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## Determination of antibacterial activities of some salicylaldimine derived-Schiffbase with their copper complexes on some pathogenic bacteria

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### Abstract

The identification of novel potent chemicals against drug-resistant bacteria is critical to combat the alarming issue of microbial resistance toward antibiotics. There is a high risk of resistant infections spreading uncontrollably due to a lack of new and efficient antibiotics. So regarding to this manner, we tested the antibacterial activity of some salicylaldimine-derived Schiff bases with their Copper (Cu) complexes and Gentamicin (Genta) as four different concentration 1  $\mu$ M, 10  $\mu$ M, 100  $\mu$ M and 1000  $\mu$ M, by our newly modified MTT assay protocol against three bacterial strains of *Streptococcus pyogenes*(*S. pyogenes*), *Klebsiella pneumonia*(*K. pneumonia*), and *Proteus mirabilis*(*P.mirabilis*)which they have high risk for drug resistance development and analyzed their Optical density(OD) data in order to get MIC<sub>50</sub> values. And as a result,the lowest and most effective MIC<sub>50</sub> value of Genta was 1.6 $\mu$ M for *P. mirabilis* followed by 1.8 and 2.4  $\mu$ M for *S. pyogenes* and *K. pneumonia* respectively. The compound ligands and their complexes had effect differently on these bacteria based on their different cellular structure and some of the complexes showed better antibacterial activity than their original ligands, regarding with observation of unique bacterial response toward our MTT protocol.

**Keywords:** Schiff base, Salicylaldimine, Antimicrobial, MIC<sub>50</sub>, MTT test

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### Introduction

Antimicrobial resistance (AMR) is a serious danger for patient care, since it increases morbidity and mortality, lengthens hospital stays, and costs the patient and country a lot of money (Rosenberger et al., 2011)and (Morales et al., 2012).Antibiotic resistant is the state in which the bacteria get the power of defense against the pre-existing antibiotics or antimicrobials and can live with the existence of these drugs. The Bacteria carry out resistant through gene level and can be interstice or acquired(Willey et al.,

2020),(MADIGAN et al., 2021, p.930- 940) and (Zaman et al., 2017).

According to WHO's figures from 2005, the global burden of patients infected and suffering from *S. pyogenes* is 18.1 million people, with (1.78 million) new cases and (500,000) death cases recorded annually (World Health Organization, 2005). However *S.pyogenes* slowly improved resistance against traditional antibiotics especially Penicillin-derived and B-Lactam class of antibiotics(Horn et al., 1998).*K. pneumoniae* is one of the members of *Enterobacteriaceae* family which is common cause of infections all over the world. According to recent studies, due to



mutational inactivation of the *mgrB* regulatory gene, multi drug resistance *K.pneumonia* showed resistance to Colistin(Kidd et al, 2017).

Schiff base is any compound with structure  $R_1R_2C=NR_3$ (Abu-Dief& Mohamed, 2015). Schiff base can be obtained naturally or through modification of natural source or by synthetically production (Da silva et al, 2011). Schiff base compounds are broadly applied in industrial field, and they have a wide range of biological effects such as antimicrobial (antibacterial, antiviral, antimalarial) as well as anticancer activity (Przybylski et al., 2009).

Metal complexes of Schiff bases have been recommended and preferred by many studies that may show better biological activity. For instance in a study Conducted by (Emmanuel et al, 2018), Copper complex from (Sulphadiazine Schiff Base bind with aniline derived Schiff base) has been synthesized and checked against (10) species of bacteria, among them are (*E. coli*, *K. pneumonia*, *Proteus vulgaris*, *Salmonella typhi* and *S. aureus*), the agar disk diffusion method has been used for testing antibacterial activity and Ampicillin has been used as positive control. Eventually, the outcome was amazing; the metal complex Schiff base has better effect and larger inhibition zone than original ligand against all bacterial species. While in another study, Schiff-base with cobalt ion complexes synthesized and investigated for their antibacterial activity and in outcome, Antibacterial activity of previous Schiff bases is higher than that of cobalt complexes, even though synthesized compound showed antibacterial activity

against some pathogenic bacteria(Saghatforoush et al. 2009).

