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MASTER OF SCIENCE (MSc) THESIS



**DETERMINATION OF ANTIBACTERIAL ACTIVITIES OF SOME
SALICYLALDIMINE DERIVED-SCHIFF BASE WITH THEIR COPPER
COMPLEXES ON SOME PATHOGENIC BACTERIA**

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ÖZET

Yüksek Lisans Tezi

BAZI SALİSİLALDİMİN TÜREVİ SCHIFF BAZI BAKIR KOMPLEKSLERİNİN BAZI PATOJENİK BAKTERİLER ÜZERİNDEKİ ANTİBAKTERİYEL AKTİVİTELERİNİN BELİRLENMESİ

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Antibakteriyel ilaçlara karşı artan mikrobiyal direnç sorunuyla mücadele etmek için günümüz dünyasının dirençli bakterilere karşı güçlü kimyasal veya doğal antibakteriyel ilaçlara yönelik acil ihtiyacı kritik öneme sahiptir. Çalışmamızda, 8 Salisilaldimin türevi Schiff baz bileşiminin (ligandlar ve bunların bakır kompleksleri) ve gentamisin'in (pozitif kontrol) antibakteriyel etkisi, *Klebsiella pneumonia*, *Proteus mirabilis* ve *Streptococcus pyogenes* gibi patojenik bakteri suşlarına karşı antibakteriyel etkisi MTT testi ile belirlendi. Bileşikler 1, 10, 100 ve 1000 μM olmak üzere farklı konsantrasyonlarda eklendikten sonra standart dozda bakteri ile kültür edilmiştir. Kültür sonrası kuyucuklara MTT boyası eklendikten sonra oluşan formazan kristallerini çözme için dimetil sülfoksit eklendi. Her bir kültür kuyucuğu için optik dansite değerleri 570 nm'de bir Microplate okuyucu ile belirlendi. Her bir bileşiğe ait MIC₅₀ değeri hesaplandı. Gentamisin'in MIC₅₀ değeri *P. Mirabilis*, *S. Pyogenes*, *K. pneumonia* için sırasıyla; 1.6 μM , 1.8 ve 2.4 μM olarak izlemiştir. Bileşiklerin *S. pyogenes* için MIC₅₀ aralığı 1.9-3.9 μM , *K. pneumoniae* için 5.7 - >100 μM ve *P. mirabilis* için 3.9 - >100 μM olarak belirlenmiştir. Çalışmamızda, gram negatif bakteriler üzerinde C3 ve *S. pyogenes* (Gram pozitif) üzerinde C1 en iyi antibakteriyel aktiviteye sahipken, C6 dışındaki bileşiklerin neredeyse tamamı orta düzeyde aktiviteye sahiptir. Bakteri bileşiklere tepkileri ile ilgili olarak, bakır komplekslerinin bakteriler üzerinde ligandlardan daha etkili olmasının nedeninin farklı yapılarından kaynaklanmış olabileceğini düşünmekteyiz.

ANAHTAR KELİMELER: Antimikrobiyal, Schiff bazı, Salisilaldimine, MIC₅₀, MTT testi.

ABSTRACT

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DETERMINATION OF ANTIBACTERIAL ACTIVITIES OF SOME SALICYLALDIMINE DERIVED-SCHIFF BASE WITH THEIR COPPER COMPLEXES ON SOME PATHOGENIC BACTERIA

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The urgent request of potent chemical or natural antibacterial drugs by the nowadays world against antibacterial-resistant bacteria is critical in order to combat the raising microbial-resistance issue toward antibacterial drugs. In our study, we checked the antibacterial action of (8) Salicylaldimine derived Schiff-bases compounds (Ligands and Copper complexes) and Gentamicin (positive control), by antibacterial MTT-assay against three pathogenic bacterial strains of: *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus mirabilis* (*P. mirabilis*) and *Streptococcus pyogenes* (*S. pyogenes*). The compounds have been prepared in different concentration of 1 μ M, 10 μ M, 100 μ M and 1000 μ M, then have been cultivated with adjusted standard number of standard bacteria, then after the application of the MTT-stain, and adding dimethyl sulfoxide for crystal dissolving, the optical density (OD) values for each well were recorded by an Microplate reader at 570 nm and MIC₅₀ value of any compound have been calculated. In result, the most effective MIC₅₀ value of Genta was (1.6 μ M) for *P. mirabilis*, followed by (1.8 and 2.4 μ M) for both *S. pyogenes* and *K. pneumoniae*, respectively. And the compounds MIC₅₀ range for *S. pyogenes* was 1.9-3.9 μ M followed by (5.7 to >100 μ M) for *K. pneumoniae* and (3.9 to >100 μ M) for *P. mirabilis*, respectively. In our study, C3 has the best activity on gram negatives and C1 on *S. pyogenes* (Gram positive), while almost the entire compounds have moderate activity except of C6. In regarding with different bacterial response, the ligands with their Copper complexes had showed better effect on these bacteria, which may be due to their different cellular structure.

KEY WORDS: Antimicrobial, Schiff-base, Salicylaldimine, , MIC₅₀, MTT test.

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LIST OF SYMBOLS and ABBREVIATIONS

AMR	Antimicrobial Resistance
ATCC	American Type Culture Collection
B-lactam	Beta lactam
CDC	Centers for Disease Control and Prevention
C	Compound
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CU	Copper
DHFR	Dihydrofolate Reductase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EC50	Effective Concentration 50 percent
ESBL	Extended Spectrum Beta Lactamase
ELIZA	Enzyme Linked Immunosorbent Assay
FQ	Fluoroquinolones
FTIR	Fourier Transform Infrared Spectroscopy
Genta	Gentamicin
mM	Milli Molarity
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
μ l	Microliter
M	Molarity
μ M	Micro Molarity
MDR	Multidrug Resistance
MRSA	Methicillin Resistance Staphylococcus Aureus
MIC	Minimum Inhibitory Concentration
MTT	Methyl Thiazolyl diphenyl-Tetrazolium bromide
OD	Optical Density
PABA	P. aminobenzoic Acid
PBP	Penicillin Binding Protein
RNA	Ribonucleic Acid
SB	Schiff Base
SD	Standard Deviation
TET	Tetracycline
TLC	Thin Layer Chromatography
UTI	Urinary Tract Infection
WHO	World Wide Organization

1. INTRODUCTION

Antimicrobial-resistance has become a serious enemy for human health, with its demand to increase morbidity and mortality, Costing Country and the patients a lot of money, with extending patient-hospital staying (Morales et al., 2012; Rosenberger et al., 2011).

Streptococcus pyogenes causes a variety of serious human illnesses, such as Streptococcal "Toxic-shock" syndrome and Septicemia (Isaacs and Dobson, 2016). Regarding to WHO's figures at 2005, all peoples infected globally are (18.1 million) people, with 1.78 million new cases, and 500,000 case deaths (World Health Organization, 2005). Depending on temporal and geographic context on northern-European setting, highly variable rate of resistance to Clindamycin and Erythromycin have been recorded among species of β -hemolytic *Streptococcus species* such as *S. pyogenes*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae*, on the other hand, the genes *lnu* and *lsa*, which cause lincosamide resistance, are occasionally reported as well. In *S. pyogenes*, all those resistance genes occur by either clonal expansion mechanism or horizontal genetic transfer mechanism (Oddvar O et al., 2020). On the other hand, Fluoroquinolones (FQ) and Tetracycline's (TET) resistance among Streptococci strains especially *S. pyogenes* are often reported in high rate (Alves et al., 2020).

Members of the *Enterobacteriaceae* family, such as *Klebsiella pneumoniae*, *E. coli*, and *Proteus spp.*, commonly develop antibiotic resistance quickly and spread across hospitals (Arias and Murray 2009). Jankauskait et al, 2016 studied that rising demand for antibiotics among general public, agricultures, hospitals and veterinary has resulted in an accumulation of antibiotics in the environment and posing a worldwide issue for mankind by developing Antibiotic resistance among previously susceptible pathogens. *E. coli*, Methicillin-resistant strain of *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *K. pneumonia* are some examples of the most problematic multidrug resistant (MDR) bacteria now encountered.

K. pneumoniae is a member of *Enterobacteriaceae* family, which is one of the common pathogenic infections agents all over the world. In regarding to recent studies, *K. pneumoniae* had shown resistance to Colistin drug, this may because of mutational-inactivation of the mgrB regulatory gene. Centers for Disease Control and Prevention (CDC) organization, WHO and the UK Department of Health have all listed Multidrug resistance *Klebsiella pneumoniae* as a serious threat to human health (Kidd et al, 2017). Fluoroquinolones are Broad-spectrum antibiotics that are widely used. According to a recent study (Geetha et al, 2020), antibiotic resistance has recently increased among *K. pneumoniae* strains against Fluoroquinolones.

Proteus is another member of the family *Enterobacteriaceae* that live commensally in human digestive system, among them, *Proteus mirabilis* and *Proteus vulgaris* are common human pathogens that have been reported, which have naturally resistance to Polymyxins (Colistin), Nitrofurans, Tetracycline and Tigecycline. *P. mirabilis* commonly cause urinary tract infection (UTI), in which the rapid spread of multidrug-resistant have been isolated, particularly those that produce ESBLs, Cephalosporinases, AmpC type B-lactamase, and Carbapenemases, which are becoming increasingly prevalent. Recently, it showed high resistant to Imipenem due to loss of Penicillin binding proteins and reduced Porins. On the other hand, *P. vulgaris* produces class A-cefuroxime that show resistance to first and second-generation Cephalosporin and Amino-penicillin, in the exception of Cefoxitin (Girlich et al., 2020).

SB is any compound with structure $R_1R_2C=NR_3$ (in which R_2 and R_3 are not hydrogen), also called imine compound, because has Carbone-Nitrogen double bond (Abu-Dief and Mohamed, 2015). SB can be found in nature or through modifying a natural source, or through synthetic synthesis (Da silva et al., 2011).

SB compounds are widely used in industrial fields, which show an enormous range of biological activity such as Antimicrobial (antimalarial, antiviral and antibacterial), along with anti-cancer actions (Przybylski et al., 2009) , on the other hands it shows other biological actions, for example, anthelmintic and anti-

tuberculosis, anti-inflammatory, antioxidant, antidepressant activities (Uddin et al., 2020). For example, 2-Salicylideneaminophenol has a MIC of 8 Micro-gram/mL and is effective against *Mycobacterium tuberculosis* bacteria. An experiment has been conducted for testing selectivity of this Schiff base compound on J774-macrophages, and there was no cytotoxic effect against macrophages and more than 80 percent of them stayed alive, even though the concentration have been increased as 1000 Microgram /mL concentration, which revealed that this compound had great selectivity (Da silva et al., 2011; Souza et al., 2007).

Metal-complexes of SB have been searched and recommended by many researches which may express higher biological effect. For example, in (Emmanuel et al, 2018) investigation, Copper-complex (Cu complex) from SB has been synthesized and tested through agar-disk diffusion assay on ten bacterial strains, which are (*K. pneumonia*, *E. coli*, *P. vulgaris*, *S. aureus* and *Salmonella typhi*), Ampicillin used as antimicrobial positive control. In results, the metal-complex Schiff base compound had better activity by looking at its larger inhibition-zone than original Ampicillin.

In the other hands, in another study, cobalt ion-complexes of SB had been synthesized and chemically checked by several techniques such as FTIR, elemental analysis, molar and electron spectra conductivity. According to the activity data, Antibacterial effect of previous Schiff-bases was better than cobalt-complexes, even though synthesized compound had shown antibacterial effect against *S. aureus*, *S. pyogenes*, *S. agalactiae*, and *B. anthracis* which are gram positive and *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* which are gram negative (Saghatforoush et al. 2009).

(Bavya et al., 2019) showed that Incorporation of gentamicin encapsulated by zein nanoparticles combined with Schiff base and prepared in chitosan gel matrix, demonstrated antibacterial synergism activity. The Schiff-Base complex with Gentamicin Cause cell lyses due to disrupting integrity and showed low toxicity

against L929 murine fibroblast cell line, and suggested to use against resistant bacterial infections.

In our study, the biological actions of Schiff base and metal complexes have been investigated through MTT-analyses method to develop new drug with salicylaldehyde derived compounds and their copper complexes against *S. pyogenes*, *K. pneumonia*, and *P. mirabilis* bacterial strains, in order to design a Schiff base complex with better antibacterial activity than previous antibacterial drugs by testing our complex against these resistance acquired pathogens.



2. LITERATURE REVIEW

2.1. Antimicrobial Drugs

2.1.1. Definition of antimicrobial

Antimicrobials by definition are diverse group of natural, semi-synthetic or synthetic compound, which have effect on different classes of microbial growth. The effects are diverse and differ from one categorical microorganism (Bacteria, Virus, Fungi or Parasite) to another category (MADIGAN et al., 2021; L, Paul et al., 2018).

Antimicrobial can be classified according to its effect against a group or multi-group of microbes as antibacterial, antiviral, antifungal and anti-parasitic drug. And further can be classified by the outcome of using desired antimicrobial, either its activity on killing microbes (with the suffix-cidal) or stop growing of microbes (with the suffix- static).(Willey et al., 2020; L, Paul et al., 2018).

2.1.2. Antibiotics and antibacterial drug

Antibacterial are the drug of choice against bacterial microbes that have selective toxicity as little as it can damage the normal human tissue and cell, and this can be accommodate by its diverse mode of action on bacterial cell structures that is unique for bacterial cell (Willey et al., 2020; L, Paul et al., 2018).

The sources of antimicrobial drug generally, and antibacterial specifically can be different. The most common and historical source to obtain antimicrobial are natural source. Naturally produced antimicrobial are the origin of molecular microbiology, the best term to describe this source is antibiotic. Antibiotics are chemical byproduct compound which are synthesized by molds or bacteria, and play important ecological role in agriculture and served as human defense against pathogenic microbes, for Example penicillin and streptomycin. On the other hand

chemically modified semi-synthetic antimicrobials that derived from previously discovered antibiotics, and chemically improved to increase spectrum of action (to have Broder spectrum) such as Ampicillin, or against specific species that got resistant against original antibiotic or naturally has resistant, such as oxacillin that have been improved from original penicillin, that previously lost its effect against penicillin-resistant strain of staphylococcus aureus. The final form source of antimicrobial is chemically synthesized compounds that historically have been used against infection, or originally synthesized for other purpose and then accidentally tested for therapeutic purpose, such as sulfonamide and Schiff-base derived compound as well (Willey et al., 2020; L, Paul et al., 2018).

2.1.3. Antibiotic and antibacterial drug classification

Classification of antibiotics rely their actions on bacteria's various targets, its cell wall synthesis, Protein synthesis (ribosomes), RNA polymerase, nucleic acid-synthesis enzymes, Cytoplasmic cell membrane, as depicted at (Figure 2.1).

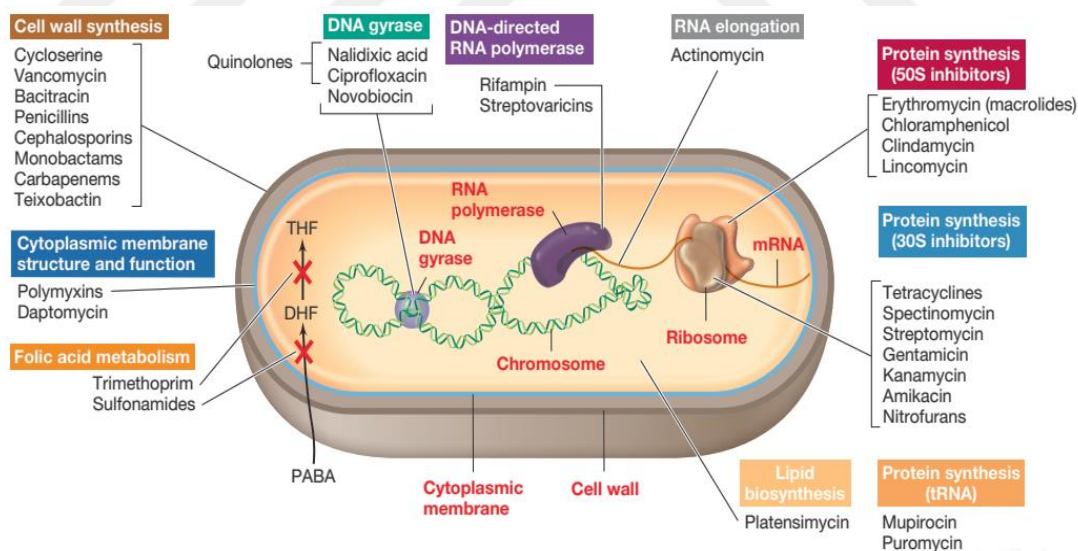


Figure 2.1. Antibacterial mechanism of action on different bacterial target (Madigan et al., 2021).

2.1.3.1. Cell wall as antibacterial target (B-Lactams)

Approximately 100,000 tons of Antimicrobial are synthesized and produced worldwide, and B-Lactams accounts the majority of them which are penicillin and

cephalosporin, approximately around 65% of whole antimicrobials. Majority used for agricultural purpose. B-Lactams are those antibiotics that have B-Lactam ring in their structure (Madigan et al., 2021).

The first B-lactam antibiotic that has been discovered is Penicillin which is produced by *Penicillium chrysogenum* a species of a magician genus of fungi *Penicillium*. Penicillin G is discovered by Alexander Fleming (1920) while worked on staphylococci variants exposed to air (Fleming A, 1929). Penicillin has B-lactam ring and a ring of Thiozolidine (Lobanovska and Pilla, 2017).

Cephalosporin is another member of B-Lactam group, which is discovered by Giuseppe Brotzu (1945), which is produced by a genus of fungi *Cephalosporium* (Nakajima S, 2003). Cephalosporin has B-Lactam ring which is bound to dihydrothiazine ring. The semi-synthetic form of cephalosporin such as ceftriaxone has broader spectrum than penicillin, and can be used against penicillin resistant-*Neisseria gonorrhoeae* strain (Madigan et al., 2021; Chaudhry et al., 2019).

B-lactam drugs target cell wall biosynthesis and interrupt its formation by binding penicillin binding proteins (PBP) which are mostly Transpeptidase enzyme that works on binding peptidoglycan cross-linking sheets together that lead to disrupt the bacterial cell and bacterial death (Georgopapadakou and Liu, 1980).

During mid-1980s, when many bacteria acquired resistant against previous B-Lactams because of B-Lactamase producing enzymes, two other class of B-Lactams have been developed which are Monobactam and Carbapenem (Willey et al., 2020).

