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Synthesis of a Novel 4,4'-[1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl)] bis(azaneylylidene) bis(methaneylylidene) diphenol and Determination of Its pharmacological and antimicrobial Activities

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Synthesis of a Novel 4,4'-[1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl)] bis(azaneylylidene) bis(methaneylylidene) diphenol and Determination of Its pharmacological and antimicrobial Activities

Ban M.S. Saeed¹, Shaker A.N. Al-jadaan¹, Basil A. Abbas^{*2}

¹College of Pharmacy, University of Basrah, Iraq

²College of Veterinary Medicine, University of Basrah, Iraq

* shakeraljadaan@yahoo.com

Abstract: The Cytotoxic activity, median lethal dose (LD₅₀) and the biological activity of new Schiff base compound was determined at 100, 150, 200 and 250 mg/ml against four types of bacteria i.e. *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Slmonella* sp. and its minimum inhibitory concentration (MIC) was 2,5,4,5 mg/ml for *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Slmonella* sp. respectively. Median lethal dose (LD₅₀) and cytotoxic activity were also investigated. The results showed that this compound did not affect the red blood until the concentration reach 100mg/ml or above. The LD₅₀ for the compound is 0.28278 gm/kg bw.

1 Introduction

Schiff base are the compound containing azomethine group (-HC=N-). They are condensation products of ketones (or) aldehydes (aldehyde and ketones) with primary amines and were first reported by Hugo Schiff in 1864 [2]. Schiff base formation usually occurs under acids or base catalysis or heat [3]. Schiff bases are used as substrates for industrial and biologically active compounds through ring closure, cycloaddition and replacement reactions [1]. The toxicity of a substance can be determined before use in humans by using in vitro tests and animal studies [4].

This compound which was synthesized in present study was a novel compound and there were no data about its biological activity, its cytotoxicity and its LD₅₀. The aim of our study is to synthesized a novel compound (Schiff base) and determined its activities such as antibacterial, MIC, cytotoxicity assay and its LD₅₀.

2 Materials and Methods

2.1. Synthesis of Schiff base compound: Synthesis of 4,4'-[1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl)] bis(azaneylylidene) bis(methaneylylidene) diphenol

This new compound was prepared according to [5] using microwave method. (1.954gm, 0.008mol.) of 4-hydroxybenzaldehyde and (1.105gm, 0.004mol.) of compound A which was prepared previously (Synthesis of 5,5'[(1,4-Phenylene) bis(1,3,4-thiadiazol-2-amine)]) [26] were mixed in a conical flask and 1ml of distilled water was added. The contents were subjected to microwave irradiation at 180 watts for 2-3 minutes, the reaction was monitored by TLC, cooled at room temperature, recrystallization from ethanol gave orange-yellow crystals with m.p. 110 °C., wt. 1.54gm, yield (80%), R_f value= 0.8 (3:7 hexane/ethyl acetate).



2.2. Physical measurements

2.2.1 Melting point: The product compound's melting point was expressed in degree (0°C). It was measured in the Department of Chemistry, University of Basrah College of Science. Use of digital SMP31 melting point device and was uncorrected.

2.2.2 FT-IR Spectra: FT-IR spectrum was recorded, using Shimadzu FT-IR-8400 affinity spectra photo meter made in Japan, in Department of Chemistry, College of Education for pure Science, University of Basrah using KBr disc, and expressed in cm^{-1} .

2.2.3 Thin Layer Chromatography: Thin layer chromatography of the starting materials and products was carried out using Eastman chromatography sheet (GERMANY) with the appropriate eluent ratio (methanol: ethyl acetate) (3:7); the spot was visualized by exposing the dry plate in UV light.

2.2.4 Elemental Analysis: Elemental micro analysis of Carbon, Hydrogen and Nitrogen were carried out in Al al-Bayt University, Al-Mafraq, Jordan using a Euro vector EA 3000A Elemental analysis (Italy).