MTT assay is a common technique which has been originally used in estimating the metabolic activity of mammalian viable cell lines. The principal of this assay is depending on application of MTT or (Methyl thiazolyl diphenyl-tetrazolium bromide) dye on the cells, in which has been undergone through enzymatic reduction reaction by Mammalian mitochondrial enzymes, and as a result it will reduce light-colored tetrazolium salt to its purple colored formazan crystals. Eventually the water insoluble formazan crystals can be dissolved in Alkaline Dimethyl sulfoxide (DMSO) and can be measured by spectrophotometer. MTT has been proposed to be used in prokaryotic quantification for a long time as they have some enzymatic mechanisms which is close to eukaryotic reduction reactions or some unique reduction mechanisms. It has been used in many microbial researches such as MIC, multidrug-resistant bacteria determination, biofilm evaluation and antimicrobial studies (Grela et al, 2018) and (Benov L, 2021).

In our study, the biological actions of eight salicylaldehyde derived compounds and their copper complexes have been investigated through modified MTT assay in order to find out their antibacterial ability toward *S. pyogenes*, *K. pneumonia*, and *P. mirabilis* bacterial strains, and find out that if the complexes have better antibacterial activity than previous ligands.

### Experimental section

Eight Salicylaldehyde derived compounds (compound 1-8) have been



prepared and provided by Harran university/Faculty of Arts and Sciences/ Chemistry Department- Inorganic Chemistry Department. Compounds chemical structures and their Molecular weight are presented in (Figure 1 and Table 1) and Genta has been used as antibacterial positive control. All compounds have been dissolved in (10mM) Absolute alcohol, then sterilized by passing through 20  $\mu\text{m}$  pore size syringe filter (Minisart<sup>®</sup>, Biotech, USA), then they had been serially diluted to get 1000  $\mu\text{M}$ , 100  $\mu\text{M}$  and 10  $\mu\text{M}$  concentration. In order to prepare culture plates, 10  $\mu\text{l}$  of Compounds were added and planted in 96-well culture plates in triplicate order as following; 10  $\mu\text{M}$ , 100  $\mu\text{M}$ , 1000  $\mu\text{M}$  and 10000  $\mu\text{M}$ . MTT or (Methylthiazolyldiphenyl-tetrazolium bromide) dye has been prepared as the concentration (10 mg/mL), which used to determine the viability and antibacterial effect of synthesized chemical compounds on three Bacterial strains of *S. pyogenes* (ATCC 19615), *K. pneumonia* (ATCC 13883), and *P. mirabilis* (ATCC 14153). Nutrient Broth media (Sigma) has been used for microbial reactivation and growth. McFarland (0.5) standard for each bacterium has been prepared by Spectrophotometer in order to adjust the

bacterial numbers as ( $1.5 \times 10^8$  CFU/ml). Microplate reader (Biotech, TS-800) has been used to measure wells Absorbance at 570nm against the Blank. All statistical data has been analyzed by SPSS version 16 (SPSS Inc., Chicago, IL, USA), and Non-linear regression to calculate MIC<sub>50</sub> performed by Graphpad (Prism 9).