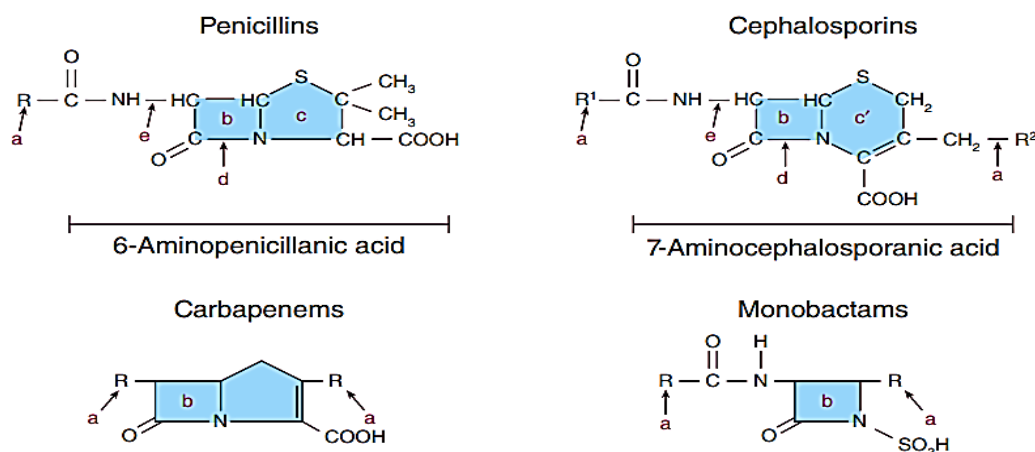


Figure 2.2. B-lactams antibiotics structure.(a)Side chains, B)Beta-lactam ring, C) Thiozolidine ring , C') Dihydrothiazine ring, D) site of B-lactamase action (L, Paul et al., 2018)

2.1.3.2. Protein synthesis inhibitor antimicrobials

Some antibiotics target protein synthesis of the bacterial cell by either act on ribosomal part (30s or 50s) or RNA polymerase enzymes. Bacteria has unique 70S ribosome which is different than human 80s ribosomes, this gives bacteria an advantage of Selective toxicity (Madigan et al., 2021).

Aminoglycosides class is amino-sugar bind together by glycosidic bond that interrupt protein translation by target 30s sub-unit part of ribosome (Madigan et al., 2021). They are produced naturally or semi-synthetically. The earliest discovered membrane is Streptomycin which is produced by *Streptomyces griseus* (Krause et al., 2016). Another Example is Gentamicin which is produced by *Micromonospora purpurea* in 1962 (Weinstein et al., 1963) and has been routinely used against Pseudomonas infections (Madigan et al., 2021).

Tetracycline is a family of protein inhibitor antibiotics that has four ring structures. They are available naturally produced by *Streptomyces spp.*, whereas some others are semi-synthetic. They inhibit and stop protein translation by targeting 30s Ribosomal unit and has broad spectrum of action against most bacteria, in which obligated intracellular pathogens such as mycoplasma, rickettsia and chlamydia (Willey et al., 2020; Grossman, 2016).

Macrolide antibiotics inhibit protein synthesis as well, but differently act on ribosomes, in which instead of targeting 30s unit, it will act on another 50s large unit as target (Madigan et al., 2021). It is produced by species of *Streptomyces*, such as Erythromycin which is isolated from *Streptomyces erythraeus* (Ann, 2004) , and has unique lactone ring in its structure that bound to sugar, and has effect against Gram-positive bacteria, mycoplasmas, and some Gram negative bacteria (Willey et al., 2020).

2.1.3.3. Nucleic acid synthesis as drug target

Quinolones are chemically-synthetic antibacterial that interfere DNA gyrase and topoisomerase (iv) enzyme function, which aid in supercoiling and twisting DNA, and lead to disrupting DNA replication (Hooper, 1998) and (Anderson and Osheroff , 2001), And Fluoroquinolones are fluorinated form of quinolones which has effect against urinary tract infections, most widely ciprofloxacin that has effect against penicillin resistant *Bacillus anthracis*, and moxifloxacin which is used as combination with other types of anti-tuberculosis drugs (Madigan et al., 2021).

Some antibiotics interact with the function of RNA-polymerase and interrupt its function as RNA transcription machine, such as Rifamycin which is isolated from bacteria *Streptomyces mediterranei*, and Longley used as Co-treatment with isoniazid against pathogenic bacterial group of Mycobacteria, in which binds with B-subunit of RNA polymerase and inhibits RNA-synthesis (National Center for Biotechnology Information, 2022; Madigan et al., 2021).

2.1.3.4. Cell membrane and metabolic antagonist as targets

Cell membrane was the last target of choice in the mind of scientists because of its almost likelihood similarity with human cells, till the Platensimycin was isolated from soil bacteria *Streptomyces platensis* in 2006 by Merck researchers (Singh et al., 2006), and it showed bacteriostatic effect against mycobacterial species, Vancomycin resistant-*Enterococci* and Methicillin resistant-*Staphylococci* (Potera, 2006). Its mode

of action is very unique, in which inhibits FabF and FabH of fatty acid synthesis type II process, which doesn't exist in human cells (Martens and Demain, 2011).

Sulfonamides and Trimethoprim's are chemically synthetic antimicrobials. Sulfonamide act as an metabolism analog, in the place of P-aminobenzoic acid (PABA), while Trimethoprim binds to dihydrofolate reductase (DHFR) enzyme, both interfere folic acid synthesis and interrupt this process which lead to inhibit DNA synthesis, because bacteria must provide folic acid and further purine nucleic acid by its own process, unlike human cells, In which can obtain folic acid from environmental source (Willey et al., 2020).

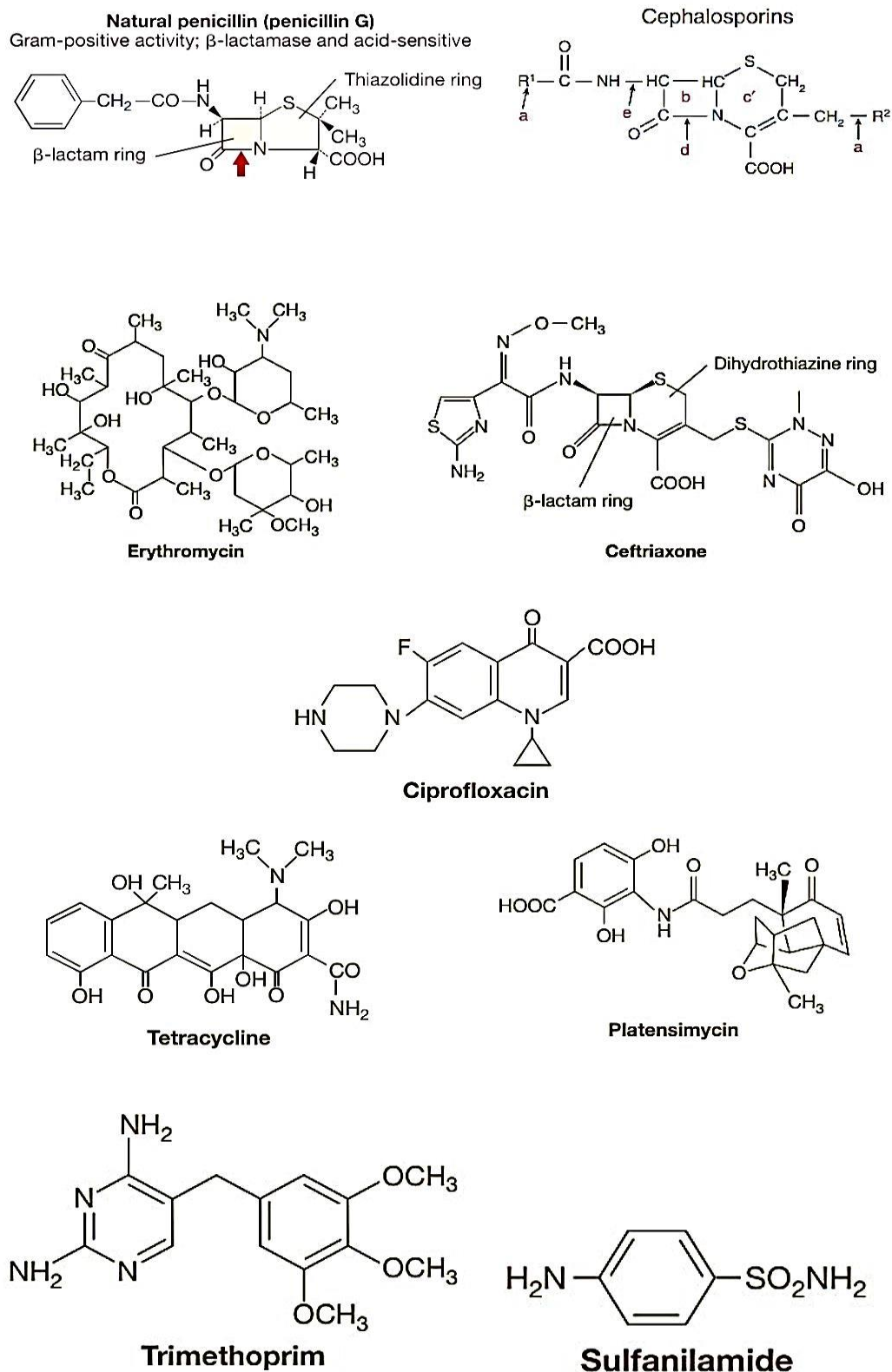


Figure 2.3. Chemical structure of some antibacterial drug (Madigan et al., 2021)

2.1.4. Antibacterial drug resistance

Antibiotics and antimicrobials are ancient human defense shield which human used as magic bullet against infections; the structure of tetracycline has been observed from an ancient human-skeletal sample, the sample has been taken from Sudanese Nubia and examined by the aid of fluorescent microscopy (Nelson et al., 2010; Bassett et al., 1980). Historically alexander Fleming was the first who aware of penicillin resistance in microbes, because of overuse of desired antibiotics (Abraham and Chain, 1988).

Antibacterial drug resistant is the bacterial defense power toward the pre-exposure antibacterial or antimicrobials, by which the bacteria can live with the presence of these compounds. Naturally, Bacteria can produce resistant through genetic level, which could be interstice or acquired. An example of interstice resistance is *Mycoplasma* that absent cell wall which is naturally resistance against B-lactam antibiotics, another example is biofilm forming bacteria like *Pseudomonas* which is impermeable to antibiotic, on the other hand bacteria acquire resistance by either mutation or horizontal gene transfer (plasmid transformation), (Zaman et al., 2017; Madigan et al., 2021; Willey et al., 2020).

The extent production and overuse of antibiotics for agriculture and anti-infections purpose provide the favorable condition for spreading resistance-plasmid(R-plasmid) across pathogenic bacteria, for example penicillin has been used against Gonorrhea infection till it obtained resistance at 1980, and later Ciprofloxacin was used in place of penicillin but unfortunately, after 10 years, it also got resistance and later replaced by ceftriaxone. Another reason is patient non-compliance, in which patients interrupt its medication after they feel better while bacteria are in dormant state, or patients get antibiotic drugs empirically, especially in non-developing country where there economic states are too bad (Madigan et al., 2021).

Historically, Alexander Fleming was the first who observe that *E.coli* has an enzyme which helps *E.coli* to live with the existence of penicillin. After six year of

development of aminoglycoside, *Staphylococcus spp.* strains which have resistant to aminoglycoside had developed, and in 1960 methicillin had been discovered and used against penicillin resistance staphylococci, but unfortunately after a while methicillin resistance staphylococcus had been reported and it was one of the serious problem all over the world till nowadays. while fluoroquinolone was discovered and used against gram negative bacteria at 1980, resistant strain have been developed as well, after all at 2002 vancomycin resistant strain of *S. aureus* has been reported ,although it was a drug of choice in the place of methicillin for a long time. The brief history of antibacterial discovery and time to get resistance is depicted at (Figure 2.2) (Zaman et al., 2017).

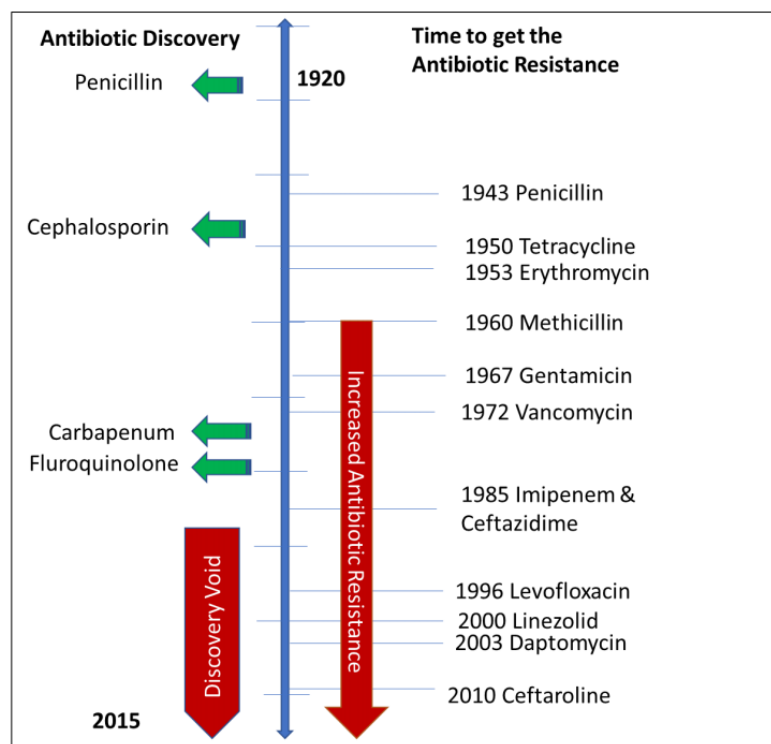


Figure 2.4. The brief history of Antibacterial discovery (Left side) and time flow to get resistance (Right side) (Zaman et al., 2017)

2.1.5. Antibacterial resistant mechanisms

Commonly Bacteria can get resistant against the antibacterial through alteration of the specific antibacterial targets, or Deactivation and changing of antibacterial chemical structure or cutting the antibacterial drug, or can decrease the drug flow and take them out of its cell through the aid of different class of Efflux pump system, the picture (2.5) illustrate three common mechanism of bacterial resistant (L, Paul et al., 2018).

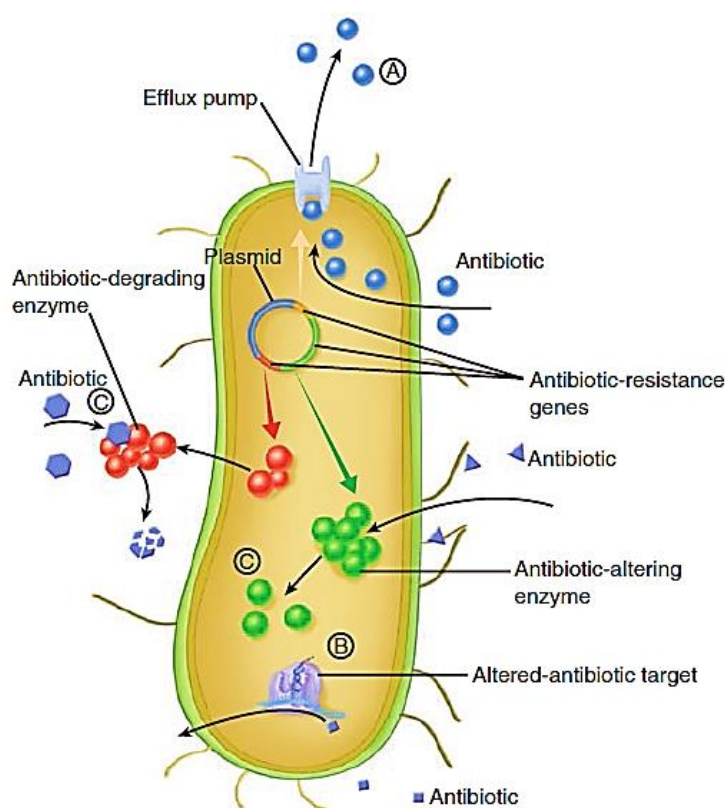


Figure 2.5. Antimicrobial resistance mechanisms. A. Efflux pump. B. Altered target. C. Enzymatic inactivation (L, Paul et al., 2018)

One of the bacterial resistance-mechanism is minimizing the concentration of intracellular antimicrobial, which is carryout by either altering membrane structure especially outer membrane of gram negative bacteria or by pumping out the interior antibiotics through transmembrane protein (Efflux pump); the most common

example of multiple drug out pump which takes out multiple class of antibiotics is AcrAB system in *E.coli* (Okusu et al., 1996; Willey et al., 2020).

Plasmids transformation among species of the same genus or different strain of bacterial species is the major bacterial strategy to achieve resistance, especially in the family of *Enterobacteriaceae* and gram positive pathogenic bacteria, in which several resistant genes which codes for enzymes such as B-Lactamase or Carbapenemase can be shared through plasmid transformation process (Schultsz and Geerlings, 2012; Bush and Bradford, 2016), in which B-Lactamase will bind to B-lactam ring of B-Lactam antibiotics and hydrolyze its structure, as illustrated in (Figure 2.6) (Madigan et al., 2021; Willey et al., 2020), on the other hand, Carbapenemase which accounts majority of a family b-lactamase, bind and cut the structure of almost all class of B-Lactam antibiotics as well. They are chromosomally and plasmid encoded proteins which causes serious problem to human health nowadays, especially Carbapenemase-producing *Enterobacteriaceae* family (L, Paul et al., 2018; Queenan and Bush, 2007).

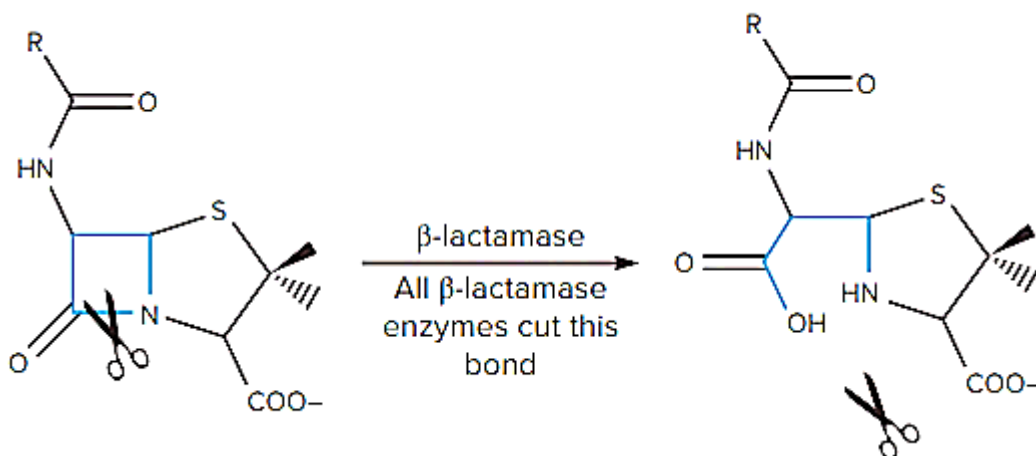


Figure 2.6. β -lactamase enzymes cleave the β -lactam ring (Willey et al., 2020)

Another resistant mechanism is antibiotic altering enzymes. Bacteria can inactivate and alter aminoglycosides by amino acid acetylation or hydroxide group phosphorylation and don't allow antibiotic to bind with 30s ribosomes. (Krause et al., 2016; L, Paul et al., 2018).