2.2.5 Nuclear Magnetic Resonance Measurements: ^1H -NMR and ^{13}C -NMR spectra of prepared compounds were recorded using BRUCKER Ultra shield spectrophotometer (300 MHz), University of Al al-Bayt, Jordan. The Chemical shift was expressed in σ (ppm) unit. Using tetramethylsilane (TMS) as internal standard and DMSO- d_6 as a solvent.

2.3 Biological activity of chemical compounds:

2.3.1 Bacterial isolates: In this test, four bacterial isolates have been used. These are *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. *Bacillus cereus* was isolated during this study. The other bacterial strains were collected from the Central Lab. of College of Veterinary Medicine University of Basrah. Bacteria were cultivated on MYP agar, manitol salt agar, EMB agar and XLD agar. Growth was transferred to a sterile tube with a normal saline of 5ml. The turbidity of the actively growing broth culture was adjusted with 0.5 McFarland standard [7].

2.3.2 Antimicrobial activity test of the prepared compounds: Different concentrations i.e. (100, 150, 200, 250) mg/ml from the synthesis compounds were prepared and were used in this study [6]. The antimicrobial susceptibility was tested by agar well diffusion method according to [8].

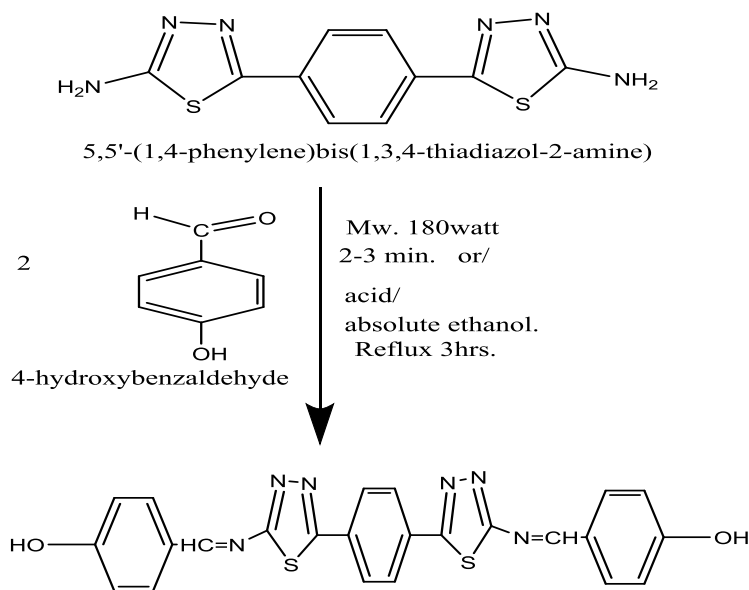
2.3.3 Minimum inhibitory concentration (MIC) for the compound: Using concentrations (5, 10, 15, 20, 25, 30, 45, 60, 75, 90) mg/ml, the four bacterial strains were subjected to the susceptibility tests to prepared compound. Well diffusion method was used to determine the MIC for prepared compounds. The prepared compounds were dissolved in DMSO into different concentrations [9]. Bacterial suspensions were adjusted to MacFarland standard $0.5(1.5 \times 10^8)$ CFU.

2.3.4 Blood cell cytotoxicity assay: The method of studying the cell toxicity of the prepared compounds [10]. A physiological saline (1.0 ml blood suspension in 20 ml saline) has been prepared. Different levels of prepared compounds have been used in DMSO. Two ml of erythrocyte suspension prepared in the first step were added to the sterile tubes, adding 0.1 ml of each concentration. Two ml of tap water with 0.1 ml of erythrocyte were used as positive control, and two of normal saline with 0.1 ml of erythrocyte were used as negative control. The turbidity was read at 10, 30 and 60 min. at 37°C . The concentrations that provided a clear solution due to RBC lysing are an indication of the degree of toxicity to the erythrocytes of the test compounds. [10].