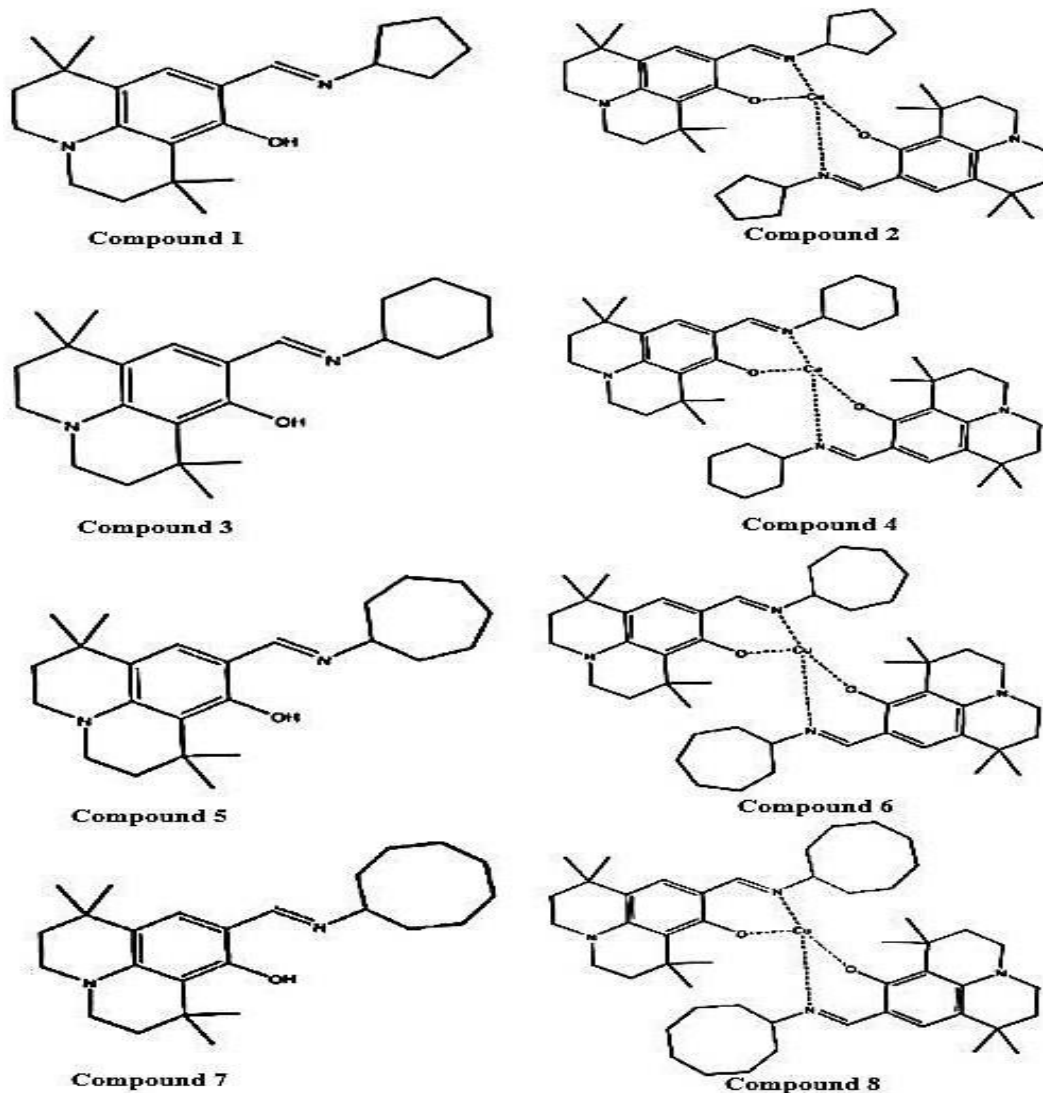
Bacterial stocks have been reactivated in 25ml of sterile Nutrient Broth (NB), and incubated at 37C for 24h. For Microdilution purpose, the number of bacterial cell in the media has been adjusted as (0.5 McFarland), then diluted in sterile nutrient Broth to get the final number of ( $5 \times 10^5$ ). Finally, 100  $\mu\text{l}$  of bacterial suspension were added into each planted, Growth and Positive controls and incubated for 18h at 37c<sup>o</sup>. After incubation, 10  $\mu\text{L}$  of yellow colored MTT solution has been added to all wells and incubated for 4 hours. During incubation, the chemical structure of MTT transformed by viable bacteria into purple colored formazan crystal which is not soluble in water. Then, in order to dissolve Formazan crystals, 100  $\mu\text{L}$  of DMSO (Sigma) has been added to each well. Eventually, formazan absorbance measured at 570nm and analyzed in order to get MIC<sub>50</sub> values.