Another common and basic strategy to combat antibiotics from bacteria is changing and altering the original antibiotic target. *S. aureus* is one of most problematic pathogen and over years improved its defense line against most antibiotics generally and beta-lactams specifically by *mec A,B,C,D* genes which encode Penicillin binding proteins with less affinity to B-lactams and inhibit binding with Cell wall-transpeptidase (Ubukata et al., 1989). *Streptococcus species* especially *S. pneumoniae* and *S. viridans* is another example of altering the PBP, however their mechanism is not clear. *S. pyogenes* slowly got resistant; however mutagenized strain of *S. pyogenes* has been developed at laboratory that has improved resistance against penicillin derived antibiotics (Horn et al., 1998; Gutmann and Tomasz, 1982).

2.2. Schiff Base

2.2.1. Definition of Schiff base

Schiff base compounds firstly discovered and synthesized by (Hugo Schiff), who published his first work at 1864 (Schiff, 1864). SB is any compound with structure $R_1R_2C=NR_3$ (in which R_2 and R_3 are not hydrogen), also called imine compound, because has Carbone-Nitrogen double bond (Abu-Dief and Mohamed, 2015). The double bonds in the SB compounds represents imine or Azomethine group, which is previously replaced with $(C=O)$ group of carbonyl compound (Da Silva et al., 2011). The structure of Schiff base is illustrated at (Figure 2.7).

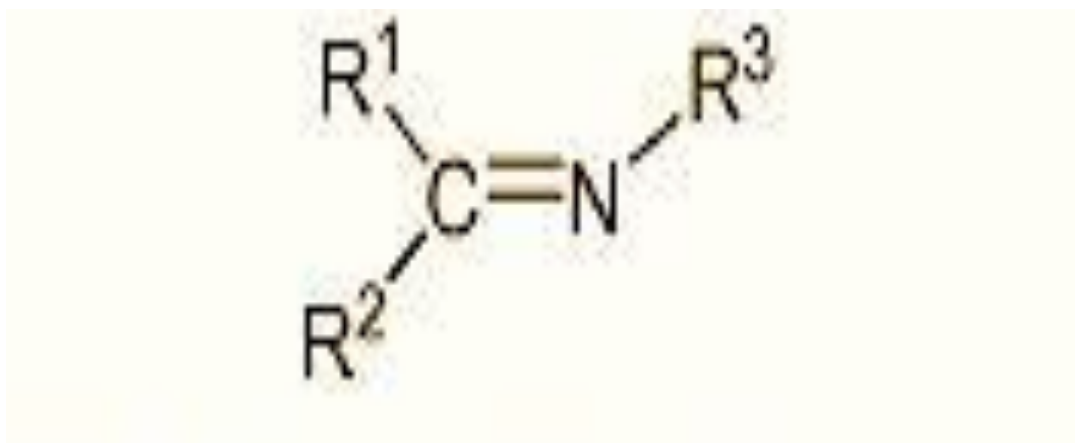


Figure 2.7. General chemical structure of Schiff base compound (R² and R³ are not hydrogen)

2.2.2. Synthesis and application of Schiff base

Schiff base as a phenol derived compounds has a wide range of biological activity and has been tested and checked for their antimicrobial activities such as (antimalarial, antibacterial, antiviral) and with anti-cancer activity (Przybylski et al., 2009), on the other hands it shows other biological activities, for instance, anthelmintic and anti-tuberculosis, anti-inflammatory, antioxidant, antidepressant activities (Uddin et al., 2020).

Schiff base Generally can be synthesized by binding of two basic compounds (primary amine or aromatic amine with carbonyl compounds such as Ketones or aldehydes) through condensation reaction, which replace oxygen in carbonyl group by nitrogen and form imine group or azomethine group (N=C) (Vinusha et al., 2019). The reaction and synthesis of ideal Schiff base has been depicted in (Figure 2.8).

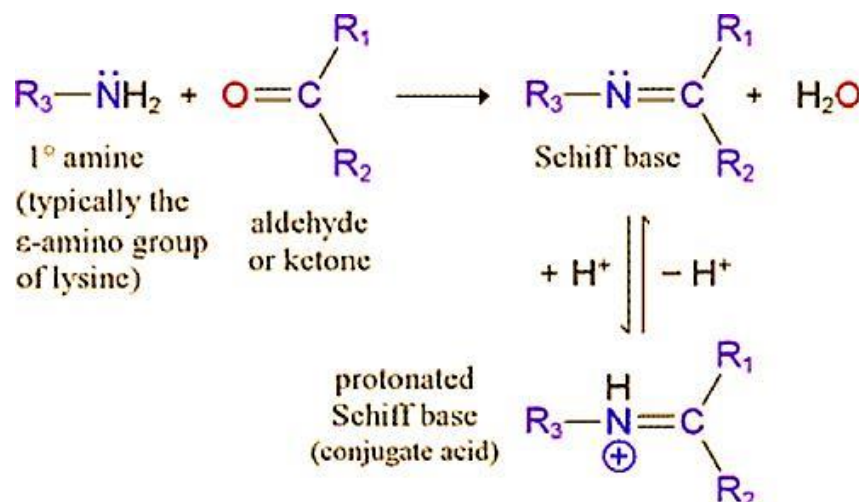


Figure 2.8. general scheme for formation of schiff base (Abu-Dief and Mohamed, 2015)

SB compounds can be present naturally in plants, for example alkaloid ancistrocladidine compound is naturally produced and isolated from species of *Ancistrocladus heyneanus*, and its structure is depicted in (figure 2.9), in which various biological activities has been observed against various species of parasites such as *Plasmodium spp.* (Malaria agent) and *Leishmania spp.* (Bringmann et al., 2004). Another example is Corrin ring from vitamin B12 (Harris, 2013).

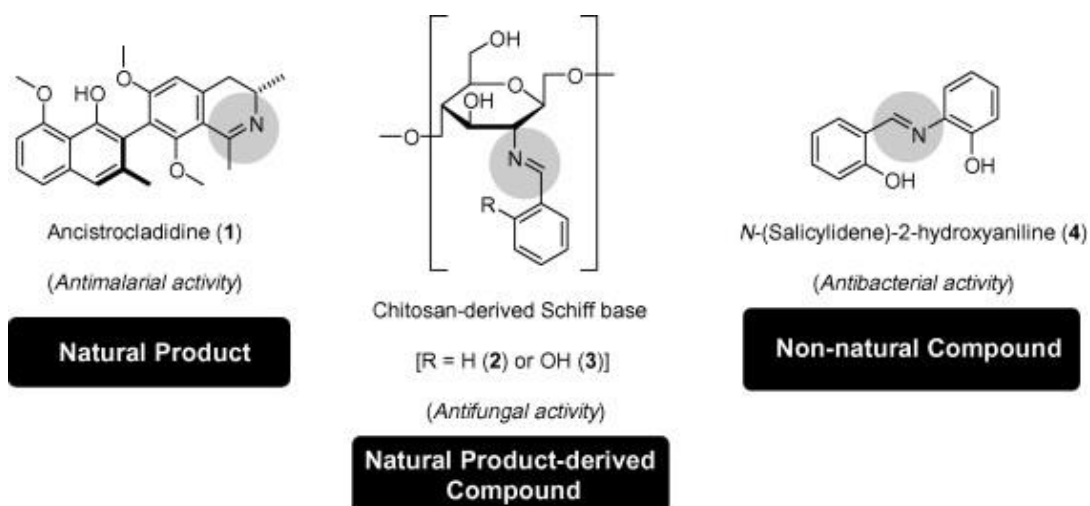


Figure 2.9. Chemical structure and examples of Natural Schiff base (left), Natural derived Schiff base (center) and Non-natural compound (Right) Photo derived from (Da Silva et al, 2011)

Some SBs can be modified after being isolated from natural source or synthesized after the isolation of primary compounds (Amines or Carbonyl), for

instance Chitosan derived Schiff base as illustrated in (Figure 2.9), that showed slightly activity against some fungal specious (Guo et al., 2007).

Mostly SBs can be easily synthesized in laboratory by binding of two basic compounds (primary amine or aromatic amine with carbonyl compounds such as Ketones or aldehydes) through condensation reaction and some of them has broad range of biological activity against microbes or against cancer cells, the most basic SB compounds are Salicylaldehyde derived Schiff base, the structure depicted at (Figure 2.9) (Qin et al, 2013).

Synthetically Schiff base can be prepared by different method. It can be prepared and synthesized by solvent based method, in which the condensation reaction of both amine and carbonyl compound occur in solvent medium such as Methanol or ethanol , or it can be prepared in catalyst medium such as SnCl_2 or acetic acid. Some of Schiff bases can be prepared without using any solvent or catalyst by mixing of both compounds in mortar and pestle (Uddin et al., 2020).

The most recent and effective and costless way to prepare Schiff base compounds is Microwave irradiation without solvent, in which the mixture of both primary compounds(amine and aldehyde or ketones) placed in beaker then it will be irradiated at 160W for some minute. The product poured in cold water and crystals will be filtered and dried (Uddin et al., 2020; Shntaif and Rashid, 2016).

2.2.3. Antimicrobial studies with Schiff base

2.2.3.1. Antibacterial study with Schiff base

Schiff bases have been proposed as antibacterial candidates. For example, 2-Salicylideneaminophenol as structurally depicted at figure (2.10) has a MIC of 8 Micro-gram/mL and is effective against *Mycobacterium tuberculosis* bacteria. An experiment has been conducted for testing selectivity of this compound of Schiff base on J774- macrophages, and there was no cytotoxic effect against macrophages and more than 80 percent of them stayed alive, even though the concentration have

been increased as 1000 Microgram /mL concentration, which revealed that compound had great selectivity (Da silva et al, 2011; Souza et al., 2007).

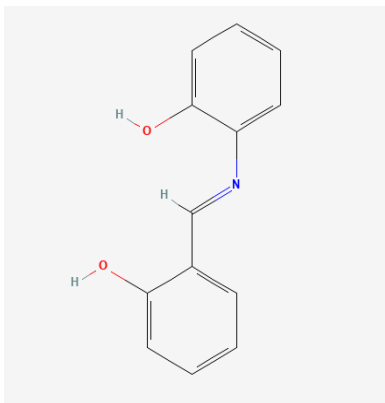


Figure 2.10. Structure of 2-Salicylideneaminophenol (National Center for Biotechnology Information, 2022)

Three ligands of Schiff base have been synthesized in (Bayeh et al, 2020) study, ligands have been depicted at (Figure 2.11), then they had been checked for their antibacterial activity by Agar disk diffusion method against four bacterial species (Gram positive: *S. aureus* and *S. epidermidis*, Gram negative: *P. aeruginosa* and *K. pneumoniae*) and the result has been compared with two positive Antibacterial-control (Chloramphenicol and Ciprofloxacin). as an outcome, all synthesized ligands showed inhibition zone against all bacterial types, and Ligand-3 has better inhibition zone than Positive control-antimicrobials against *S. epidermidis* and *P. aeruginosa*, while Ligand-1 showed the best inhibition zone than all synthesized ligands and positive control against *S. aureus* (Bayeh et al., 2020; Ceramella et al., 2022).

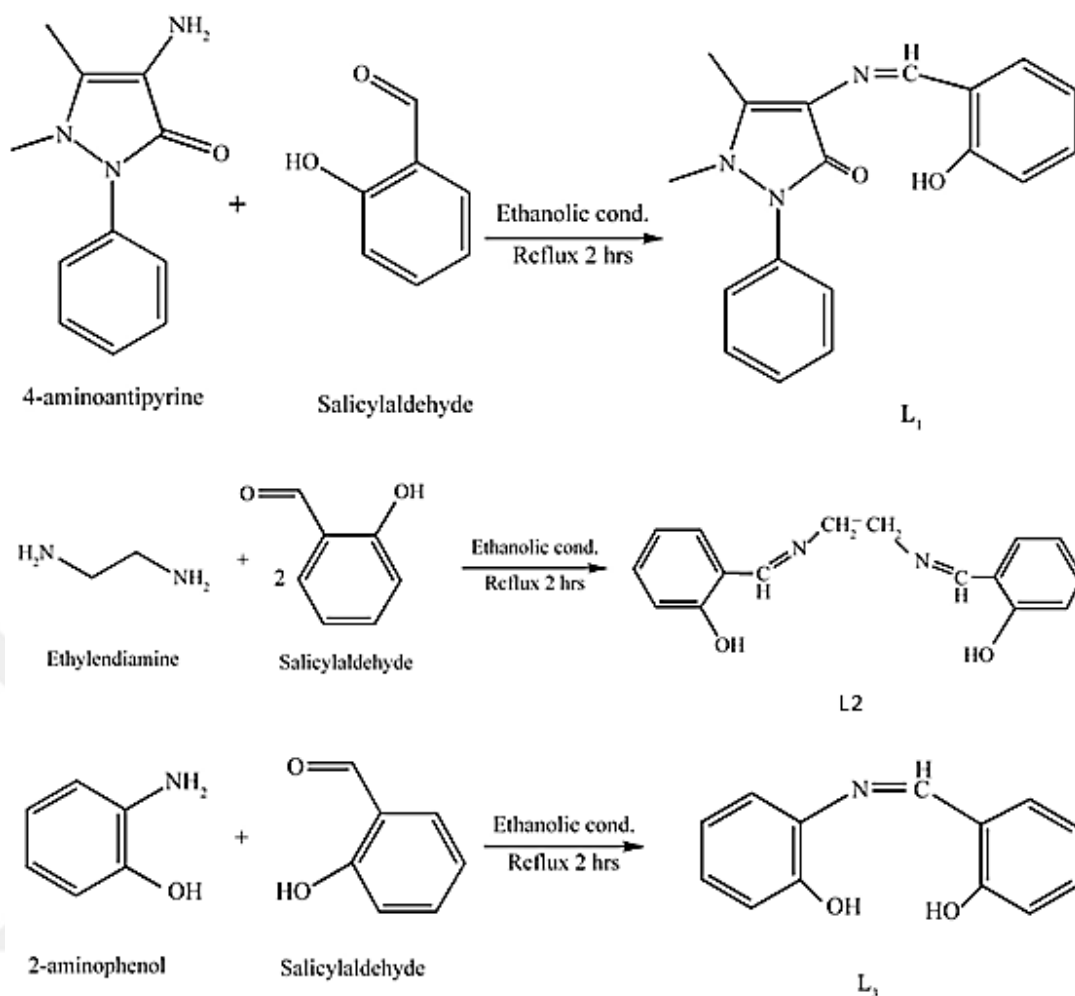


Figure 2.11. Three ligands of Schiff base (L₁, L₂ and L₃) that show antibacterial activity (Bayeh et al., 2020)

Many studies prefer that Metal complex of Schiff bases ligands may show better biological activity than previous ones, although some of Schiff-metal complexes failed, but struggling to create a Metal complex still in progress. In (Emmanuel et al., 2018) investigation, Copper-complex (Cu complex) from SB has been synthesized as illustrated at (Figure 2.12), and tested through agar-disk diffusion assay on ten bacterial strains, which are (*K. pneumonia*, *E. coli*, *P. vulgaris*, *S. aureus* and *Salmonella typhi*), Ampicillin used as antimicrobial positive control. In results, the metal-complex Schiff base compound had better activity by looking at its larger inhibition-zone than original Ampicillin.

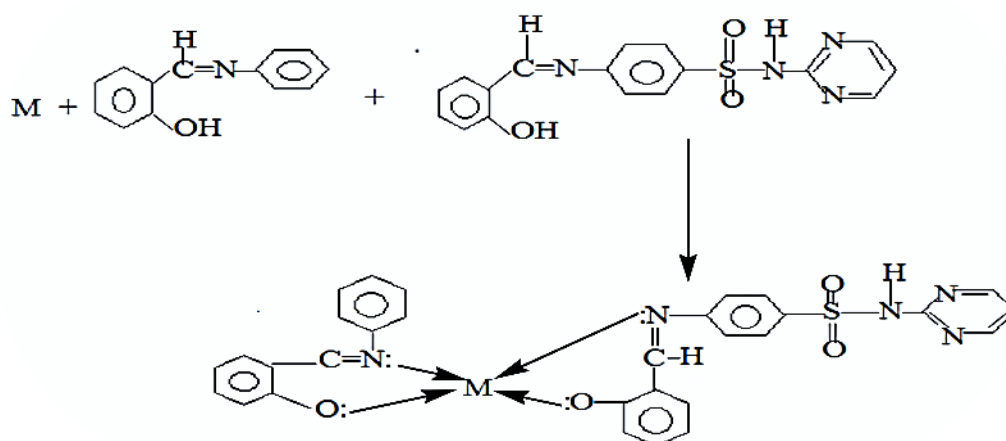


Figure 2.12. Copper complex from Sulphadiazine Schiff Base bind with aniline derived Schiff base (Emmanuel et al., 2018).

2.2.3.2 Antifungal study with Schiff base

(Emmanuel et al, 2018) study showed that N-Salicylideneaniline and N-Salicylidenesulphadiazine have better antifungal effect and larger inhibition zones than metal complexes against 10 different species of fungus among them (*Candida albicans*, *Candida krusei*, *Aspergillus niger*, *A. flavus*, *Fusarium oxysporium* and *Rhizopus stolonifera*), by using Agar disk diffusion method and Mycotine has been used as positive control, in outcome, both ligands showed antifungal activity against all 10 species. On the other hand, Copper complex of both (L1+L2) showed better activity and larger inhibition zone than both ligands alone and positive control (Mycotine), especially against *Candida albicans*, *Candida Krusei*, *Aspergillus fumigatus* and *A. niger*.

Patients with immune disorder faced a huge health problem due to easily be infected by Pathogenic microorganisms and especially those that got multidrug resistant against common drugs. Among them are invasive fungal infections (Mycoses). The scientists tried and checked various Compounds to be alternative for previous Anti-fungal drugs, one of the attempts was by (Hamad et al, 2021) study, in which they synthesized Some Sulphonamide-Schiff base compounds, and tested them against different *Candida species* and strains especially Multi-drug resistant *C. auris* and *C. glabrata*, then the minimum inhibitory concentration were

taken by Tube-dilution method, eventually Two of the compounds (which has been depicted at figure 2.13) showed interesting activity against *Candida* species with generally MIC value of (4-32 microgram/ml), and especially against *C.auris* strains with Mic value of (16-32 microgram/ml).this study opened the new class of drugs which can be called the future antifungal class drugs (Ceramella et al., 2022).