2.3.5 Median Lethal Dose (LD_{50}) assay: To determine the LD_{50} for the compound, a total number of seven male and female rats (*Rattus norvegicus*) were used. In the animal house of the College of Veterinary Medicine / University of Basrah, the animals aged 8-10 weeks and their body weight between 190-200 gm, each animal was isolated in one cage in a good air condition room. The animals fed on dried bread, pellet and given R.O. water. The LD_{50} steps used in this experiment was "up and down" method described by [11].

3 Results

3.1 Synthesis of 4,4'-[1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl)]bis(azaneylylidene) bis(methaneylylidene) diphenol. Compound B: In this study, new Schiff base compound was synthesized. The reaction scheme for the synthesis of this compound is shown in Figure1.



4,4'-(((1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl))bis(azaneylylidene))bis(methaneylylidene))diphenol
Figure 1: Synthesis pathway for preparing new Schiff base compound

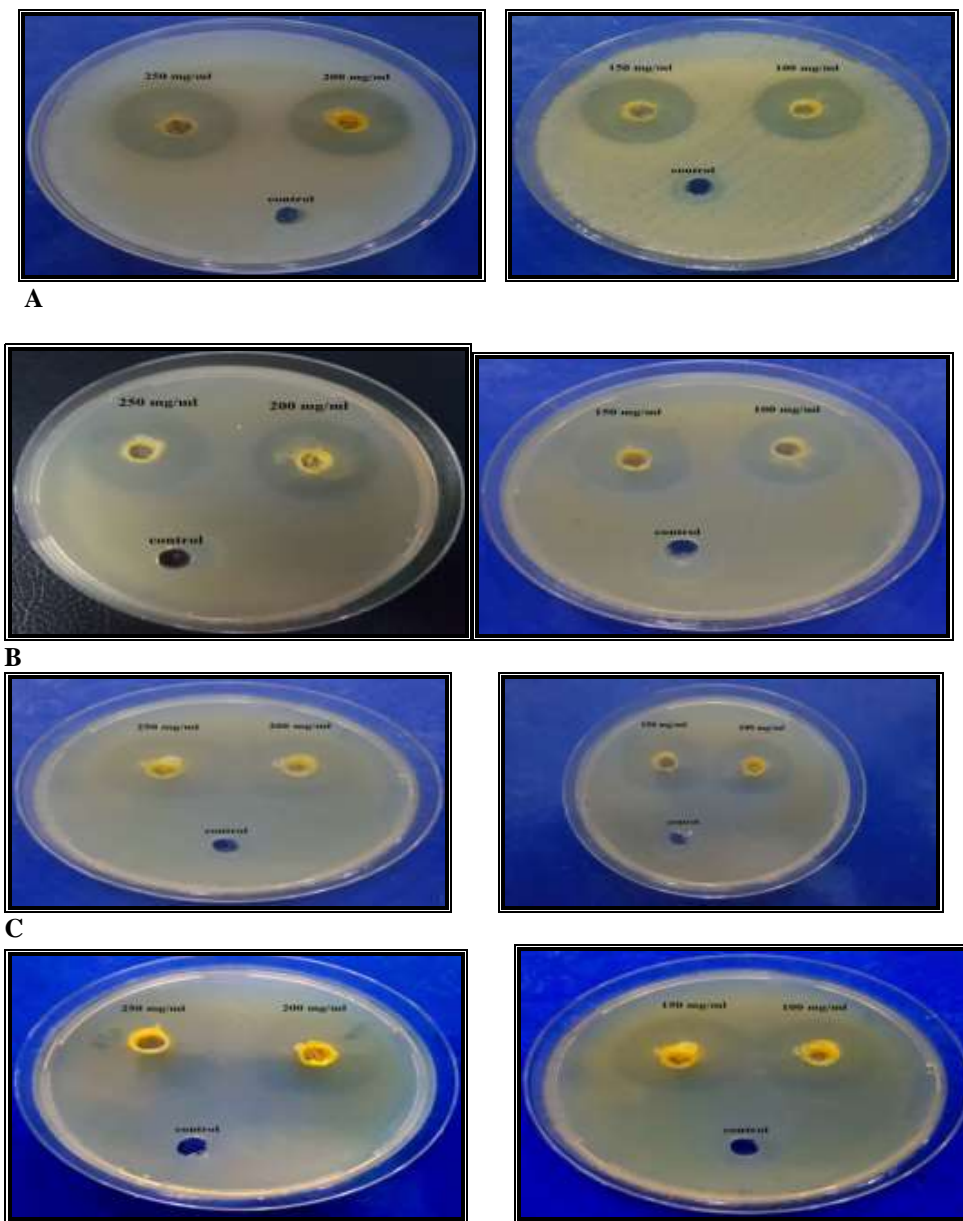
Elemental analysis; Found (calculated)=**C**: 59.61(59.49), **H**: 3.45(3.33), **N**: 17.47(17.35), **S**: 13.33(13.24). The FT.IR spectrum for compound (**B**) using KBr disk: $\nu_{\text{OH}_{\text{assym}}}$ 3209 cm^{-1} , $\nu_{\text{OH}_{\text{sym}}}$ 3172 cm^{-1} , $\nu_{\text{C-H}_{\text{arom}}}$ 3090 cm^{-1} , $\nu_{\text{C=N}}$ 1666 cm^{-1} , $\nu_{\text{OH}_{\text{bending}}}$ 1597 cm^{-1} , $\nu_{\text{C=C}_{\text{asym}}}$ 1517 cm^{-1} , $\nu_{\text{C=C}_{\text{sym}}}$ 