**Table 1:** Chemical compound names and 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 of Compound 1	742.55
3	1,1,7,7-tetra-methyl-julolidine-N-hexyl-Salicylaldehyde	354.53
4	Copper complex of Compound 3	770.59
5	1,1,7,7-tetra-methyl-julolidine-N-heptyl-Salicylaldehyde	368.56
6	Copper complex of Compound 5	798.64
7	1,1,7,7-tetra-methyl-julolidine-N-octyl-Salicylaldehyde	382.58



8	Copper complex of Compound 7	826.69
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**Figure 1:** Salicylaldimine derived Chemical compound structures (C1-C8).

### Result and Discussion

Optical densities obtained from dissolved Formazan crystals have been measured at 570nm by Microplate reader against the Background(Blank), then plotted on (x and y) axes for each Compound and analyzed by statistical Software programs to obtain (Mean±Standard Deviation) and MIC<sub>50</sub> value which is the minimum amount of tested compounds that inhibit 50% of all isolated Bacterial cells. MIC<sub>50</sub> values of all compounds against Gentamicin (Positive control) were presented in (Table 2) in Micro molarity.



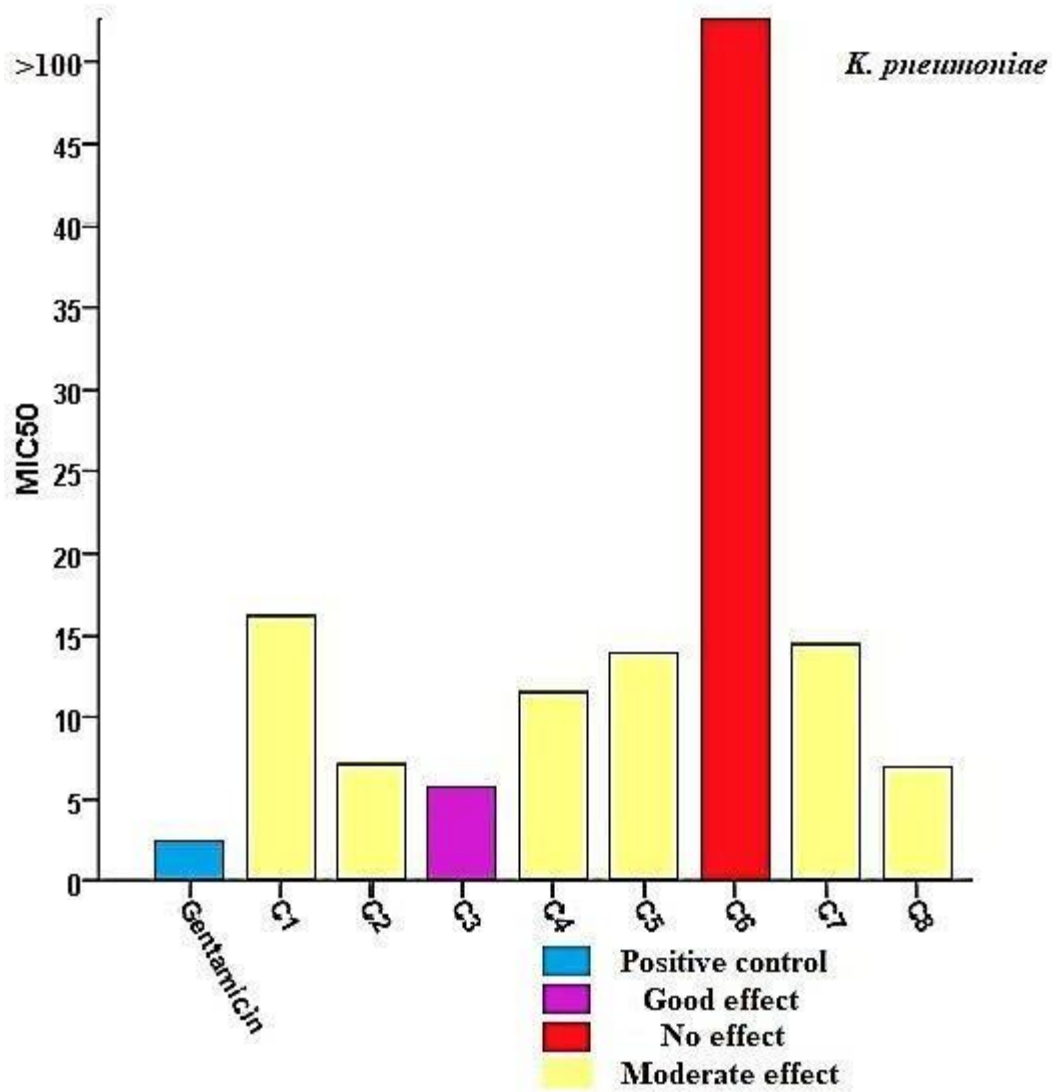
**Table 2:**BacterialMIC<sub>50</sub>values obtained from Formazan Optical density.