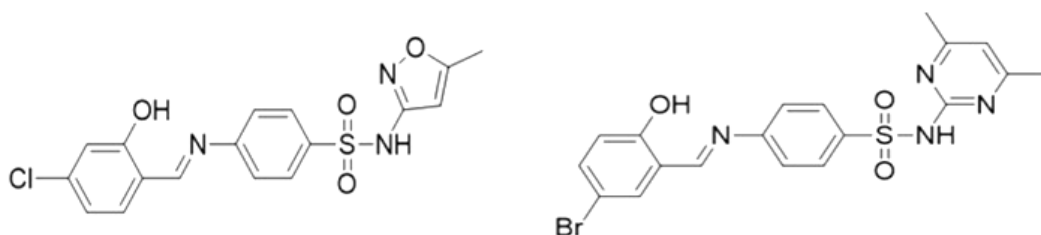


Figure 2.13. Two compounds of Sulphonamides derived Schiff base compounds (Hamad et al., 2021)

As a wish to find and design new antimicrobial drugs, SB derives from totally different compounds have been synthesized and Checked as a hope against various bacterial species and Fungal species (*C. albicans* and *A. niger*), which was Dehydro-acetic acid condensed with aromatic-amines, then the zone of inhibition has been recorded, eventually, the outcome effect against fungal was higher than bacterial species, especially by two of DHA derived compounds(L2 and L5) which is shown at (Figure 2.14) with the zone of inhibition 21 and 19 against *C. albicans* and *A. niger* respectively, even though there is a range of effect on different bacterial species (Bhagwatrao et al., 2021; Ceramella et al., 2022).

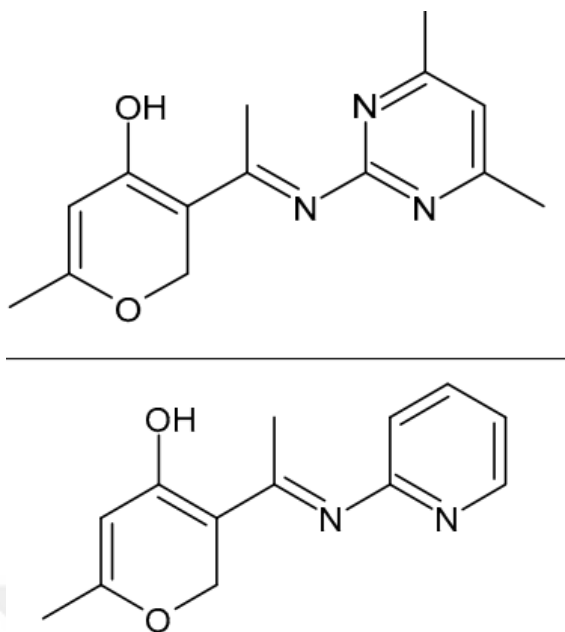


Figure 2.14. DHA (Dehydroacetic acid derived Schiff bases), Up (L2) and down (L5) (Ceramella et al., 2022)

2.2.3.3 Anti-malarial study with schiff bases

Malaria is a risky disease transmitted by a female mosquito (*Anopheles spp.*), and caused by a small protozoan parasite of genus *Plasmodium spp.*, especially by four common species (*P.falciparum*, *P.knowlesi*, *P. malariae*, *P.vivax* and *P.ovale*). According to WHO reports in 2017, Malaria causes approximately 435,000 deaths among 219 million infected patients globally. Incidence of recently resistant plasmodium parasites against many antimalarial drugs, and increasing morbidity and mortality encourage researchers by working hard toward finding alternative antimalarial drugs and facing the future resistance problems of this parasite (Talapko et al., 2019).

Some SB compounds are Known for their Anti-parasitic activity, for example alkaloid Ancistrocladidine compound which is naturally produced and isolated from species of *Ancistrocladus heyneanus*, and its structure is depicted in (Figure 2.9), in which various biological activities have been observed against various species of parasites such as *Plasmodium spp.* (Malaria agent) with the MIC value of 0.3 and

1.9 $\mu\text{g/mL}$ against both *P. falciparum* (K1 and 3D7) strains, respectively (Bringmann et al., 2004).

Another interesting study in the synthetization of newly derived Schiff bases was in (Rathelot et al., 1995) study, in which Isoquinoline-derived Schiff bases has been synthesized and checked against Chloroquine resistant type of *P. falciparum* (as shown at figure (2.15), and the IC_{50} was 0.7 $\mu\text{g/mL}$ which was better and more effective than chloroquine itself which was 0.1 $\mu\text{g/mL}$ (Da Silva et al., 2011).

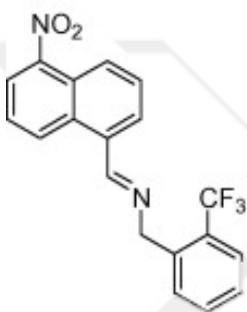


Figure 2.15. Isoquinoline derived Schiff base (5-Nitroisoquinolines). (Rathelot et al., 1995)

2.2.3.4 Anti-viral study with schiff bases

Incidence of viral resistant toward many antiviral agents encourage researchers to try, check and synthesize new compounds in order to face the fast incidence of multi-drug resistance viruses. In a research carried out by (Kumar et al., 2010), Quinazoline which already known as active compound against (Microbes, Virus, Cancer, Inflammation, Histamine and Tuberculosis) activity was used in synthetization of Quinazoline derived Schiff base compounds (34a-34i) which have been depicted at (Figure 2.16), then the Cytotoxicity and antiviral activity was checked against various virus species, as a result, compound (34a) has better antiviral activity than all compounds.

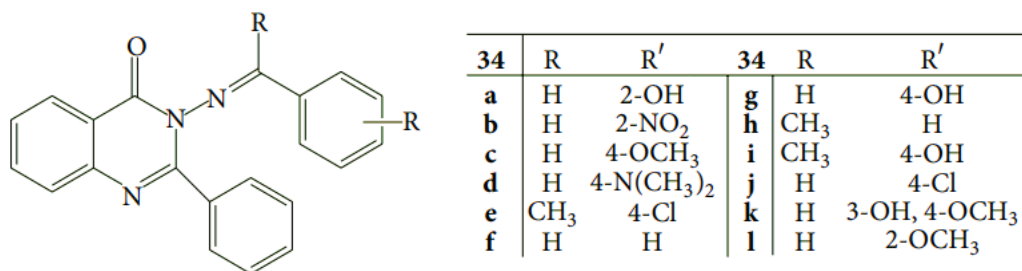


Figure 2.16. Quinazoline derived Schiff bases (34a to 34l) (Kajal et al., 2013)

Interestingly, another recent study conducted by (Sriram et al., 2006), in which Schiff base compounds have been synthesized from Abacavir, which is nucleoside analog and inhibit Reverse transcriptase of Retroviruses, especially in case of (HIV) virus. Compounds (55-60) as shown in picture (2.17) are Abacavir derived Schiff bases which synthesized and checked against (HIV) virus. Finally, compound (57) has EC₅₀ of 0.05 μ M, which is 32 times more effective than Abacavir (EC₅₀=1.6 μ M) and has CC₅₀ >100 μ M.

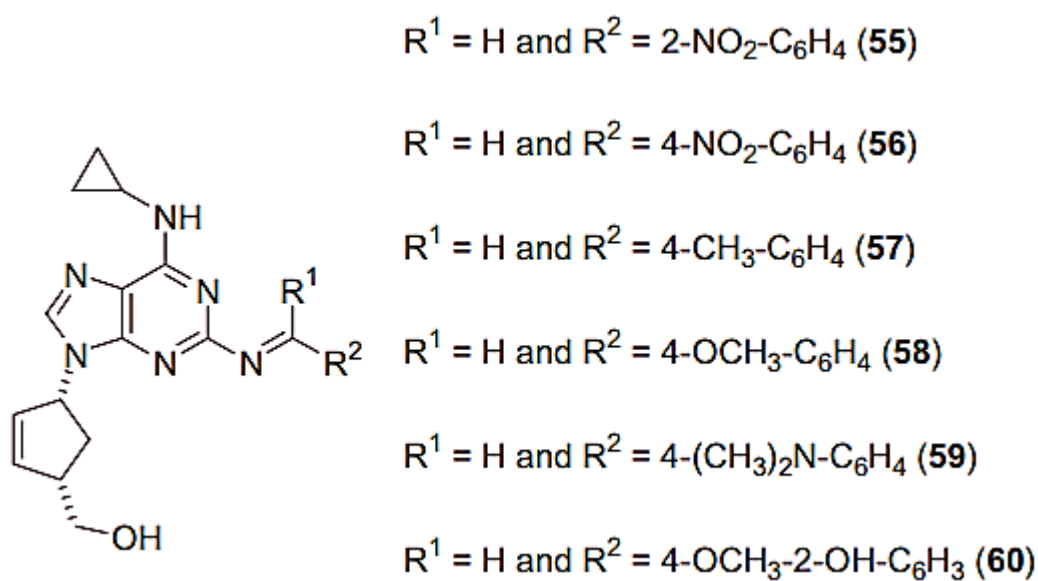


Figure 2.17. Abacavir derived Schiff bases compounds (55-60) (Da Silva et al., 2011)

2.3. Antimicrobial Research Methods

Since the discovery of the first Antimicrobial-antibiotic drug (Penicillin), and development of resistance mechanisms from Pathogenic microbes during that moment up to that point, and Attempting to find new and alternative drugs in place of previously resistant drugs have posed a huge challenge to humanity, particularly in Clinical microbiology era against dangerous Pathogenic microbes that have demanded a huge sacrifice from the human race and have remained our constant defender enemy until now.

In order to win this challenge against microbes, Scientists developed Techniques to test and discover new antimicrobial drugs from Natural, semi-synthetic or synthetic sources. Following are the common Antimicrobial Susceptibility methods especially for Bacteria, which also have been shown in (Table 2.1).

Table 2.1. Antimicrobial Susceptibility Techniques with sub-methods

Antimicrobial Susceptibility Techniques	Sub-methods
Diffusion method	- Disk diffusion method - Well diffusion method - Plug diffusion method
Dilution method	- Broth Macrodilution - Broth Microdilution - Agar dilution method
TLC-Bioautography	- Contact TLC-Bioautography - Direct TLC-Bioautography - Agar overlay- TLC-Bioautography

2.3.1. Agar diffusion technique

2.3.1.1. Disk diffusion technique

This method was introduced in 1940, and the idea was taken from diffusion phenomenon through agar medium which has the ability of diffusion through itself.

In this technique a disk of about standard (6mm) diameter is impregnated with the desired amount of the Extract (Plant or Microbial) or tested compound, in which the concentration is adjusted by either mg/ml or $\mu\text{g/ml}$, then dried and will be placed over a petri-plate with Mueller Hinton agar medium inoculated by an adjusted amount of tested bacteria, yeast or fungi and incubated at desired temperature, depend on the protocol and guideline which has been published and updated nearly annually by CLSI which is abbreviated for Clinical and Laboratory Standards Institute (CLSI, 2012; CLSI, 2004). After addition of the disks, the compound that impregnated on disks starts to diffuse into agar medium in circle motion and the concentration starts to decrease as it diffuses far away from the disks (Benkova et al., 2020; Balouiri et al., 2016; Jorgensen and Ferraro, 2009) .

Disk diffusion is a Standardized quality test with the sensitive, resistance and intermediate outcome.in this case, the effect of compounds will be determined by appearing clear inhibition zones around disks after incubation, and the largest inhibition zone is the most effective compound against microbes, So its quality test with yes or no information result, an example of Antibiotic susceptibility test by disk diffusion has been shown in figure (2.18). It's recommended to do further quantity test, In order to determine the minimum inhibition concentration (MIC) of the effective compounds (Balouiri et al., 2016).

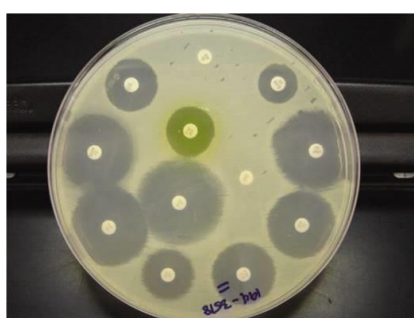


Figure 2.18. A disk diffusion test for an isolate of *E. coli* from a urine culture (Jorgensen and Ferraro, 2009)

2.3.1.2. Well diffusion technique

It's an old common and easy technique which is widely used to test the antimicrobial effect of extracts (Plant or microbial) or chemical compounds on

microbes. The principle is quite similar with disk-diffusion method; the only difference is that we have to dig wells instead of using disk papers. The wells are created by Cork borer (mainly 6-8mm) after inoculation of agar plate (mainly Mueller Hinton agar) with adjusted turbid of bacterial suspension (Mainly 1×10^8 CfU/ml and its equal to 0.5 McFarland standard), then the wells will be filled with appropriate Concentration of the tested compounds (each well can take 20 to 100 μ l of the compound), then incubated with appropriate temperature and time, finally similar to disk diffusion method, the result will be qualitative based on the clear zone which appear around the wells, which indicate that the compound has antimicrobial activity. an example of well diffusion technique has been shown at (Figure 2.19) (Bubonja-Šonje et al., 2020; Balouiri et al., 2016).

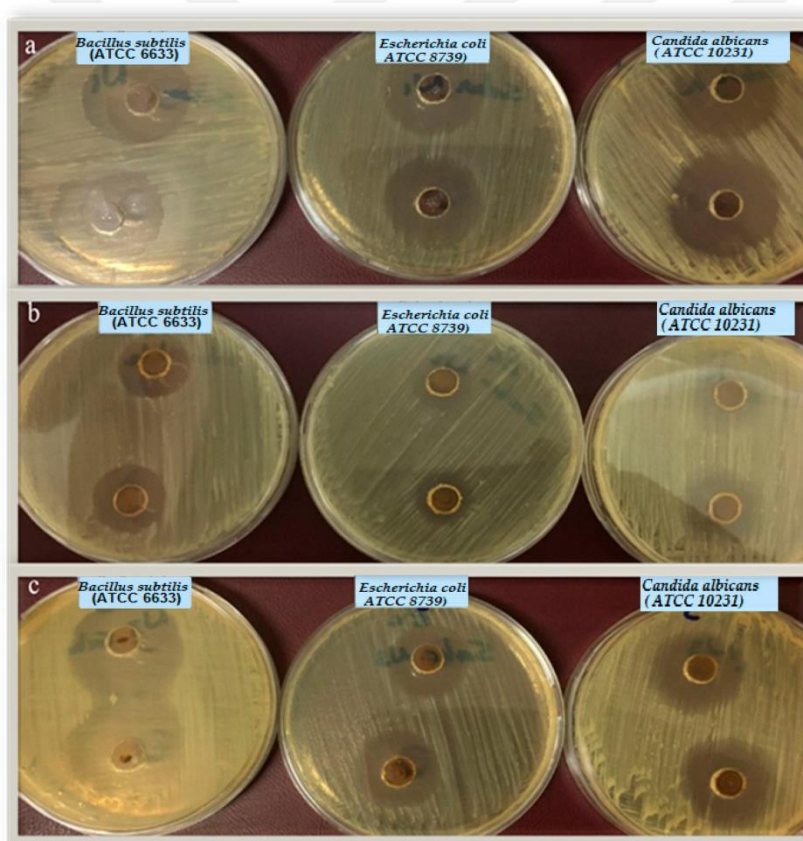


Figure 2.19. The agar well diffusion method to test antimicrobial activity on *Candida albicans*, *E. coli*, and *Bacillus subtilis*) (Farag et al., 2019)

2.3.1.3. Plug diffusion technique

This technique mostly used to determine microbial antagonisms, and antibiotic secretion observation in the form of secondary product by one microbe, which causes to kill a wide range of microbes (Jiménez-Esquilín and Roane, 2005).

The idea is simple, first the microbe which has suspected to secrete antimicrobial molecule has to be tightly cultured on proper agar plate media, and then during growth time, the secreted-molecules of this microbe diffuse into agar medium. After incubation, a plug of the cultured media containing the microorganisms will be taken and placed over another plate containing inoculated agar medium by the microbe that we are looking for the effect of antagonism over, and then it will be incubated at the proper temperature and for the suitable amount of time. Finally similar to disk-diffusion and well diffusion method, the result is qualitative and will be indicated by forming inhibition zone around the plugs such as shown at figure (2.20) (Balouiri et al., 2016; Balouiri et al., 2015).

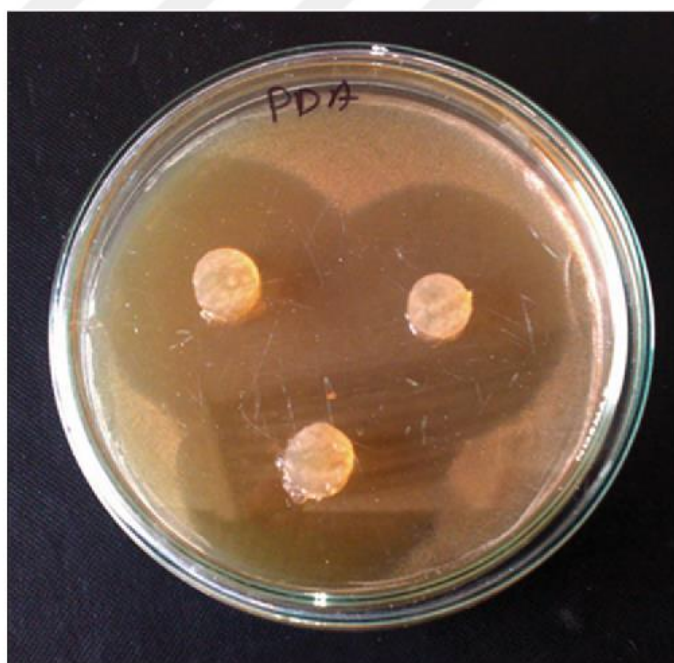


Figure 2.20. Antifungal screening of *Bacillus* spp. over *Candida albicans* by using agar-plug diffusion method (Balouiri et al., 2015)

2.3.2. Dilution method

It's the most standardized quantitative technique which has been used to measure the (MIC) of antimicrobials, which stand for Minimum inhibitory concentration. It is the smallest amount of extract (either plant or microbes), antibiotic or any chemical compound that stops the growth of inoculated microbes (Bubonja-Šonje et al., 2020).

2.3.2.1. Broth-Dilution method

This technique is considered as one of the oldest quantitative antibiotic susceptibility techniques, in which the extracts or the chemical compounds are serially diluted in Mueller Hinton broth by two-fold, then an adjusted turbid of microbial suspension will be added to all tubes equally, either in normal Test-tubes (in Tube-Microdilution assay) or in 96 well- Microplate (in Microdilution assay), then the MIC will be measured through turbidity observation method, which is either by eyes in Macro-dilution assay, or by one of the turbidity measurement device (such as Microplate spectrophotometer) after adding tetrazolium salt, and the MIC is demonstrated as $\mu\text{g/ml}$ or mg/ml (Gajic et al., 2022).

In order to measure Minimum bactericidal concentration (MBC) or Minimum fungicidal concentration (MFC) after measuring MIC which are the latest extract concentration that kill nearly (99.9 percent) of all microbes, the last tubes with MIC can be inoculated on sterile agar petri-dish, if there is no growth then the MBC or MFC will be determined and demonstrated at that point as $\mu\text{g/ml}$ or mg/ml (Schumacher et al., 2018).

