (OD 600nm) after treatment.

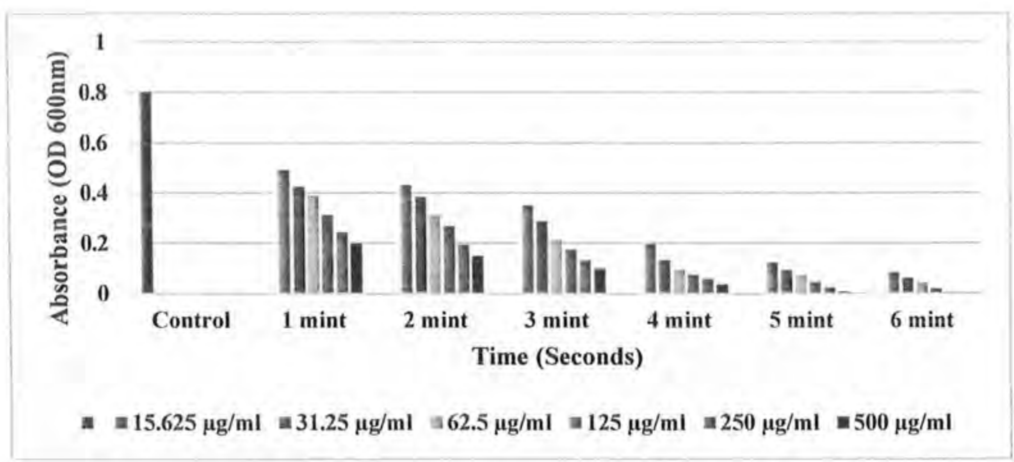


Figure 4-15: Demonstrate *S. aureus* optical density (OD 600nm) after treatment.

4.7. Emission spectra of for detection of bacterial inhibition

The emission spectra of *P. aeruginosa* and *S. aureus* were recorded immediately after aPDT with a 635nm diode laser at 300mW/cm^2 to determine the inhibition of the bacteria strains. By using the FluoroMax-4 spectrofluorometer (HORIBA Scientific, Jobin Yvon), the emission spectra were reported before and after photosensitization with MB and a diode laser, from 285 nm to 525 nm, with 270 nm of excitation. The 0.5 nm interval was set between emission and excitation. Numerous cellular biological compounds found in living bacteria have distinct excitation and emission spectra, which excite between 250 and 450 nm while their emission is between 280 and 540 nm in wavelength. Tryptophane is a highly fluorescent compound in bacteria cells that excites at 270 and 280 nm and emits at 330-350 nm (Ammor, 2007). This compound's strong spectral peak indicates the viability of bacterial cells (Du et al., 2022).

In this study, before diode laser treatment, both *Pseudomonas aeruginosa* and *Staphylococcus aureus* showed a strong emission peak between 340 and 350 nm with a 270nm excitation wavelength and an intensity greater than 1200000 CPS. According to the literature, this wavelength indicates the tryptophane compound present in both bacterial cells. Subsequently, right after every treatment, a complete spectrum was recorded, and a significant reduction was observed in this emission peak of both bacterial strains after photosensitization.