Chemicals	MIC <sub>50</sub> (μM)		
	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>S. pyogenes</i>
Genta	2.4	1.6	1.8
C1	16.2	11.2	1.9
C2	7.2	4.9	2.6
C3	5.7	3.9	3.3
C4	11.5	7.9	2.4
C5	13.9	9.6	3.9
C6	>100	>100	2.2
C7	14.5	10	3.5
C8	7	4.8	3.7

In order to compare our result with positive antibacterial control, we used Genta as Broad spectrum antibiotic. In our study the Minimum MIC<sub>50</sub> of Genta for all Bacteria was (1.6μM) for *P. mirabilis* followed by 1.8 and 2.4 μM for *S. pyogenes* and *K. pneumoniae* respectively as presented in (Table 2).

Out of Eight compounds which have been tested against *K. pneumoniae*, the Lowest MIC<sub>50</sub> value that has reported was for C3 which was (5.7 μM), on the other hand, the Highest MIC<sub>50</sub> was (>100) for C6. The Bar Graph of the compounds MIC<sub>50</sub> values which have been obtained by the MTT assay at the end of the 18 h incubation of *K. pneumoniae* culture with Genta and all compounds were presented in Figure 2

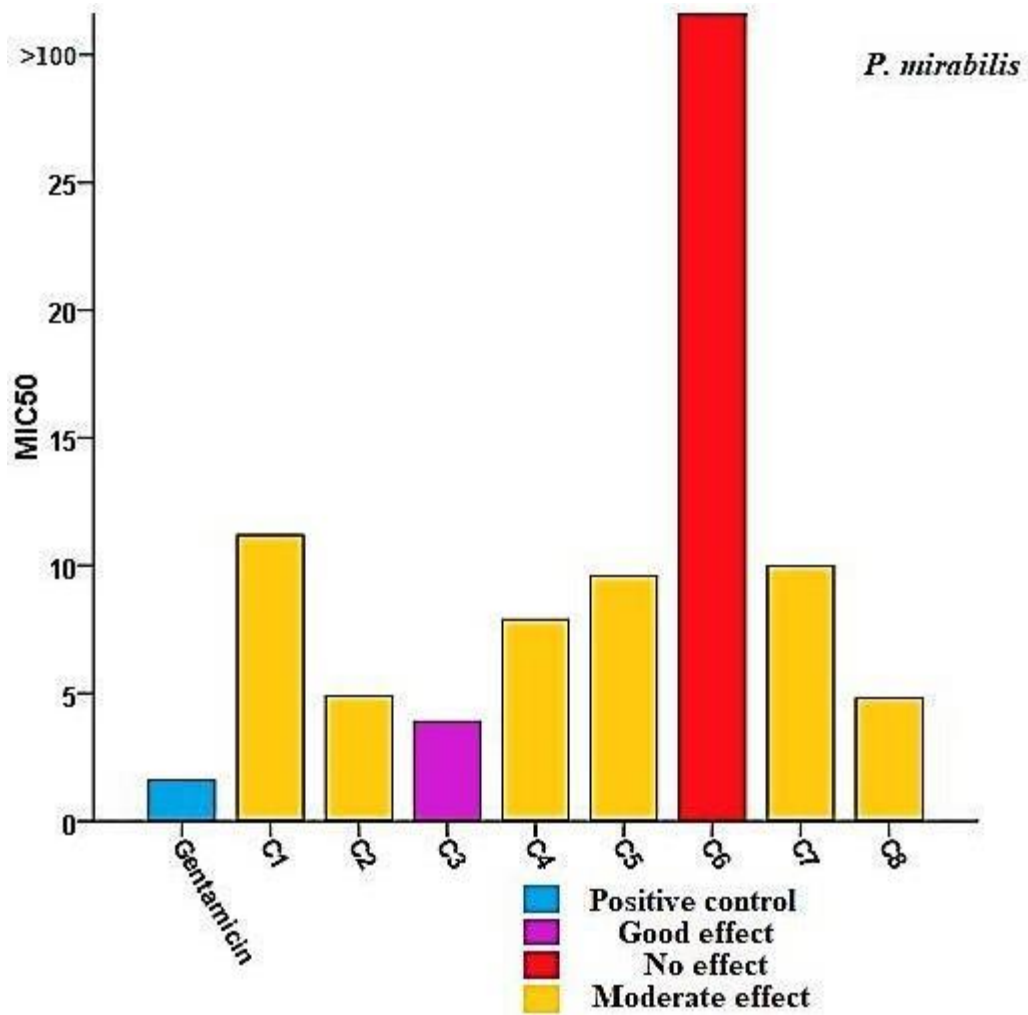




**Figure 2:** MIC<sub>50</sub> values of Genta and all salicylaldimine compounds on *K. pneumoniae*

Out of Eight compounds which have been tested against *P. mirabilis*, the Lowest MIC<sub>50</sub> value that has been reported was for C3 which was (3.9  $\mu$ M), on the other hand, the Highest MIC<sub>50</sub> was (>100) for C6. The Bar Graph of the compounds MIC<sub>50</sub> values which have been obtained by the MTT assay at the end of the 18 h incubation of *P. mirabilis* culture with all compounds and Genta were presented in Figure 3.