The main benefit of Macro-dilution is its quantitative outcome and being standardized by CLSI, but on the other hand, the disadvantage is that it needs more than 24-48 hour and numerous tubes should be used. The protocol and processes is shown in figure (2.21) (Jorgensen and Ferraro 2009).

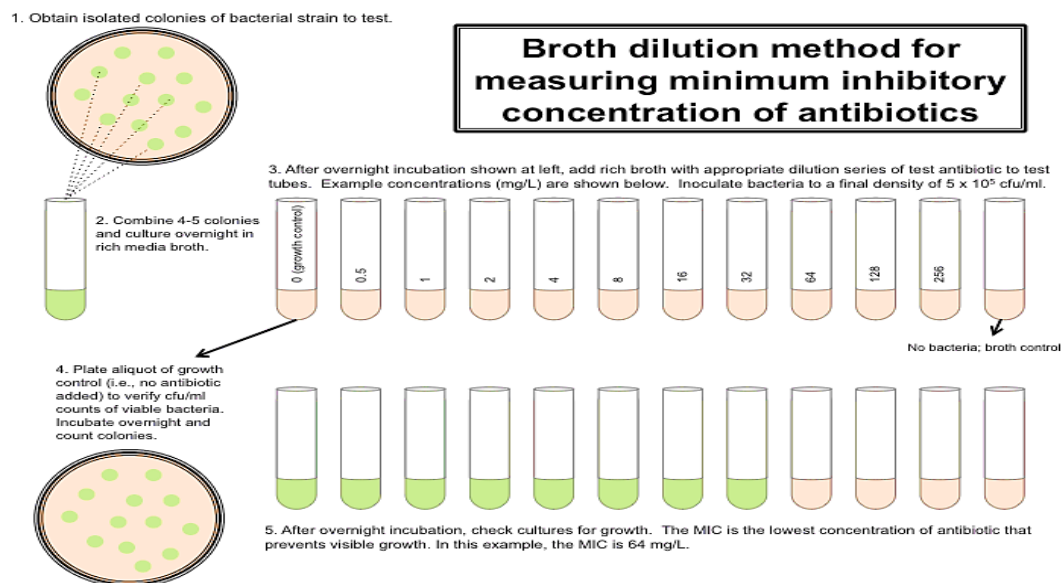


Figure 2.21. Broth Macro-dilution assay (Microbeonline, 2013)

In order to make the dilution easier and cheaper than traditional way, the idea of using 96 microplate trays came into challenge, finally Microdilution has been standardized and improved by Increasing visualization which can be done by adding some sort of dye such as Tetrazolium salt. An image of microplate application in microdilution assay is shown in (Figure 2.22) (Jorgensen and Ferraro 2009).

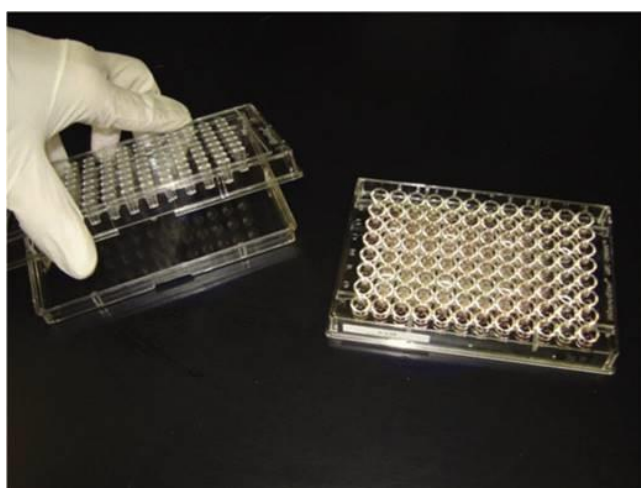


Figure 2.22. A Broth Microdilution susceptibility panel containing 96 reagent wells and a disposable tray inoculator (Jorgensen and Ferraro, 2009)

2.3.2.2. Agar-Dilution method

In this technique, the different set of extract concentration is prepared by dilution, then mixed with molten inoculated Mueller Hinton agar medium with the final turbidity of (0.5 McFarland), then incubated and the MIC can be taken by observing no-growth on the plate. The main advantage of agar dilution over broth dilution is that it can be used for colorful extract in which inhibition might be difficultly seen by Broth dilution assay (Bubonja-Šonje et al., 2020).

2.3.3. TLC-Bioautography

This technique is a combination between chemical isolation and biological detection of extracts (either plants or microbes), by using thin layer chromatographic plate for separation of active compounds and later testing separated compounds by one of the three bioautographic assays (Contact, Direct and Agar overlay) for antibacterial and antifungal detection as illustrated in (Figure 2.23). It's firstly introduced by Goodall and Levi in 1946 to detect antimicrobial activity of some penicillin extracts (GOODALL and LEVI, 1946; Bubonja-Šonje et al., 2020).

Contact TLC-Bioautographic assay is done by placing of developed chromatogram plate after isolation and drying over an inoculated agar medium with tested bacteria or fungi for nearly 15 to 60 minute. During this period, the compounds which trapped in silica powder on the chromatogram plate start to diffuse into agar medium. After incubation clear zone will appear around Active-isolated compound bands. This technique is illustrated in (Figure 2.23) (Bubonja-Šonje et al., 2020; Grzelak et al., 2016).

Direct TLC-Bioautographic assay is the most frequently used assay over other three assays, and it's the easiest and more sensitive for antimicrobial detection, which can be done by spraying or dipping of developed Chromatogram plate into inoculated broth medium with adjusted turbidity of Bacterial cells or Molds spores. Then after incubation, the visualization will be done by adding Tetrazolium salt solution and re-incubation, which is converted by viable cells to Purple colored

formazan crystals. An inhibition zone will appear around active compound in contrast to purple zone(viable cells). This technique is illustrated in (Figure 2.23) (Bubonja-Šonje et al., 2020; Grzelak et al., 2016; Jesionek et al., 2013).

Agar-immersion Bioautography or Agar-overlay TLC-Bioautography is a combination between both previous assays. The idea was taken by placing the developed chromatogram plate in a sterile petri-plate, then pouring of molten Mueller Hinton agar mixed with an adjusted turbid of tested microbial suspension on the TLC plates. The effect can be observed through appearance of inhibition zones around active compounds, either by naked eyes, or by adding Tetrazolium salt, or by using bioluminescent microbes. This technique is illustrated in (Figure 2.23) (Bubonja-Šonje et al., 2020; Grzelak et al., 2016).

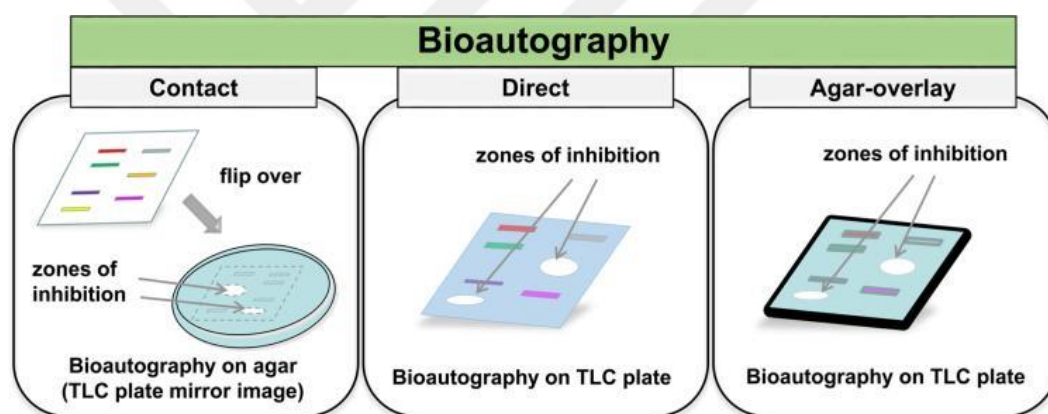


Figure 2.23. Bioautography combination with TLC techniques (Grzelak et al., 2016)

2.4. Bacterial Types Used in Our Study

2.4.1. *S. pyogenes*

S. pyogenes is facultative anaerobic and belong to the family *Streptococcaceae*, it is Gram positive coccus (circular) shaped bacteria that arranged in chain while stained with Gram-stain. It is catalase negative and secret Exotoxins, by which has the ability to lyse red blood cells when it is grown on blood agar medium (blood agar base with 5% horse or sheep blood). *S. pyogenes* is classified as Group-A Streptococci by the Lancefield group system, which divides the genus into 20 groups

based on different Carbohydrate-antigens on the surface of *Streptococcus species* (Willey et al., 2020).

Regarding to WHO's figures at 2005, all peoples infected globally are (18.1 million) people, with 1.78 million new cases, and 500,000 case deaths (World Health Organization, 2005) . It can be transmitted by direct contact from person to person through saliva, nasal secretion, fomites (such as towel) or by Respiratory droplets in the case of Pharyngitis (Strep throat) and impetigo, or by skin contact in case of cellulitis (Ralph and Carapetis, 2013). Even though this bacteria slowly improved resistance toward previously antibiotics especially (B-Lactam antibiotics), because of extensive production of DNase enzyme by which directly consumes Plasmid-DNA pieces without using them for Bacterial transformation (Horn et al., 1998). On the other hand, Incidence of Macrolide-Penicillin resistant *S. pyogenes* strains have been recorded among pets, in which all the isolated Macrolide-resistant samples have resistant to Penicillin as well (Samir et al., 2020).

S. pyogenes routinely isolated from upper respiratory tract, and nearly half of the pharyngitis cases are caused by *S. pyogenes* which commonly known as Strep throat, that appears as small red spots covered by yellow-white exudate at the back of the throat (Nakhoul & Hickner, 2013). If Strep-throat is untreated it leads to severe secondary infections which are Scarlet fever, Toxic shock syndrome, or Rheumatoid fever. Some *S. pyogenes* strains are infected with Bacteriophages that carry genes for *S. pyogenes* Exotoxin A, B, C, or D, which are Superantigens that cause massive lymphatic T-Cell activation and production, leading to massive cytokines production (Sriskandan et al., 2007). As a result, it will either cause cellular and tissue damage by a severe inflammatory reaction, as in scarlet fever and toxic shock syndromes, or it will activate an autoimmune response, as in Rheumatic fever (Madigan et al., 2021).

2.4.2. *K. pneumonia*

Klebsiella is a genus of the *Enterobacteriaceae* family that is mostly found in soil and water environment, and mostly has the ability to fix nitrogen (Madigan et al., 2021). When stained with Gram stain, *Klebsiella* is Gram negative bacilli which can be distinguished from other members of its family (*Enterobacteriaceae*) by lack of movement, Positive reaction for Lysine decarboxylation and Negative for Ornithine, Voges-Prokauer is negative, and the presence of more than 70 polysaccharide capsules which give the colony on the plate (Media in petri-dish) a mucoid character, and through high virulence and pathogeny to animal host. *K. pneumoniae* is the most common and dangerous species which is one of the Pneumonia agents that cause lobar pneumonia (Edwards and Ewing, 1986) and (L, Paul et al., 2018), especially those that have k1 and k2 polysaccharide capsule which now appear to be associated with liver abscess in community-acquired infections (Wu et al., 2009).

Gastrointestinal tract, nasopharynxes and personal hands at hospital are the original shelter for *K. pneumoniae*, which can cause Urinary tract, Respiratory tract (pneumonia), Blood (septicemias) and soft tissue infections. Currently, outbreaks of hospital-acquired *klebsiella* infections are facilitated by a high resistant rate of this isolated bacterium, in which *K. pneumonia* has obtained multidrug-resistant (MDR) and extended-spectrum-beta-lactamase (ESBL) strains. It's now regarded as one of the most dangerous resistant pathogens among bacterial pathogens in general, and its family in particular (Jankauskait et al., 2016; Montgomerie, 1979; Podschun and Ullmann, 1998).

The ESBL-strain of *K. pneumonia* is resistant to a wide range of antibiotics, which are obtained through plasmid transformation and spread among members of the *Enterobacteriaceae* family, resulting in multidrug resistance and limited therapy options against this pathogen. However, the drug of choice was Carbapenem such as Meropenem and Imipenem, unfortunately Imipenem-resistant strain has been raised for *K. pneumonia* (Bradford et al., 1997; Podschun and Ullmann 1998).

According to recent studies, due to mutational inactivation of the *mgrB* regulatory gene, multidrug resistance *K. pneumonia* showed resistance to Colistin. Centers for Disease Control and Prevention (CDC) organization, The World Health Organization (WHO) and the UK Department of Health have all listed Multidrug resistance *Klebsiella pneumoniae* as a serious threat to human health (Kidd et al., 2017). Fluoroquinolones are Broad-spectrum antibiotics that are widely used. According to a recent study (Geetha et al., 2020), antibiotic resistance has recently increased among *K. pneumoniae* strains against Fluoroquinolones. Several mechanisms contribute to fluoroquinolone resistance. Chromosomal-mutation in the quinolone-resistance regions which encoded by topoisomerase IV and DNA gyrases is the main mechanism. Plasmid-mediated quinolone resistance, which was originally identified in a clinically isolated *K. pneumoniae* in 1998, is another mechanism of resistance (Geetha et al., 2020).

2.4.3. *P. mirabilis*

Proteus is a genus of the family *Enterobacteriaceae*, which is Gram negative with Bacilli shape after Gram-stain application, and Lactose non-fermenter on MacConkey agar media. They are highly motile that swarming motility on the plate media is one of famous *Proteus*'s colony characters. *Proteus spp.* have the ability to hydrolyze urea in urine and produce ammonia with the aid of urease enzyme, because of this, it can live in urinary tract and cause urinary tract infection (Madigan et al., 2021; O'Hara et al., 2000; Williams and Schwarzhoff, 1978).

The most isolated species is *Proteus mirabilis* which is Indole negative that still has susceptibility to penicillin G, but *Proteus vulgaris* which is indole positive is naturally resistant to cephalosporin's and ampicillin. The highly motility aids the bacteria to move into urinary catheter and cause urinary tract infection (pyelonephritis and cystitis) and formation of urinary stone. *P. mirabilis* accounts for 1-10% of UTI cases, which vary depend on geographic location. Furthermore, it can cause Respiratory tract infection and bacteremia (L, Paul et al., 2018; O'Hara et al., 2000; Schaffer and Pearson, 2015).

P. mirabilis commonly cause urinary tract infection (UTI), in which the rapid spread of multidrug-resistant have been isolated, particularly those that produce ESBLs, Cephalosporinases, AmpC-type B-lactamase, and Carbapenemases, which are becoming increasingly prevalent. Recently, it showed high resistant to Imipenem due to loss of penicillin binding proteins and reduced Porins (Girlich et al., 2020), and against aminoglycosides, Tetracyclines, Fosfomycin, and also developed resistance against Fluoroquinolones, Nitrofurantoin and Sulfonamides worldwide (Schaffer and Pearson, 2015).



3. MATERIALS and METHODS

3.1. Materials

3.1.1. Compounds

(8) Salicylaldehyde-derived SB compounds (Compound 1 to 8) have been obtained by Harran university-Faculty of Arts and Sciences-Chemistry Department. The Gentamicin has been used as Antibacterial Positive control and for comparing our result with it. SB Compounds were dissolved in 10 mM alcohol, then have been sterilized through 20 μm pore of syringe filter. Chemical structures of Compounds with their Molecular-weight are shown in (Table. 1 and Figure 3.1). The compounds were dissolved in (10 mM) alcohol, and have been sterilized by syringe filter (20 μm pore).

Table 0.1. SB compound names with their molecular-weight

Compound	Name	Molecular-weight (g/mole)
1	1,1,7,7-tetra-methyl-julolidine-N-pentyl-Salicylaldehyde	340.5
2	Copper-complex from Compound (1)	742.5
3	1,1,7,7-tetra-methyl-julolidine-N-hexyl-Salicylaldehyde	354.5
4	Copper-complex from Compound (3)	770.5
5	1,1,7,7-tetra-methyl-julolidine-N-heptyl-Salicylaldehyde	368.5
6	Copper-complex from Compound (5)	798.6
7	1,1,7,7-tetra-methyl-julolidine-N-octyl-Salicylaldehyde	382.5
8	Copper-complex from Compound (7)	826.6

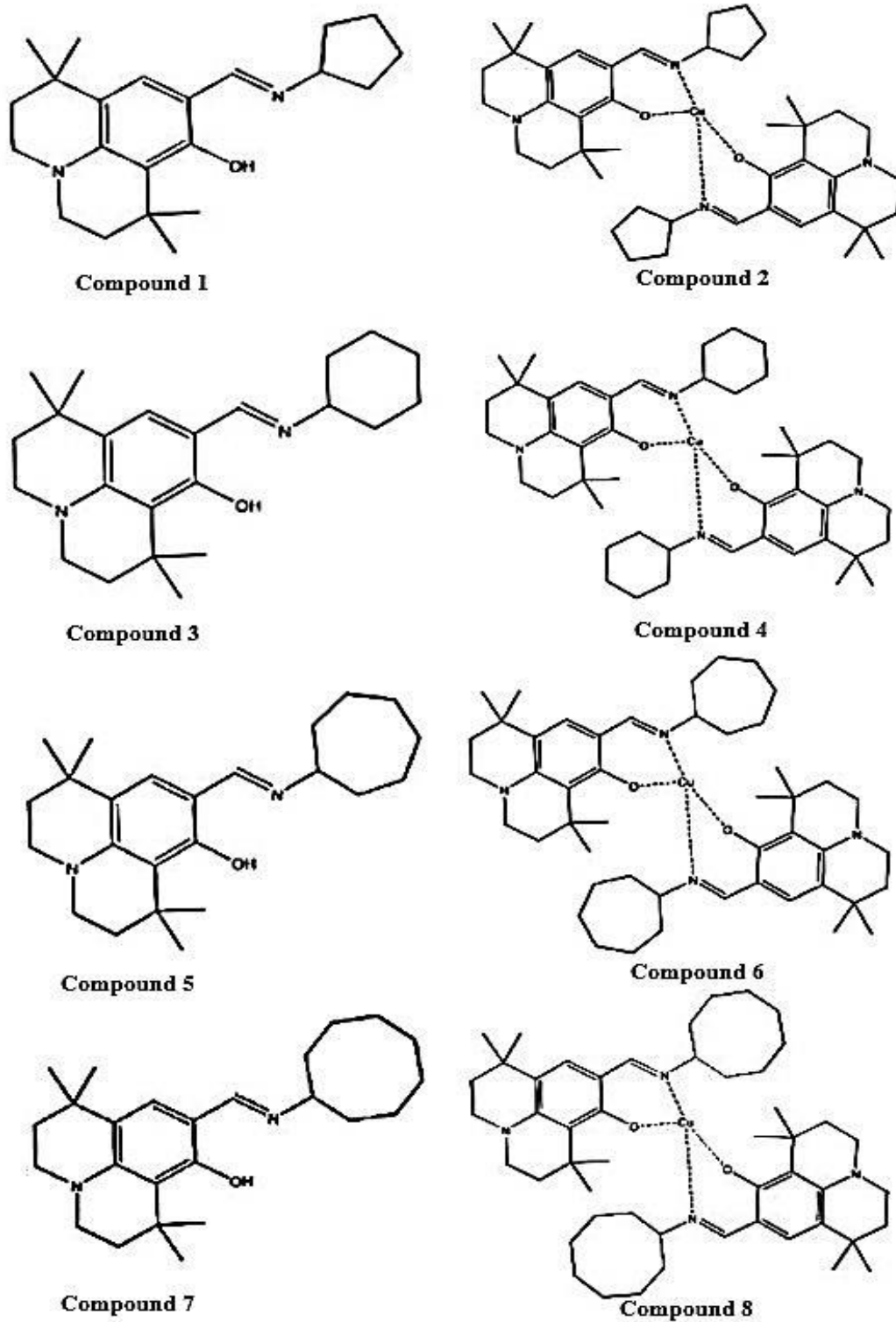


Figure 3.1. Salicylaldehyde-derived Chemical structures (C1 to C8)

3.1.2 . MTT stain

10 mg/mL of MTT or (Methyl thiazolyl diphenyl-tetrazolium bromide) dye has been prepared and used to detect the Antibacterial activity and cellular viability of our SB compounds against *Streptococcus pyrogens*, *K pneumonia*, and *P mirabilis*.