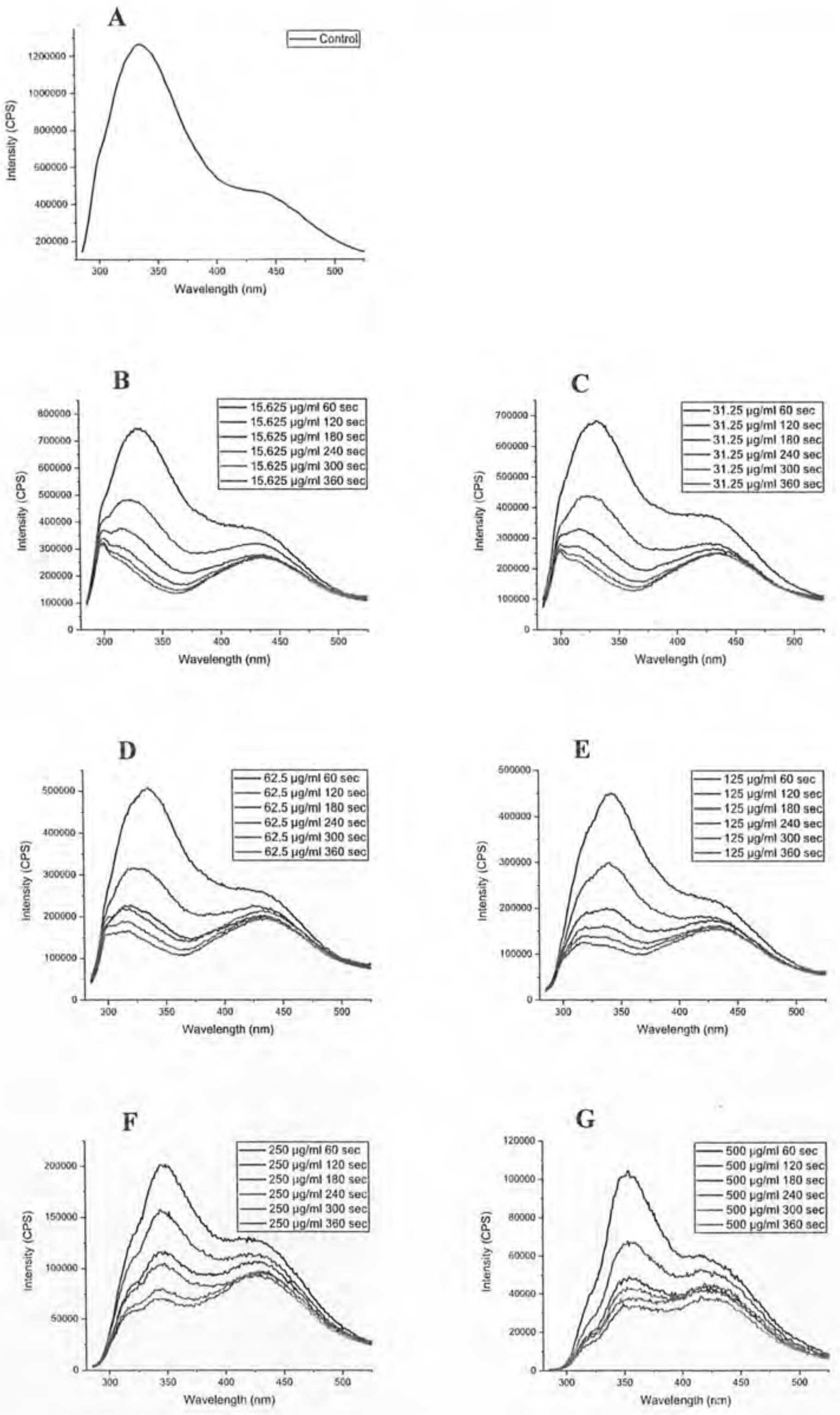


Figure 4-16: Emission spectra of *P. aeruginosa* before and after aPDT

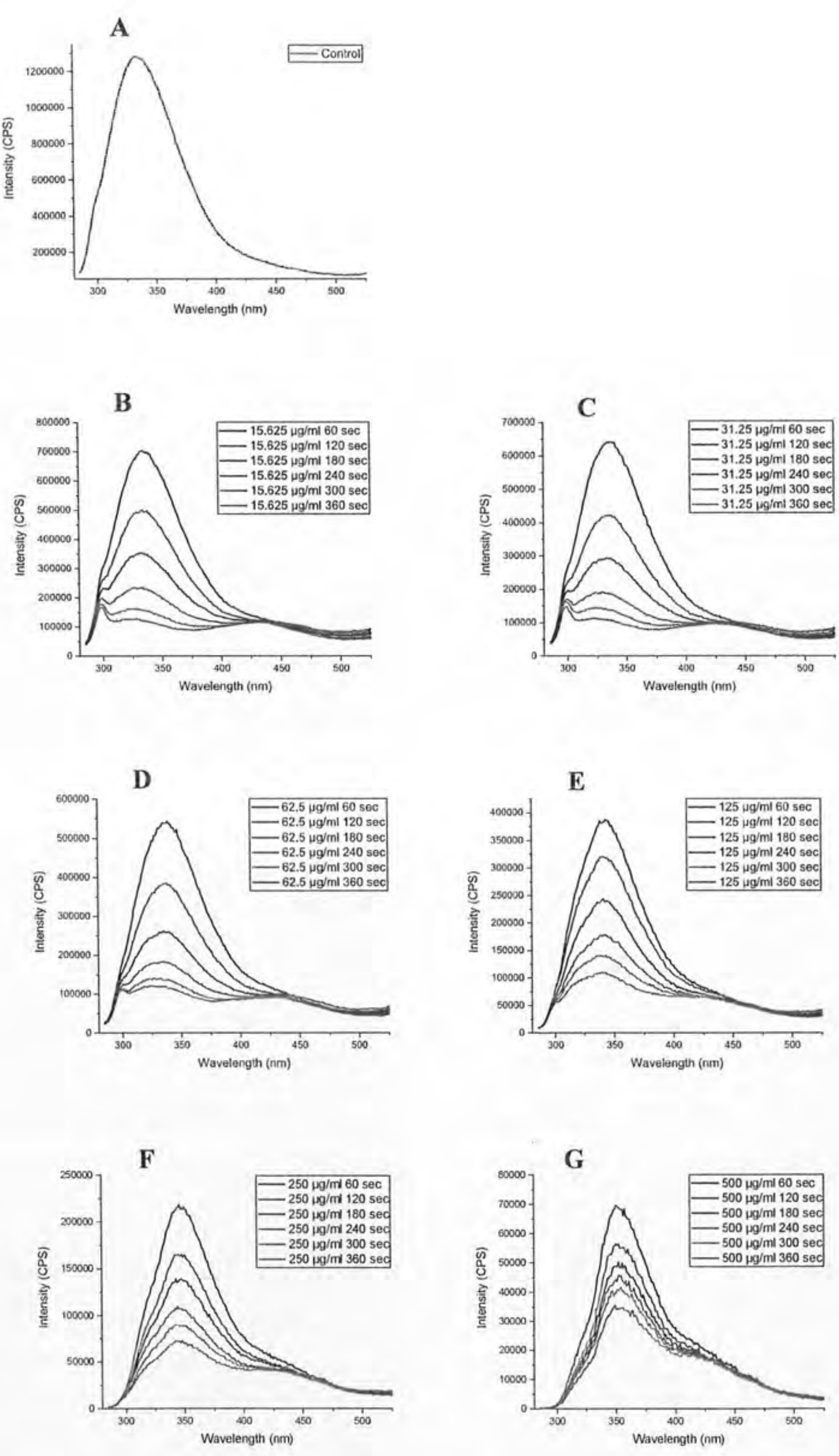


Figure 4-17: Emission spectra of *S. aureus* before and after aPDT

The tryptophane emission spectra of *P. aeruginosa* were reduced by 74% to 98% after treatment, while *S. aureus* was reduced by 84.8% to 99.9% from 15.625 to 500 μg/ml of methylene blue at 300mW/cm2, as shown in Figures 4.16 and 4.17. In addition, as compared to *S. aureus,* along with the tryptophane peak, another emission peak in *P. aeruginosa* was also documented. which had an emission range of 440–460 nm and an excitation wavelength of 410 nm. According to the literature, pyoverdines (siderophores) produced by *Pseudomonas aeruginosa* are indicated by their peaks at 450 and 460 nm (Alimova et al., 2003). Pyoverdines are virulence components required for *P. aeruginosa* toxicity and pathogenicity (Wendenbaum et al., 1983). After treatment with methylene blue and a diode laser, a significant reduction was also reported in this pyoverdine emission peak. As compared to *P. aeruginosa*, *Staphylococcus aureus* was more susceptible to aPDT according to the reduction in its emission spectra indicated in Figures 4.16 and 4.17.

4.8. Evaluation of bacterial viability using confocal microscopy

(Results are awaited)

Chapter 5 Discussion

One of the greatest accomplishments in the new global scenario was the identification of novel antimicrobial drugs, which boosted the expectancy of life. The discovery of penicillin was a massive advancement in antimicrobial medicine, but a year later, due to its extensive and inappropriate use against bacterial strains like *Staphylococcus aureus*, it showed resistance to them that grew up to about 50% after a decade (Alanis, 2005). In addition, the majority of antibiotics remain ineffective against many bacterial infections, which leads to illness that lasts longer than expected, disability, and mortality (Wise, 2002). Different types of antimicrobial resistance, including (MDR, XDR, PDR), can be classified; these include *S. aureus* and *P. aeruginosa*, etc. (Basak et al., 2016). As a result of the sequential introduction of antibiotics to microorganisms, they develop resistance to them, and ultimately antibiotics lose their efficiency against pathogenic microbes. The chance of spreading increases when infections remain in the human body (Zhu et al., 2022). According to the report of O'Neill et al., the mortality rate increased to 700,000 annually as a result of resistant infectious diseases (JIM O'NEILL, 2016).