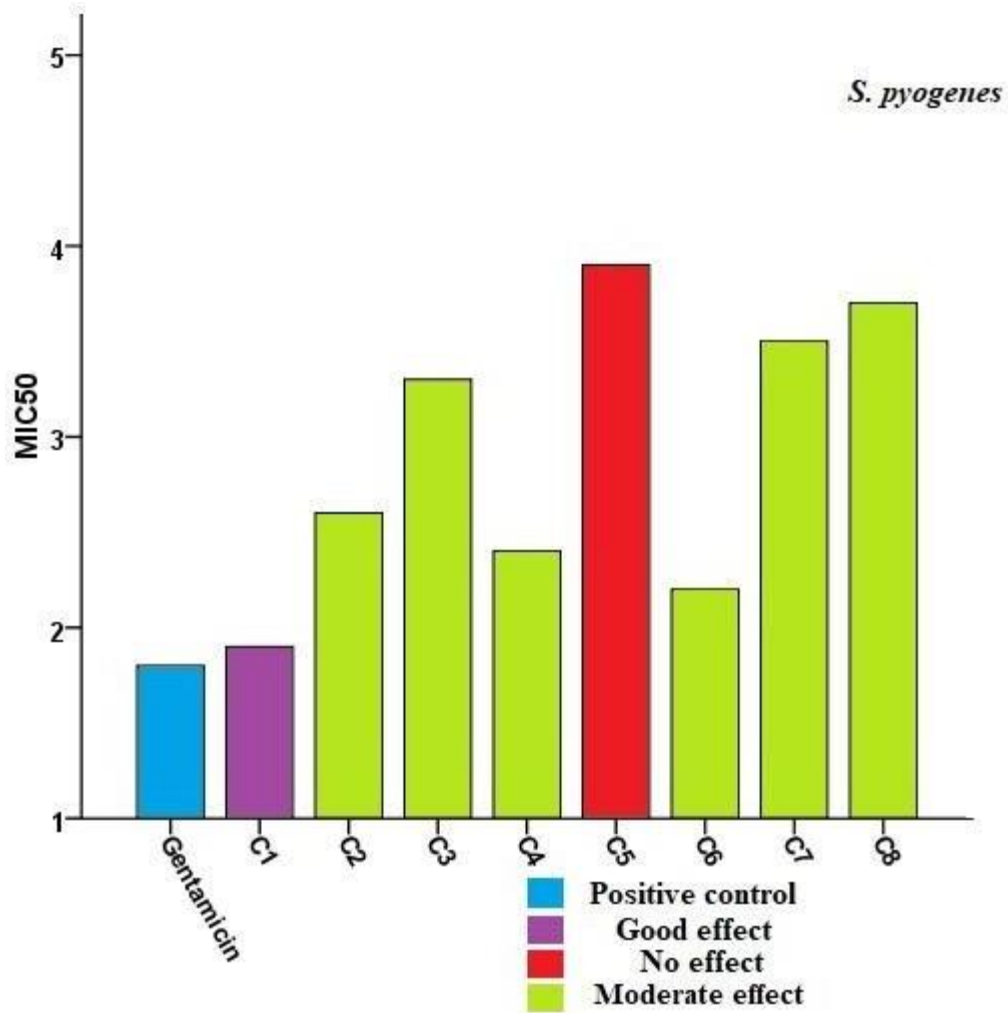




**Figure 3:** MIC<sub>50</sub> values of Genta and all salicylaldehyde compounds on *P. mirabilis*.

Out of Eight compounds which have been tested against *S. pyogenes*, the Lowest MIC<sub>50</sub> value that has been reported was for C1 which was (1.9  $\mu$ M), on the other hand, the Highest MIC<sub>50</sub> was (3.9  $\mu$ M) for C5. The Bar Graph of the compounds MIC<sub>50</sub> values which have been obtained by the MTT assay at the end of the 18 h incubation of *S. pyogenes* culture with Genta and all compounds were presented in Figure 4.





**Figure 4:** MIC<sub>50</sub> values of Genta and all salicylaldimine compounds on *S. pyogenes*.

Microbial resistance toward nowadays clinical antibacterial has increased researchers interest in order to find a new strategy or new Compound, especially from plant extract or synthetic compounds which have antibacterial activity. Despite of traditional usage of agar diffusion especially (Disk or well diffusion) and dilution techniques, these methods have a lot of limitations and we cannot use those methods for all colorful and nonpolar compounds as describes by the (Eloff J.N, 2019) study, so in order to get better and more accurate result we

used one of the tetrazolium salt (MTT) in our study.