3.1.3. Bacteria strains

Three Stains of bacteria which were *P. mirabilis* (ATCC 14153), *K. pneumoniae* (ATCC 13883) and *S pyogens* (ATCC 19615) has been used in this study.

3.1.4. Bacterial culture media

Bacterial reactivation and reproduction has been done by the aid of Nutrient Broth media(NB), and a portion of grown bacteria has been stored and stocked for future application by both Plate and glycerol stock(Glycerol + Broth mixture) preparation.

3.1.5. McFarland standard

McFarland turbidity standard (0.5) turbidity has been prepared by Spectrophotometer for all bacterial strains in order to adjust the number of bacteria (1.5×10^8 CFU / ml) in the medium.

3.1.6. Microplate reader

Optical density (OD) measurement for all wells has been recorded against the Blank by Microplate-reader (Biotech, TS-800) at 570nm.



Figure 3.2. Microplate reader (Biotech, TS-800)

3.2. Methods

3.2.1. Compounds preparation

All Compounds has been dissolved in Absolute Alcohol as the concentration of 10 mM, and then has been sterilized by filtering into (20 μ m pore size) syringe filter (Minisart®, Biotech, USA), then serially has been diluted to get different concentration of 10 μ M, 100 μ M and 1000 μ M.

3.2.2. Preparation of culture plates

(10 μ l) of all SB Compounds with Genta have been added into 96-well micro plates in triple order as following concentration; 1 μ M, 10 μ M, 100 μ M and 1000 μ M.

3.2.3. Bacterial culture preparation

Frozen Stocks of *P. mirabili*, *K. pneumonia* and *S. pyogens* and bacteria has been activated in (25ml) of sterile NB medium, and been incubated at 37C and for 24h.

After incubation, A portion of grown bacteria has been stored and stocked for future application by both Plate and glycerol stock (Glycerol + Broth mixture)

preparation, while on the other hand in order to apply Microdilution assay, the bacterial number in the media has been diluted into (0.5 McFarland) standard turbidity which is equal to (1.5×10^8 CFU/ml) of bacterial cells, then they have been diluted again in sterile NB medium in order to reach the final number of (5×10^5). At the end, 100 μ l of each suspension has been add into all wells except negative control, and incubated for 18 h and at 37 c.

3.2.4. MTT assay application

(10 μ L) of MTT solution (Yellow-colored) has been applied into each wells (planted, Growth and Positive controls) and the culture plates were incubated at 37 c and for 4 hours. At the incubation time, the chemical structure of MTT molecule would be converted by life bacteria (Viable) into formazan-crystal (purple colored), which is not being dissolved in water. Eventually, (100 μ L) of solvent DMSO (dimethylsulfoxide, Sigma) was applied into each well, in order to dissolve Formazan-crystals.

Depend on the color change, the optical density (OD) values have been determined for the quantitation of dissolved formazan within DMSO, by scanning the culture plates in the ELIZA plate reader (Biotech, TS-800) at 570 nm wavelength. The background absorbance has been subtracted from all wells include positive and Growth controls.

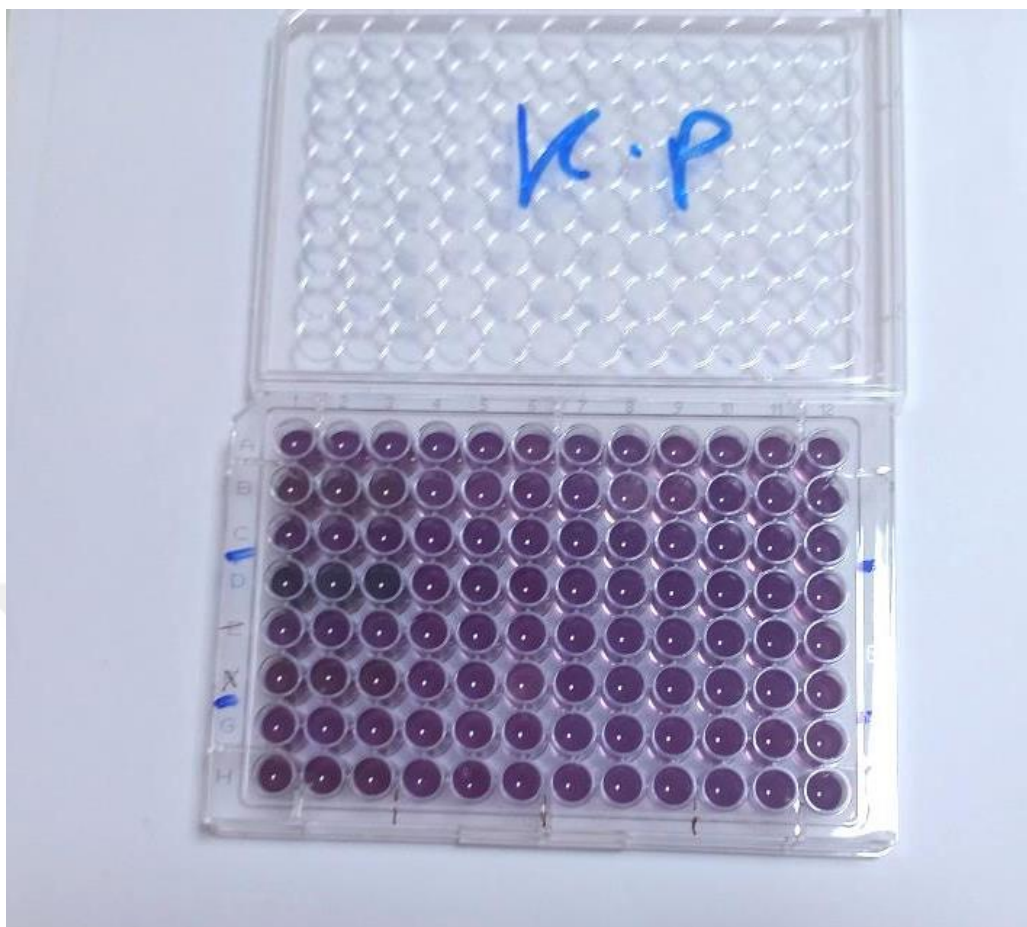


Figure 3.3. Purple colored formazan production after MTT reduction by bacteria

3.2.5. Statistical analysis

All statistical data with their consistency between different doses of genta and any compounds have been done by Homogeneity of variance, which has been interpreted by SPSS version 16 application (SPSS Inc., Chicago, IL, USA).

After variance homogeneity, and plotting Optical density (OD) in Y- axis and Doses at X-axis, Nonlinear regression to calculate MIC₅₀ was performed by Graphpad (Prism 9).

4. RESULT and DISCUSSION

4.1. Result

4.1.1. Antimicrobial activity on *K. pneumonia*

4.1.1.1. Antimicrobial activity of Gentamicin on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of Gentamicin presented in Table 4.1.

Table 0.1. OD values from the incubation of *K. pneumonia* with gentamicin and MIC₅₀ values

		Doses (μM)					Homogeneity of
		Control	1	10	100	1000	variance
Genta	OD values	0.481±0.01	0.34±0.012	0.234±0.01	0.127±0.00	0.088±0.0	P= 0.1
		MIC ₅₀ : 2.37 μM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with Gentamicin is presented in Figure 4.1.

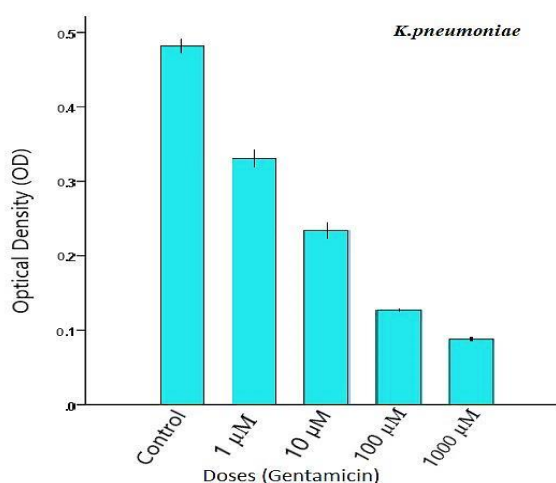


Figure 4.1. Antibacterial effects of gentamicin on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of Gentamicin on *K. pneumonia* was found to be 2.37 μ M.

4.1.1.2. Antimicrobial activity of Compound 1 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 1) presented in Table 4.2.

Table 4.2. OD values from the incubation of *K. pneumonia* with (Compound 1) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
Compound 1	OD values 0.48 $\pm$ 0.01	0.355 $\pm$ 0.00	0.319 $\pm$ 0.00	0.239 $\pm$ 0.02	0.216 $\pm$ 0.00	P= 0.002
	MIC ₅₀ : 16.20 μ M					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 1) is presented in Figure 4.2.

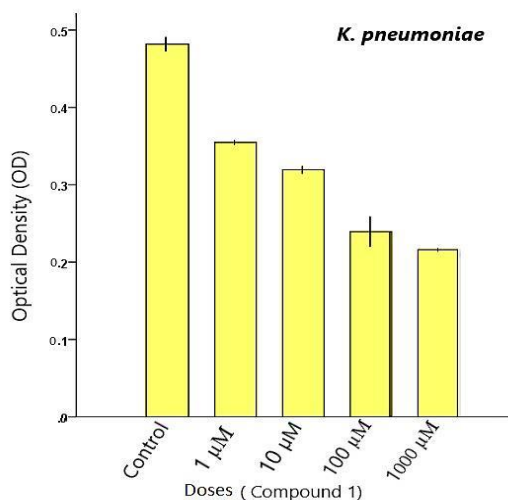


Figure 4.2. Antibacterial effects of Compound 1 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of Compound 1 on *K. pneumonia* was found to be 16.20 μ M.

4.1.1.3. Antimicrobial activity of compound 2 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 2) presented in Table 4.3.

Table 0.3. OD values from the incubation of *K. pneumonia* with (Compound 2) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.48 $\pm$ 0.01	0.473 $\pm$ 0.01	0.457 $\pm$ 0.00	0.395 $\pm$ 0.02	0.362 $\pm$ 0.02	
Compound 2	MIC ₅₀ : 7.19 μ M					P= 0.049

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 2) is presented in Figure 4.3.

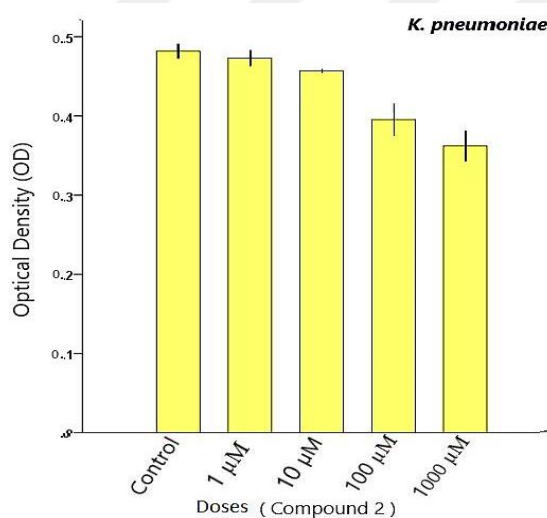


Figure 4.3. Antibacterial effects of Compound 2 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of Compound 2 on *K. pneumonia* was found to be 7.19 μ M.

4.1.1.4. Antimicrobial activity of compound 3 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 3) presented in Table 4.4.

Table 0.4. OD values from the incubation of *K. pneumonia* with (Compound 3) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.436 $\pm$ 0.00	0.421 $\pm$ 0.01	0.343 $\pm$ 0.01	0.355 $\pm$ 0.013	
Compound 3	MIC ₅₀ : 5.67 μ M					P= 0.12

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 3) is presented in Figure 4.4.

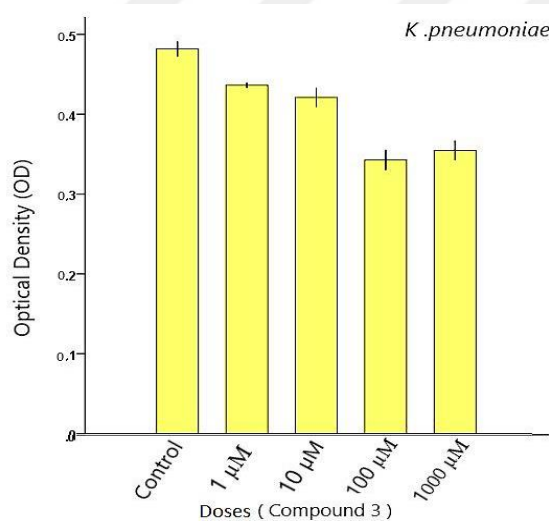


Figure 4. 4. Antibacterial effects of Compound 3 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of Compound 3 on *K. pneumonia* was found to be 5.67 μ M.

4.1.1.5. Antimicrobial activity of compound 4 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 4) presented in Table 4.5.

Table 4.5. OD values from the incubation of *K. pneumonia* with (Compound 4) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.48 $\pm$ 0.01	0.469 $\pm$ 0.00	0.469 $\pm$ 0.01	0.448 $\pm$ 0.02	0.365 $\pm$ 0.00	
Compound 4	MIC ₅₀ : 11.50 μ M					P= 0.007

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 4) is presented in Figure 4.5.

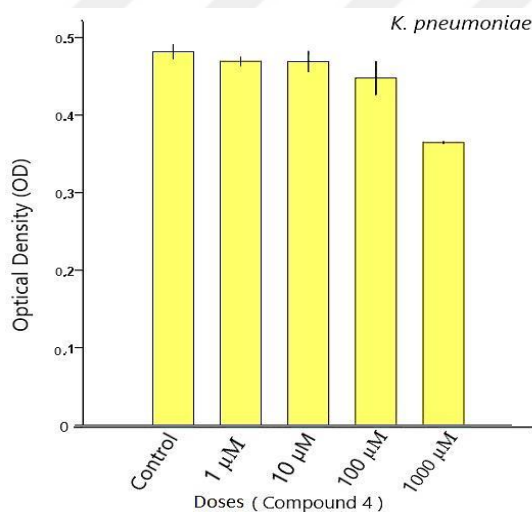


Figure 4.5. Antibacterial effects of Compound 4 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 4) on *K. pneumonia* was found to be 11.50 μ M.

4.1.1.6. Antimicrobial activity of compound 5 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 5) presented in Table 4.6.

Table 4.6. OD values from the incubation of *K. pneumonia* with (Compound 5) and MIC₅₀ values

Doses (μM)							Homogeneity of
	Control	1	10	100	1000		variance
Compound 5	OD values	0.48±0.01	0.365± 0.01	0.351±0.01	0.320±0.008	0.246±0.028	P= 0.003
		MIC ₅₀ : 13.90 μM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 5) is presented in Figure 4.6.

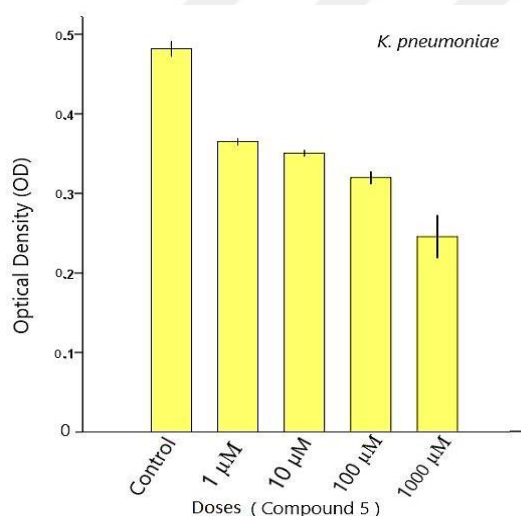


Figure 4.6. Antibacterial effects of Compound 5 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 5) on *K. pneumonia* was found to be 13.90 μ M.

4.1.1.7. Antimicrobial activity of compound 6 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 6) presented in Table 4.7.

Table 4.7. OD values from the incubation of *K. pneumonia* with (Compound 6) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.48 $\pm$ 0.01	0.480 $\pm$ 0.02	0.445 $\pm$ 0.01	0.426 $\pm$ 0.01	0.370 $\pm$ 0.010	
Compound 6	MIC ₅₀ : >100 μ M					P= 0.178

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 6) is presented in Figure 4.7.

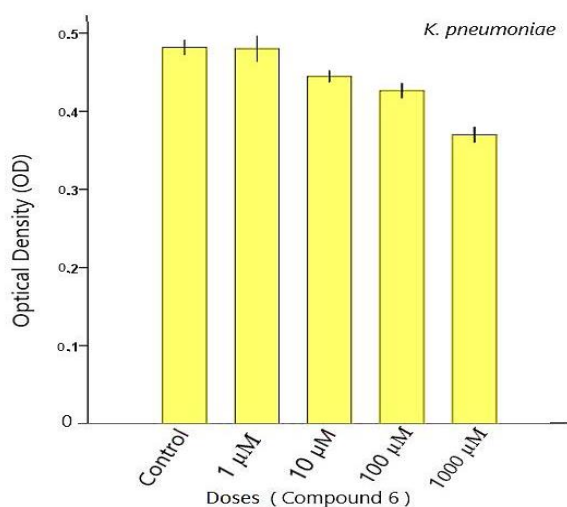


Figure 4.7. Antibacterial effects of Compound 6 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 6) on *K. pneumonia* was found to be (>100 μ M).

4.1.1.8. Antimicrobial activity of compound 7 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 7) presented in Table 4.8.

Table 0.8. OD values from the incubation of *K. pneumonia* with (Compound 7) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
Compound 7	OD values 0.48 $\pm$ 0.01	0.459 $\pm$ 0.01	0.375 $\pm$ 0.01	0.351 $\pm$ 0.009	0.326 $\pm$ 0.011	P= 0.67
	MIC ₅₀ : 14.50 μ M					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 7) is presented in Figure 4.8.