The term "superbugs" refers to microbes that are becoming resistant to most antibacterial substances (Hofer, 2019). In bacteria strain the development of antibiotic resistance is a natural phenomenon, and patients with compromised immune systems, such as HIV-infected patients, diabetes patients, organ transplant recipients, and severely burned individuals, are particularly vulnerable to antimicrobial resistance and hospital-acquired infections (Tanwar et al., 2014). Antibiotic resistance poses a huge concern for treating patients with infectious diseases under the present conditions, which increases the risk of severe infection and death rates while also causing a major economic issue throughout the globe (Nathan, 2020). Consequently, the development of new treatment approaches that can tackle antimicrobial-resistant strains and infectious diseases without affecting the host tissues and cells is an immediate need (Tanaka et al., 2012). As a substitute for conventional antibiotic treatment, antimicrobial photodynamic therapy (aPDT) has evolved, which produces toxic reactive oxygen species by photosensitizing pathogenic microbes through photosensitizer and light. The efficiency of PDT for the treatment of

chronic infectious diseases caused by bacteria is emerging (Hamblin & Hasan, 2004; Misba et al., 2017).

In the current study, the main focus was to inactivate antibiotic-resistant bacteria by antimicrobial photodynamic therapy (aPDT). Methylene blue (MB) as an antibiotic alternative was used in combination with a 635nm red light diode laser at 300mW/cm^2, which exhibited a great effect against MDR *P. aeruginosa* and *S. aureus* as compared to antibiotics. As compared to other antimicrobial drugs, antimicrobial photodynamic therapy (aPDT) provides a number of benefits, such as no toxic effect for a long time, the capability to quickly eliminate pathogens, and the ability to be repeatedly used without leading to resistance (Tao et al., 2019).

In order to check the efficiency of the diode laser, methylene blue photodegradation was performed for specific exposure times and doses without the help of any secondary chemicals, which showed a significant result after irradiation. Best dye degradation and decolorization from 100% to 80% were reported at 360, 300, 240, and 180 seconds with 54, 72, 80, and 102 J/cm^2 at 300mW/cm2, but no activity was observed in the dark, as shown in Figure 4.6. In the study of Rather et al., the MB degradation activity was observed after 60 minutes by the use of specific visible light in combination with Ag, Au, Cu, and Tio_2 compounds (Rather et al., 2017). Ren et al., used a 100mW/cm^2 AM 1.5 G Xenon lamp for 10 to 50 minutes in combination with TiO_2, and 97.2% of methylene blue degradation was reported at 20 minutes (Ren et al., 2015). *P. aeruginosa* and *S. aureus* were employed for the evaluation of their efficiency in the biodegradation of methylene blue, after 24 hours of incubations, a significant change was reported in the decolorization of MB. According to the outcomes, it is proved that *P. aeruginosa* and *S. aureus* have the capability of 88-98% to degrade MB in liquid form (Ayed et al., 2022; Eslami et al., 2016).

The cytotoxicity of methylene blue alone (+MB -L) at concentrations ranging from 500 to 15.625 µg/ml and diode laser alone (+MB -L) was studied at various time durations and doses, but no activity of MB or laser alone was observed at any concentration, time exposure, or dose, as shown in Figures 4.7, 4.8 and 4.9. In contrast to this, the study by

Freitas et al., reported that 500 μg/ml exhibited toxicity against some bacterial strains (Freitas et al., 2019). Also, Briggs et al., show that methylene blue alone and laser showed little inhibition activity against *S. aureus* as compared to *P. aeruginosa,* where no activity was observed at any concentration or dose of MB or laser alone (Briggs et al., 2018). Methylene blue in combination with a 635nm diode laser at concentrations (15.625, 31.25, 62.5, 125, 250, and 500 μg/ml) and at a specific exposure time (60, 120, 180, 240, 300, and 360 seconds) and dose (18, 36, 54, 72, 90, and 108 J/cm2) of 300mW/cm^2 showed significant inhibition in both bacterial strains (*P. aeruginosa* and *S. aureus*) as depicted in Table 3.1.

In the case of *P. aeruginosa*, aPDT inhibited growth by 39.86% to 83.57% at 15.625 μg/ml, 54.89 to 94.48% at 31.25 μg/ml, 66.44 to 97.89% at 62.5 μg/ml, 78.47 to 99.43% at 125 μg/ml, 89.04 to 100% at 250 μg/ml, and 94.26 to 100% at 500 μg/ml. and the reported inhibition of *S. aureus* growth was confirmed by 49.22 to 87.19% at 15.625 μg/ml, 59.08 to 96.29% at 31.25 μg/ml, 70.05 to 98.50% at 62.5 μg/ml, 83.95 to 99.93% at 125 μg/ml, 92.61 to 100% at 250 μg/ml, 94.81 to 100% at 500 μg/ml. Consequentially, MB + Laser in photoinactivation showed great potency at 500, 250, 125, and 65.5 μg/ml at 360, 300, 240, and 180 seconds as shown in Figure 4.10 and 4.11, Table 4.3 and 6. In a study, a 664nm red diode laser in combination with methylene was used against *S. aureus* and *P. aeruginosa,* and after a single therapy, a 99.9% reduction was reported in both bacterial strains (Biel, Sievert, Usacheva, Teichert, Wedell, et al., 2011). Peloi et al., observed a 70 to 85% Of reduction in the growth of *S. aureus* by using methylene blue at concentrations from 7 to 14 μM with light emitting diode (LED: 6 J/cm^2) for 30 minutes of exposure (Peloi et al., 2008). The morphological differences between the two species can account for this variation in sensitivity and resistance. Gram-positive bacteria are more susceptible to aPDT than gram-negative bacteria due to structural differences. Gram-positive bacteria have an outer membrane that is permeable and porous, but gram-negative bacteria have a complex outer membrane (Pereira et al., 2014).