Genta is a member of the aminoglycoside class of antibiotic which acts on bacteria through protein inhibition, and has a wide range of antibacterial effectivity, So regarding to this, most of the researchers and also in our study; we used Genta as positive control in order to compare their results with it such studies is done by (Thammawithanet al., 2021) and (Warad I et al., 2020).MIC<sub>50</sub> value of Genta as positive control was 1.6µM for *P. mirabilis* followed by 1.8 and 2.4 µM for *S. pyogenes* and *K. pneumonia* respectively;



the Statistical analyses showed that the Gentamicin has work significantly different on each bacterial species.

In our study, the dissolving agent which is DMSO has been added after MTT stain application in order to improve Optical density reading and dissolve Formazan crystal as a study conducted by (Wang et al., 2007), in contrast to previous studies on *P. mirabilis* which increased incubation time instead of adding dissolving agent such studies conducted by (Grela et al., 2015) and (Grela et al., 2016).

Colorimetric device has been used for measuring Optical density in our study as it is promotional to viable bacterial number as study conducted by (Shi et al., 2007), in contrast to studies which used MTT stain as visibly endpoint color assay in order to measure MIC. In our study instead of taking MIC as the concentration measurement tool which is the minimum concentration of chemicals that cause inhibition, the modified method of MIC<sub>50</sub> measurement has been taken which is considered the minimum amount of compound that cause 50% inhibition of bacterial number.