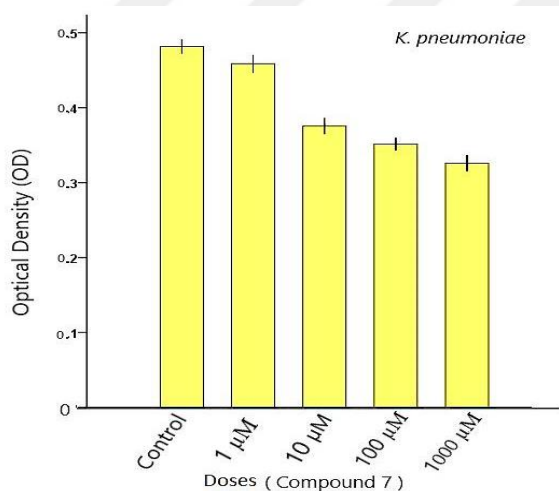


Figure 4.8. Antibacterial effects of Compound 7 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 7) on *K. pneumonia* was found to be 14.50 μ M.

4.1.1.9. Antimicrobial activity of compound 8 on *K. pneumonia*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 8) presented in Table 4.9.

Table 0.9. OD values from the incubation of *K. pneumonia* with (Compound 8) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.48 $\pm$ 0.01	0.445 $\pm$ 0.02	0.419 $\pm$ 0.01	0.375 $\pm$ 0.011	0.358 $\pm$ 0.014	
Compound 8	MIC ₅₀ : 6.99 μ M					P= 0.137

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *K. pneumonia* with (Compound 8) is presented in Figure 4.9.

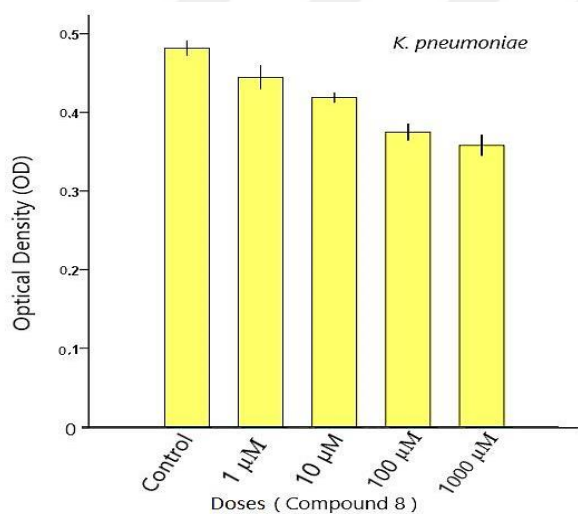


Figure 4.9. Antibacterial effects of Compound 8 on *K. pneumonia*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 7) on *K. pneumonia* was found to be 6.99 μ M.

4.1.2. Antimicrobial activity on *P. mirabilis*

4.1.2.1. Antimicrobial activity of Gentamicin on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of Gentamicin presented in Table 4.10.

Table 4.10. OD values from the incubation of *P. mirabilis* with Gentamicin and MIC₅₀ values

		Doses (μM)					Homogeneity of variance
		Control	1	10	100	1000	
Genta	OD values	0.491±0.00	0.224± 0.01	0.208±0.01	0.128 ± 0.01	0.077 ± 0.01	P= 0.22
		MIC ₅₀ : 1.63 μM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with Gentamicin is presented in Figure 4.10.

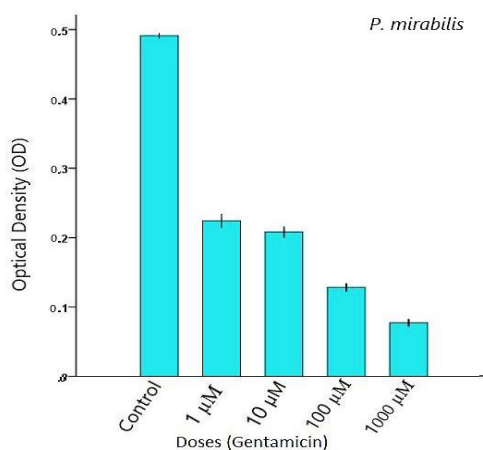


Figure 4. 10. Antibacterial effects of Gentamicin on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of Gentamicin on *P. mirabilis* was found to be 1.63 μ M.

4.1.2.2. Antimicrobial activity of compound 1 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 1) presented in Table 4.11.

Table 0.11. OD values from the incubation of *P. mirabilis* with (Compound 1) and MIC₅₀ values

Doses (μM)						Homogeneity of
	Control	1	10	100	1000	variance
Compound 1	OD values 0.491±0.01	0.474±0.01	0.394±0.00	0.385± 0.01	0.352± 0.002	P= 0.295
	MIC ₅₀ : 11.20 μM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 1) is presented in Figure 4.11.

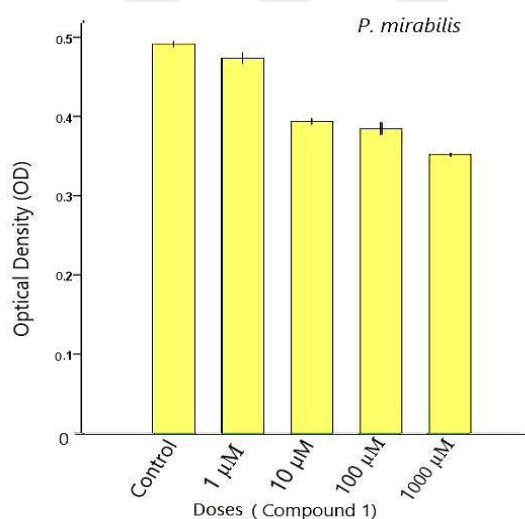


Figure 4. 11. Antibacterial effects of Compound 1 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 1) on *P. mirabilis* was found to be 11.20 μ M.

4.1.2.3. Antimicrobial activity of compound 2 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 2) presented in Table 4.12.

Table 0.12. OD values from the incubation of *P. mirabilis* with (Compound 2) and MIC₅₀ values

Doses (μM)							Homogeneity of
	Control	1	10	100	1000		variance
Compound 2	OD values	0.491±0.01	0.457±0.01	0.373±0.01	0.320± 0.01	0.286± 0.006	P= 0.61
		MIC ₅₀ : 4.95 μM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 2) is presented in Figure 4.12.

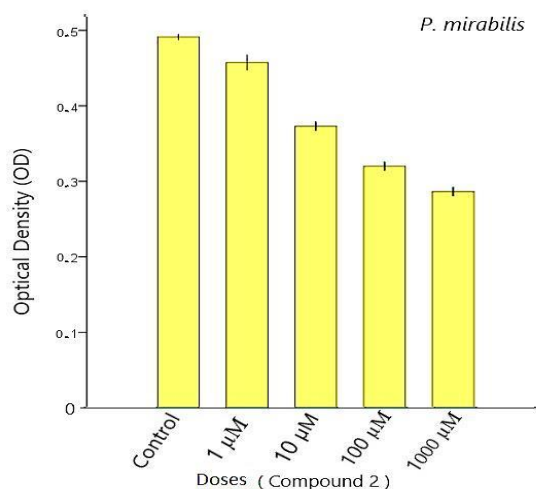


Figure 4.12. Antibacterial effects of Compound 2 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 2) on *P. mirabilis* was found to be 4.95 μ M.

4.1.2.4. Antimicrobial activity of compound 3 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 3) presented in Table 4.13.

Table 4.13. OD values from the incubation of *P. mirabilis* with (Compound 3) and MIC₅₀ values

		Doses (μM)					Homogeneity of
		Control	1	10	100	1000	variance
Compound 3	OD values	0.491±0.01	0.397±0.01	0.360±0.01	0.284±0.013	0.268±0.01	P= 0.122
		MIC ₅₀ : 3.90 μM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 3) is presented in Figure 4.13.

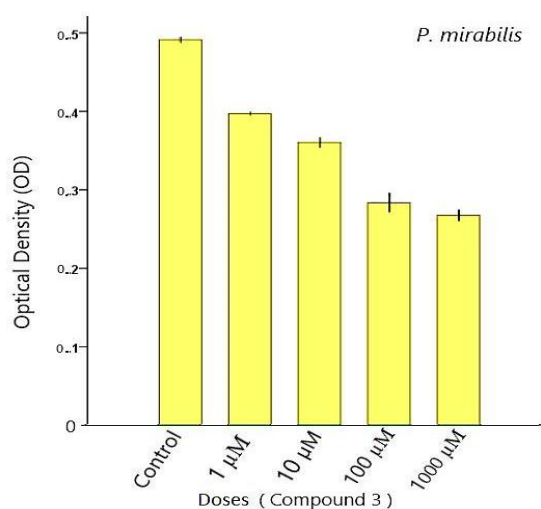


Figure 4.13. Antibacterial effects of Compound 3 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 3) on *P. mirabilis* was found to be 3.90 μ M.

4.1.2.5. Antimicrobial activity of compound 4 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 4) presented in Table 4.14.

Table 4.14. OD values from the incubation of *P. mirabilis* with (Compound 4) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
Compound 4	OD values 0.491 $\pm$ 0.01	0.46 $\pm$ 0.003	0.419 $\pm$ 0.01	0.367 $\pm$ 0.01	0.319 $\pm$ 0.01	P= 0 .167
	MIC ₅₀ : 7.93 μ M					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 4) is presented in Figure 4.14.

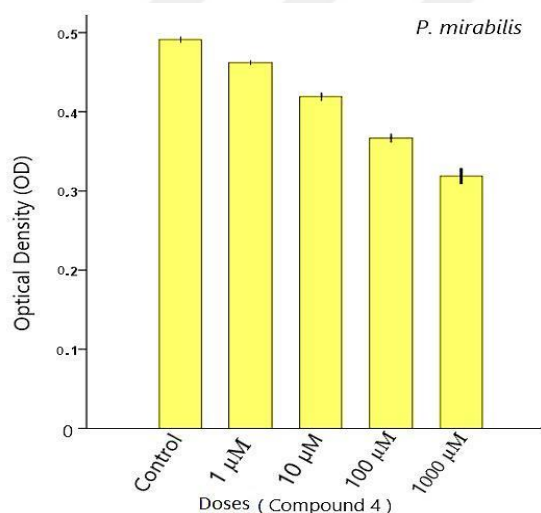


Figure 4.14. Antibacterial effects of Compound 4 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 4) on *P. mirabilis* was found to be 7.93 μ M.

4.1.2.6. Antimicrobial activity of compound 5 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 5) presented in Table 4.15.

Table 4.15. OD values from the incubation of *P. mirabilis* with (Compound 5) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.491 $\pm$ 0.01	0.451 $\pm$ 0.01	0.389 $\pm$ 0.01	0.369 $\pm$ 0.006	0.35 $\pm$ 0.01	
Compound 5	MIC ₅₀ : 9.56 μ M					P= 0.077

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 5) is presented in Figure 4.15.

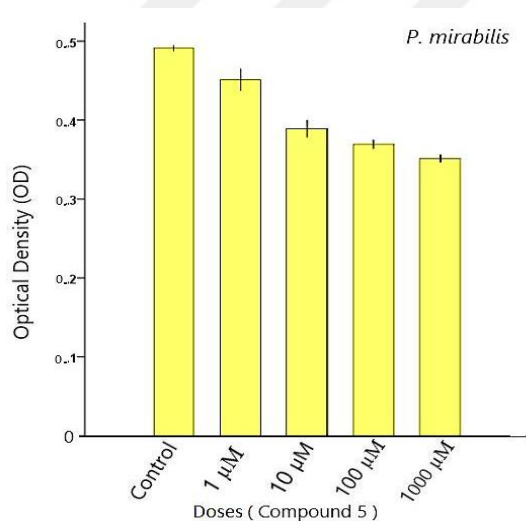


Figure 4. 15. Antibacterial effects of Compound 5 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 5) on *P. mirabilis* was found to be 9.56 μ M.

4.1.2.7. Antimicrobial activity of compound 6 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 6) presented in Table 4.16.

Table 4.16. OD values from the incubation of *P. mirabilis* with (Compound 6) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.491 $\pm$ 0.01	0.48 $\pm$ 0.017	0.445 $\pm$ 0.01	0.426 $\pm$ 0.01	0.370 $\pm$ 0.01	
Compound 6	MIC ₅₀ : >100 μ M					P= 0.178

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 6) is presented in Figure 4.16.

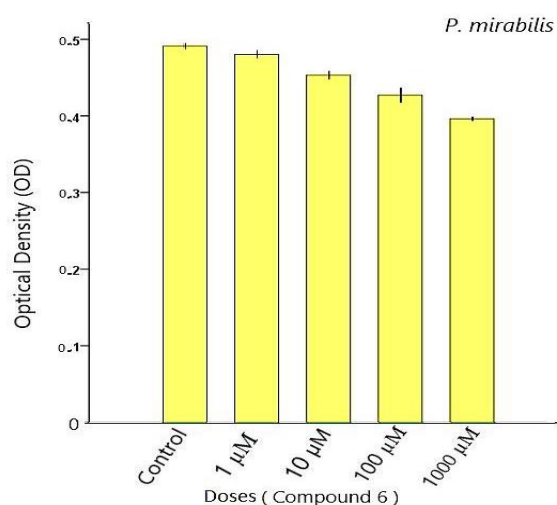


Figure 4.16. Antibacterial effects of Compound 6 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 6) on *P. mirabilis* was found to be (>100) μ M.

4.1.2.8. Antimicrobial activity of compound 7 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 7) presented in Table 4.17.

Table 4.17. OD values from the incubation of *P. mirabilis* with (Compound 7) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.491 $\pm$ 0.01	0.459 $\pm$ 0.01	0.375 $\pm$ 0.01	0.351 $\pm$ 0.009	0.326 $\pm$ 0.01	
Compound 7	MIC ₅₀ : 14.50 μ M					P= 0.671

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 7) is presented in Figure 4.17.

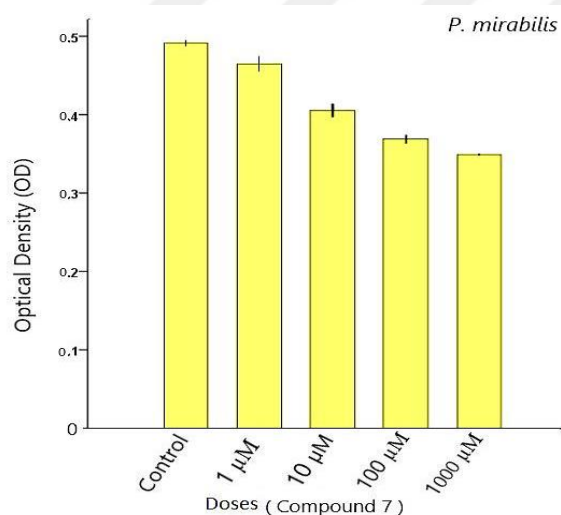


Figure 4.17. Antibacterial effects of Compound 7 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 7) on *P. mirabilis* was found to be 14.50 μ M.

4.1.2.9. Antimicrobial activity of compound 8 on *P. mirabilis*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 8) presented in Table 4.18.

Table 4.18. OD values from the incubation of *P. mirabilis* with (Compound 8) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
Compound 8	OD values 0.491 $\pm$ 0.01	0.445 $\pm$ 0.02	0.419 $\pm$ 0.01	0.375 $\pm$ 0.011	0.358 $\pm$ 0.01	P= 0.137
	MIC ₅₀ : 6.99 μ M					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *P. mirabilis* with (compound 8) is presented in Figure 4.18.

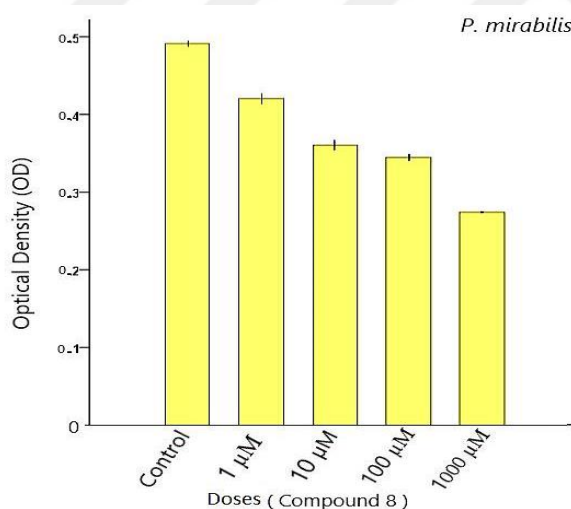


Figure 4.18. Antibacterial effects of Compound 8 on *P. mirabilis*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 8) on *P. mirabilis* was found to be 6.99 μ M.

4.1.3. Antimicrobial activity on *S. pyogenes*

4.1.3.1. Antimicrobial activity of Gentamicin on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of Gentamicin presented in Table 4.19.

Table 4.19. OD values from the incubation of *S. pyogenes* with Gentamicin and MIC₅₀ values

		Doses (µM)					Homogeneity of
		Control	1	10	100	1000	variance
Genta	OD values	0.481±0.01	0.343± 0.01	0.234±0.01	0.163±0.023	0.086±0.002	P= 0.001
		MIC ₅₀ : 1.82 µM					

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with Gentamicin is presented in Figure 4.19.

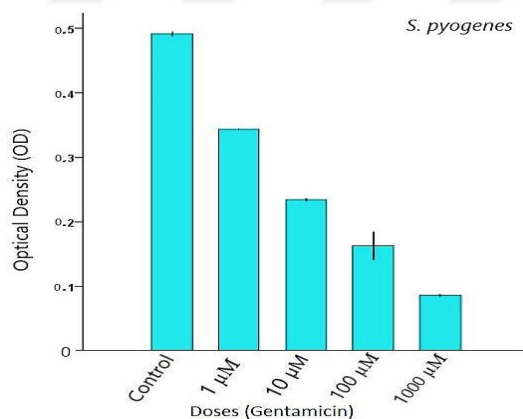


Figure 4.19. Antibacterial effects of Gentamicin on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of Gentamicin on *S. pyogenes* was found to be 1.82 μ M.

4.1.3.2. Antimicrobial activity of compound 1 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 1) presented in Table 4.20.

Table 0.20. OD values from the incubation of *S. pyogenes* with (Compound 1) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.224 $\pm$ 0.02	0.212 $\pm$ 0.01	0.195 $\pm$ 0.014	0.108 $\pm$ 0.026	
Compound 1	MIC ₅₀ : 1.86 μ M					P= 0.053

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 1) is presented in Figure 4.20.