The efficiency of aPDT was demonstrated by optical density at 600nm and fluorescence spectroscopy, the emission spectra were reported from 285 nm to 525 nm, with 270 nm of excitation. A significant reduction was reported in these two methods. Two emissions

peaks were demonstrated, first, 340 to 350nm with 270 excitation and second from 440-460 with 405nm excitation. The first emission peak represented tryptophane and the second was a pyoverdine compound in *Pseudomonas aeruginosa.* The second peak was absent in *S. aureus* as shown in Figures 4.16 and 4.17. The literature discussed that Tryptophane is a fluorescent compound in bacteria that excites at 270 and 280 nm and emits at 330-350 nm, which indicates the viability of bacterial cells (Ammor, 2007; Du et al., 2022). Also, Wendenbaum et al., discussed that pyoverdines are virulence compounds also known as siderophores which are produced by *Pseudomonas aeruginosa* and are indicated by their peaks at 450 and 460 nm and required for toxicity and pathogenicity (Alimova et al., 2003; Wendenbaum et al., 1983). In a study, Pérez-Lagun et al., observed 6log10 inhibition of *P. aeruginosa* and 3 log10 inhibition of *S. aureus* by photosensitization of methylene blue (20 μg/ml and 0.62 μg/ml) with 635nm red light LED for 18 J/cm^2 at $7mW/cm^2$ intensity (Pérez-Laguna et al., 2020).

Our study revealed that aPDT is a best substitute to antimicrobial drugs and promising approach for coping antimicrobial resistance.

Chapter 6 Conclusions

This study demonstrates the best alternative approach to antibiotics, which can eliminate multi-drug-resistant pathogenic bacterial strains very efficiently and can cope with antimicrobial resistance. We conducted the inactivation of antibiotic-resistant bacteria by antimicrobial photodynamic therapy (aPDT) experiments in our lab, which showed that methylene blue in conjugation with laser light is a potential substitute for antibiotics that effectively inactivate MDR *Pseudomonas aeruginosa* and *Staphylococcus aureus*. For this purpose, we use different concentrations of methylene blue, from 15.625 to 500 µg/ml, for specific exposure times and laser doses with an intensity of 300mW/cm2, treated by a 635 nm diode laser. All the results were reported by CFU/ml, optical density, fluorescence spectroscopy, and confocal microscopy, which show a significant 3-log to 6-log reduction and inhibition of bacterial strains that were resistant to antibiotics. There was no resistance observed when compared to antibiotics, and *S. aureus* was more susceptible than *P. aeruginosa* to aPDT, as there was a 39.86% to 100% reduction in *P. aeruginosa* and a 49.22% to 100% reduction in *S. aureus*. This activity proves that aPDT is the future of clinical medicines that can decrease the level of antimicrobial resistance (AMR) around the globe.

Future Perspectives

Antimicrobial photodynamic therapy is the best approach for coping with antimicrobial resistance to eliminate antibiotic-resistant bacterial strains, but to make it more proficient and selective, we have to follow the following steps in the future.

1. Optimize the toxicity and lethal effect of photosensitizers by using cell lines and animal models to avoid damage to human tissue and cells.
2. To improve the efficacy and toxicity of photosensitizers, different nanoparticles and drugs must be conjugated with them.
3. To improve microbial selectivity, photosensitizers must be coated with bacterial-compatible proteins that attach to cell wall of bacteria and outer layer (peptidoglycan), causing bacteria to be disrupted.
4. For *in vivo* drug delivery, photosensitizers should conjugate with self-assembled, degradable, PH, and enzyme-tolerant protein-based nanocomposites, which can protect PSs.
5. The anti-viral, anti-fungal, and anti-parasitic potential of PDT can be uncovered to extend its use in environmental decontamination.

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