In a study conducted by (Wang et al, 2007), the number of bacteria was proportionally decreased by increasing Compound concentration as linear relationship, while in our study the microbial growth decreased in nonlinear relation with increasing Concentration in regarding with many differences such as applying (10 times) dilution of Chemical compounds in our study and (2 fold) dilution in previous study and many modification which we applied in our study.

Recently, Metal complexes of Schiff bases and Salicylaldehyde have been

increasingly under researcher's attention. Some studies showed that the metal complexes of previously tested Ligands would increase chemicals effect potential on living cells, In a study conducted by (Xiao et al., 2017), in which Cobalt complexes of two different Schiff base have been synthesized and checked against human skin cancer cells and they showed that the anticancer effect of Schiff bases have been improved after combined in Cobalt complexes, another study which Support this hypotheses was in (Emmanuel et al, 2018) study, in which Copper complex from (Sulphadiazine Schiff Base bind with aniline derived Schiff base) has been synthesized and checked by agar Disk diffusion method against (10) species of bacteria, and in the outcome the metal complex Schiff base has better antibacterial activity than previous ligands, on another hand there is some studies which showed that the metal complex has changed the effect of previous ligands differently on different bacterial species, one of such studies is conducted by (Saghatfroush et al. 2009). So in our study despite of having anticancer effect of some compounds, we predicted that some of the Copper complexes of Compound (1, 3, 5 and 7) would have better MIC<sub>50</sub> values on each individual Bacterial species, and in outcome of our study after applying eight salicylaldehyde derived compounds ( four complex) on both Gram negative (*K. pneumoniae* and *P. mirabilis*) and taking out their MIC<sub>50</sub> values, the result showed that the minimum MIC<sub>50</sub> value was for C3 which was 5.7 and 3.9 μM respectively, and the Highest MIC<sub>50</sub> was (>100) for C6 on both bacteria, while on Gram positive bacteria (*S. pyogenes*) the minimum MIC<sub>50</sub>



value was for C1 which was (1.9  $\mu\text{M}$ ), and the Highest  $\text{MIC}_{50}$  was (3.9  $\mu\text{M}$ ) for C5. In regarding with Metal complex activity on bacterial inhibition, our study showed that all four Complexes had effect similarly on both gram negative bacteria, in which Compound (2 and 8) had better activity in contrast to their ligand compounds (C1 and 7), while other Complexes (C4 and C6) had lower activity in compare with their ligands (C3 and C5). On the other side of our experiment, the Metal complex compounds had opposite effect on the gram positive bacteria (*S. pyogenes*) which may due to structurally different cell wall than the other gram negative bacteria.

### Conclusion and Outlook

Microbial resistance toward clinical antibacterial drugs has increased recently as public health enemy, therefore researchers have a lot of interest in order to find a new strategy or new Compound, especially from plant extracts or synthetic chemical compounds which have antibacterial activity, So in regarding to this manner, we tested and checked (4) Salicylaldimine-Derived Schiff base compounds with their Copper complexes against (3) Standard strains of Bacteria through newly modified MTT technique and newly measurement  $\text{MIC}_{50}$ , in order to evaluate their antibacterial strengths toward tested bacteria and Compare the ligand compounds with their copper complexes, and in outcome we found out that some of the complexes had better antimicrobial activity than previous ligands and the others vice versa, and all the compounds worked differently on both different Gram stained bacteria.

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### List of Abbreviation

AMR : Antimicrobial resistance  
ATCC : American Type Culture Collection  
B-lactam :Beta lactam  
CDC :Centers for Disease Control and Prevention  
C : Compound



CU :Copper  
DMSO : Dimethyl sulfoxide  
mM : MilliMolarity  
 $\mu$ l : Microliter  
M : Molarity  
 $\mu$ M : Micro molarity  
MIC : Minimum Inhibitory Concentration  
MTT : Methyl thiazolyl diphenyl-tetrazolium bromide  
OD : Optical density  
SD : Standard deviation  
UTI : Urinary Tract Infection  
WHO : World Wide Organization