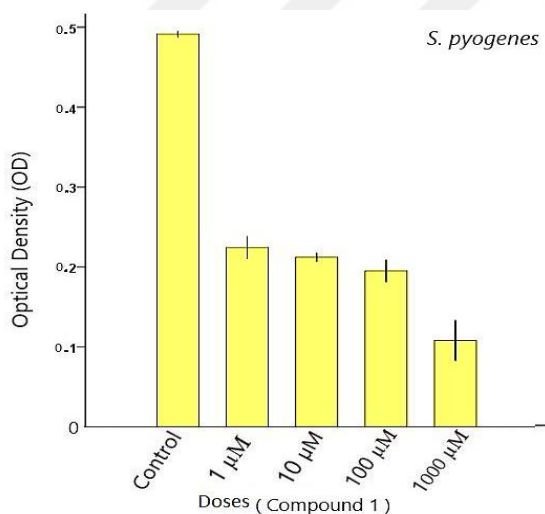


Figure 4.20. Antibacterial effects of Compound 1 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 1) on *S. pyogenes* was found to be 1.86 μ M.

4.1.3.3. Antimicrobial activity of compound 2 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 2) presented in Table 4.21.

Table 4.21. OD values from the incubation of *S. pyogenes* with (Compound 2) and MIC₅₀ values

	Control	Doses (μ M)				Homogeneity of variance
		1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.307 $\pm$ 0.01	0.25 $\pm$ 0.028	0.232 $\pm$ 0.001	0.217 $\pm$ 0.005	
Compound 2		MIC ₅₀ : 2.57 μ M				P= 0.051

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 2) is presented in Figure 4.21.

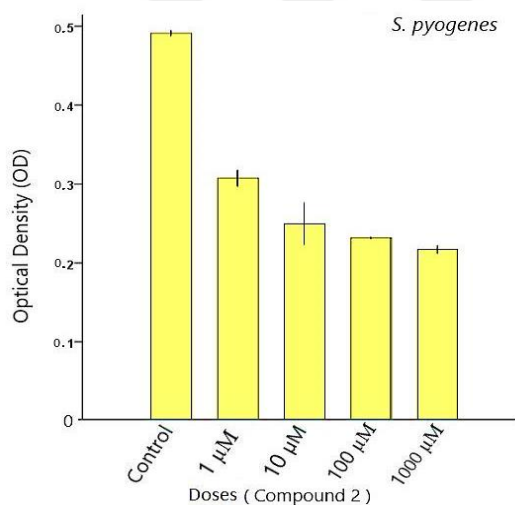


Figure 4.21. Antibacterial effects of Compound 2 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 2) on *S. pyogenes* was found to be 2.57 μ M.

4.1.3.4. Antimicrobial activity of compound 3 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 3) presented in Table 4.22.

Table 4.22. OD values from the incubation of *S. pyogenes* with (Compound 3) and MIC₅₀ values

	Control	Doses (μ M)				Homogeneity of variance
		1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.385 $\pm$ 0.01	0.296 $\pm$ 0.01	0.304 $\pm$ 0.029	0.229 $\pm$ 0.014	
Compound 3		MIC ₅₀ : 3.25 μ M				P= 0.029

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 3) is presented in Figure 4.22.

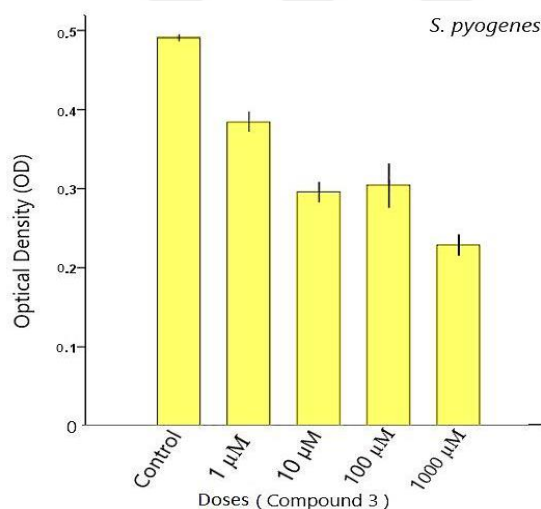


Figure 4.22. Antibacterial effects of Compound 3 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 3) on *S. pyogenes* was found to be 3.25 μ M.

4.1.3.5. Antimicrobial activity of compound 4 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 4) presented in Table 4.23.

Table 0.23. OD values from the incubation of *S. pyogenes* with (Compound 4) and MIC₅₀ values

	Control	Doses (μ M)				Homogeneity of variance
		1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.318 $\pm$ 0.01	0.273 $\pm$ 0.01	0.253 $\pm$ 0.031	0.157 $\pm$ 0.004	
Compound 4		MIC ₅₀ : 2.37 μ M				P= 0.007

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 4) is presented in Figure 4.23.

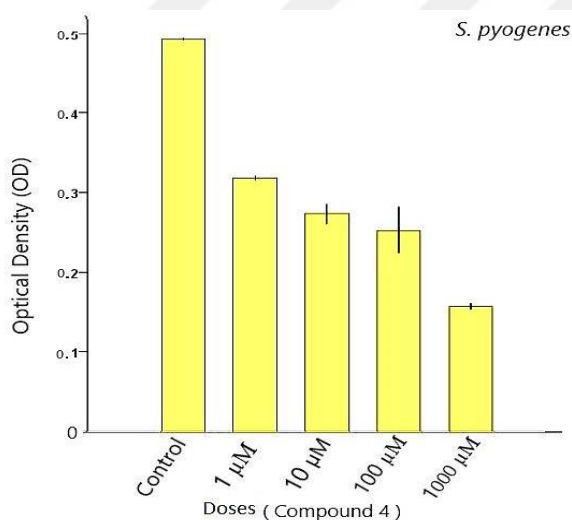


Figure 4.23. Antibacterial effects of Compound 4 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 4) on *S. pyogenes* was found to be 2.37 μ M.

4.1.3.6. Antimicrobial activity of compound 5 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 5) presented in Table 4.24.

Table 4.24. OD values from the incubation of *S. pyogenes* with (Compound 5) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.318 $\pm$ 0.01	0.314 $\pm$ 0.02	0.312 $\pm$ 0.012	0.284 $\pm$ 0.008	
Compound 5	MIC ₅₀ : 3.88 μ M					P= 0.381

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 5) is presented in Figure 4.24.

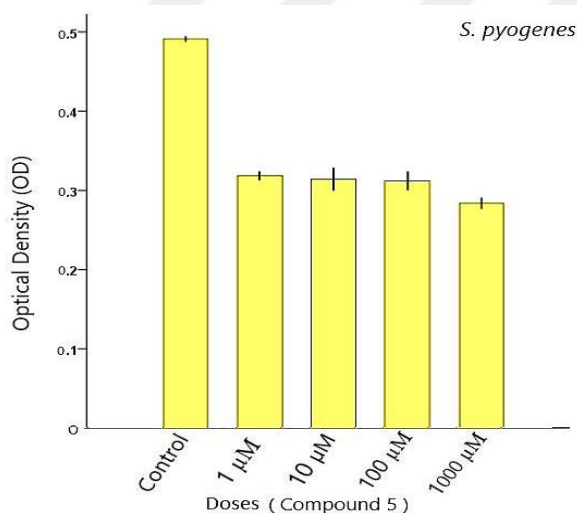


Figure 4.24. Antibacterial effects of Compound 5 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 5) on *S. pyogenes* was found to be 3.88 μ M.

4.1.3.7. Antimicrobial activity of compound 6 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 6) presented in Table 4.25.

Table 0.25. OD values from the incubation of *S. pyogenes* with (Compound 6) and MIC₅₀ values

	Control	Doses (μ M)				Homogeneity of variance
		1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.313 $\pm$ 0.01	0.254 $\pm$ 0.01	0.234 $\pm$ 0.003	0.151 $\pm$ 0.023	
Compound 6		MIC ₅₀ : 2.24 μ M				P= 0.004

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 6) is presented in Figure 4.25.

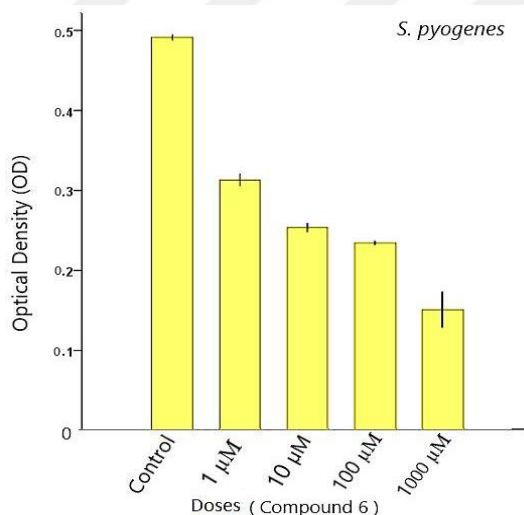


Figure 4. 25. Antibacterial effects of Compound 6 on *S. pyogenes*.

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 6) on *S. pyogenes* was found to be 2.24 μ M.

4.1.3.8. Antimicrobial activity of compound 7 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 7) presented in Table 4.26.

Table 4.26. OD values from the incubation of *S. pyogenes* with (Compound 7) and MIC₅₀ values

	Control	Doses (μ M)				Homogeneity of variance
		1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.364 $\pm$ 0.01	0.332 $\pm$ 0.01	0.308 $\pm$ 0.006	0.243 $\pm$ 0.003	
Compound 7		MIC ₅₀ : 3.51 μ M				P= 0.078

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 7) is presented in Figure 4.26.

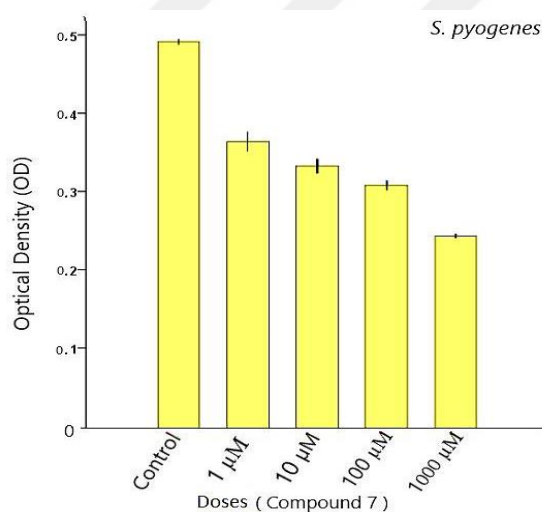


Figure 4.26. Antibacterial effects of Compound 7 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 7) on *S. pyogenes* was found to be 3.51 μ M.

4.1.3.9. Antimicrobial activity of compound 8 on *S. pyogenes*

The mean $\pm$ SD values of OD data obtained from MTT test, homogeneity of variance analysis results (P values) and MIC₅₀ value of (Compound 8) presented in Table 4.27.

Table 0.27. OD values from the incubation of *S. pyogenes* with (Compound 8) and MIC₅₀ values

	Doses (μ M)					Homogeneity of variance
	Control	1	10	100	1000	
OD values	0.481 $\pm$ 0.01	0.421 $\pm$ 0.01	0.382 $\pm$ 0.01	0.285 $\pm$ 0.013	0.244 $\pm$ 0.006	
Compound 8	MIC ₅₀ : 3.65 μ M					P= 0.483

The Graphic Bar chart of the OD values which was recorded after the MTT-assay application, and after Co-cultivation of *S. pyogenes* with (Compound 8) is presented in Figure 4.27.

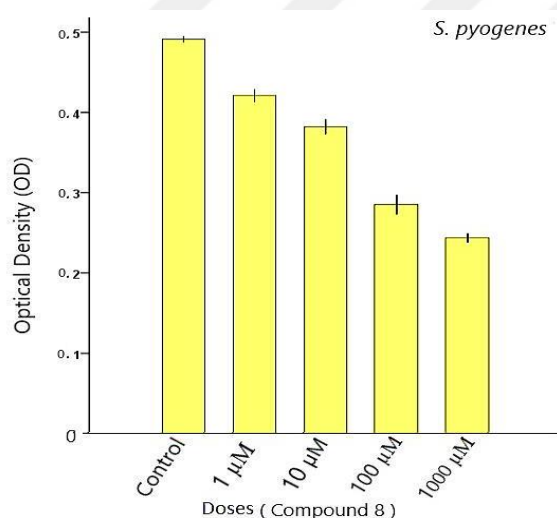


Figure 4.27. Antibacterial effects of Compound 8 on *S. pyogenes*

Depend on regression analysis by using OD values obtained from MTT assay, the MIC₅₀ value of (Compound 8) on *S. pyogenes* was found to be 3.65 μ M.

4.2. Discussion

Researchers' interest in developing novel tactics or compounds, particularly those derived from plant extracts or synthetic substances with antibacterial action, has increased due to the rise in microbial resistance to modern clinical antibacterials. Despite the widespread use of agar diffusion, particularly (Disk or well diffusion), and dilution techniques, these approaches have many drawbacks, and we are unable to utilize them for all colored and nonpolar substances as mentioned by the (Eloff J.N, 2019) study, so regarding to this in our investigation, we applied one of the tetrazolium salt stain (MTT-stain) in order to obtain better and more precise data.

Genta belongs to the class of antibiotics known as aminoglycosides, which work against bacteria by inhibiting their ability to make proteins; giving it a broad spectrum of antibacterial efficacy. So in order to compare their findings with Genta, the majority of researchers, as well as in our study, have used it as a positive control, such studies have carried out by (Warad I et al., 2020) and (Thammawithan et al., 2021). Gentamicin's MIC₅₀ value as a positive control was 1.6 μ M for *P mirabilis*, 1.8 μ M for *S pyogenes*, and 2.4 μ M for *K pneumonia*, respectively. Our Statistical data revealed that Genta behaves considerably differently against each type of bacterial specie.

In contrast to earlier researches on *P. mirabilis* that prolonged incubation period rather than adding a dissolving agent, such as studies investigated by (Grela et al., 2015) and (Grela et al., 2016), in our study the DMSO (dissolving agent) was added after the application of MTT-stain for the purpose of OD reading improvement and Formazan-crystal dissolving improvement, such as the investigation carried out by (Wang et al., 2007) study.

In contrast to common researches that employed MTT assay as the visually endpoint color test as aiming to record MIC, in our investigation one of the Colorimetric device was used to measure OD, because it is proportional to the number of living bacteria, such as the research has carried out by (Shi et al., 2007). In this investigation, MIC₅₀ which is the smallest quantity of compound that causes

50% inhibition of bacterial population value has been used to measure chemical compound inhibition concentration instead of using MIC, which is the lowest concentration of chemicals that induce inhibition.

In one of the research carried out by (Wang et al, 2007), the viable bacterial number had decreased in proportional with increasing chemical concentration in a linear relationship, whereas in our investigation there is nonlinear relation between the bacterial growth and increasing compound Concentration, in regards to many modification which have been conducted in our study.

Recently, researchers' focus has been growing more and more on metal complexes of SB and salicylaldehyde. According to some studies, previously tested ligands' metal complexes have a greater potential to affect viable cells than their basic ligands, In (Xiao et al., 2017) study, in which Cobalt complexes of two different Schiff bases were created and tested against human skin cancer cells and the results demonstrated that the combination of SBs with Cobalt improved their anticancer effect, An additional study that supports these hypotheses was that by (Emmanuel et al, 2018), which synthesized a copper complex from SBs and tested it against ten different species of bacteria through the agar disk-diffusion method. The results showed that the metal complex of SB had superior antibacterial activity to earlier ligands, However, Some other researches has indicated that the metal complex has altered the impact of earlier ligands on various bacterial species in a variety of ways, one such investigation is carried out by (Saghatforoush et al. 2009). Therefore, in our experiment, despite the fact that some compounds had anticancer effects, we anticipated that some of our Copper-complexes may would have had better MIC₅₀ values than original ligand compounds on each specific bacterial strains, and the results of our study showed that after testing (8) salicylaldehyde-derived SB on *P. mirabilis* and *K. pneumoniae* (both Gram negative), and recording their MIC₅₀ values, C3 had the lowest MIC₅₀ value which was 3.9 µM and 5.7 µM respectively, and C6 had the Highest MIC₅₀ value which was more than 100 µM on both gram negative bacterium, while against *S. pyogenes* (Gram positive), C1 had the lowest MIC₅₀ value of 1.9 µM, and C5 had maximum MIC₅₀ value of 3.9 µM. with

respecting to Metal complex action on microbe inhibition activity, Our research revealed that all four Complexes had an identical activity against both our gram negative strains, wherein SB complex (C2 and C8) had better effect in compare with their ligand (C1 and C7), while other SB Copper Complexes(C6 and C4) had less effect in contrast to their ligand SBs (C3 and C5). Our gram positive strain (*S. pyogenes*) on the other hand, responded differently to the metal complex SBs in our experiment, which may be because their cell walls differ structurally from those of the other gram negative bacteria.



5. CONCLUSIONS and RECOMMENDATIONS

5.1. Conclusions

The urgent request of potent chemical or natural antibacterial drugs by the nowadays world against antibacterial-resistant bacteria is critical in order to combat the raising microbial-resistance issue toward antibacterial drugs. In our study, we checked the antibacterial activity of (8) Salicylaldehyde derived Schiff-bases compounds (Ligands and Copper complexes) and Gentamicin (positive control), by antibacterial MTT-assay against three pathogenic bacterial strains, in aiming to find their differences antibacterial effect (Ligands and complex) on these bacteria.

MTT which is one of tetrazolium salt derivative stain has been applied as bacterial viability indicator as it has potential to undergo reduction reaction and changed to Formazan crystal, thus its color is changed from purple to yellow colored formazan which is soluble easily in DMSO solvent.

The number of bacteria inside wells is proportional to viable bacterial cells which has been recorded colorimetrically by Microplate reader and then analyzed to obtain MIC₅₀, which is regarded as the smallest amount of compound required to inhibit bacterial growth by 50%, and in outcome, Gentamicin's MIC₅₀ for *P. mirabilis*, *S. pyogenes* and *K. pneumonia* were 1.6 μ M, 1.8 μ M and 2.4 μ M, respectively.

In outcome we found out that C3 has most effective MIC₅₀ which was 5.7 and 3.9 for *K. pneumoniae* and *P. mirabilis* respectively, while C1 had more effect against *S. pyogenes* which was 1.9 MIC₅₀. And we discovered that some of the complexes had better antimicrobial action than earlier, while others had the opposite effect, and all of the compounds also had varying effects on the two types of Gram-stained bacteria.

5.2. Recommendation

Traditionally, the most common used technique in order to test the chemical compounds or extracts (Plant, insect and animals), which are diffusion (Disk and Well) and dilution (Typical macro or Microdilution) which have been standardized by many worldwide organization like CLSI and EUCAST, in regarding with their highly benefit inside antibacterial field, they have their limited usage and disadvantage, so it seeks to find newly standardized method such as our MTT assay antibacterial method.

Our study is one of the newly advanced researches in regarding to Antimicrobial research methods, in which we applied MTT modified unstandardized assay to measure antibacterial activity of synthesized salicylaldehyde derived Schiff bases in order to improve this technique with better approach for future researches and researchers.

Our research is a apart of many research projects which have been done recently, which they use MTT assay as antibacterial assay, but further standardization will has to be attempted by other researchers in order to get fully standardized assay for one future generation.

C3 and C1 which had nearly very close activity to Genta have to be put in consideration and checked against more bacterial strains as a candidate compound for future antibacterial drug chemical compound.

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