1425 cm^{-1} , $\nu_{\text{C-N}}$ 1315 cm^{-1} , $\nu_{\text{N-N}}$ 1161 cm^{-1} , $\nu_{\text{C-S}}$ 705 cm^{-1} . See figure 2. The $^1\text{H-NMR}$ spectrum for new (**B**) compound was recorded using DMSO- d_6 as a solvent (300Mz). In general, $^1\text{H-NMR}$ spectrum shows band at 6.94ppm(d) attributed to 4H (C31, C29 and C24, C26), ($J=8.49\text{Hz}$), band at 7.77ppm(d) may assigned to 4H (C28, C23 and C27, C32), ($J=8.56\text{Hz}$), band at 8.03ppm(s) may assigned to 4H (C3, C6 and C2, C5), the band at 9.79ppm(s) may attributed to 2H (C19, C20) and broad band 10.4ppm(s) may assigned to 2OH (C33, C34). See figures 3 and 4.

The $^{13}\text{C-NMR}$ spectrum for new (**B**) compound was recorded using DMSO - d_6 115.80ppm (C29, C31, C26, C24), 124.06ppm (C2, C3, C5, C6), 128.39(C22, C21), 129.31ppm (C32, C27, C28, C23), 131.71ppm (C4, C1), 155.39ppm (C25, C30), 163.29ppm (C12, C7), 169.59ppm (C19, C20), 173.46ppm (C10, C14). See figure 5.

3.2 Biological activity of chemical compounds: The antimicrobial activity was determined against four types of bacteria in the concentrations 100, 150, 200 and 250 mg/ml Table (1), Figure (1).

Table (1) Antimicrobial activity of Schiff base compound

Chemical compounds	Types of bacteria	Inhibition zone (mm)			
		100 mg/ml	150 mg/ml	200 mg/ml	250 mg/ml
	<i>Bacillus cereus</i>	23	25	27	29
Schiff-base compound	<i>Staphylococcus aureus</i>	22	24	28	29
	<i>Escherichia coli</i>	23	25	25	26
	<i>Salmonella sp.</i>	23	24	27	29



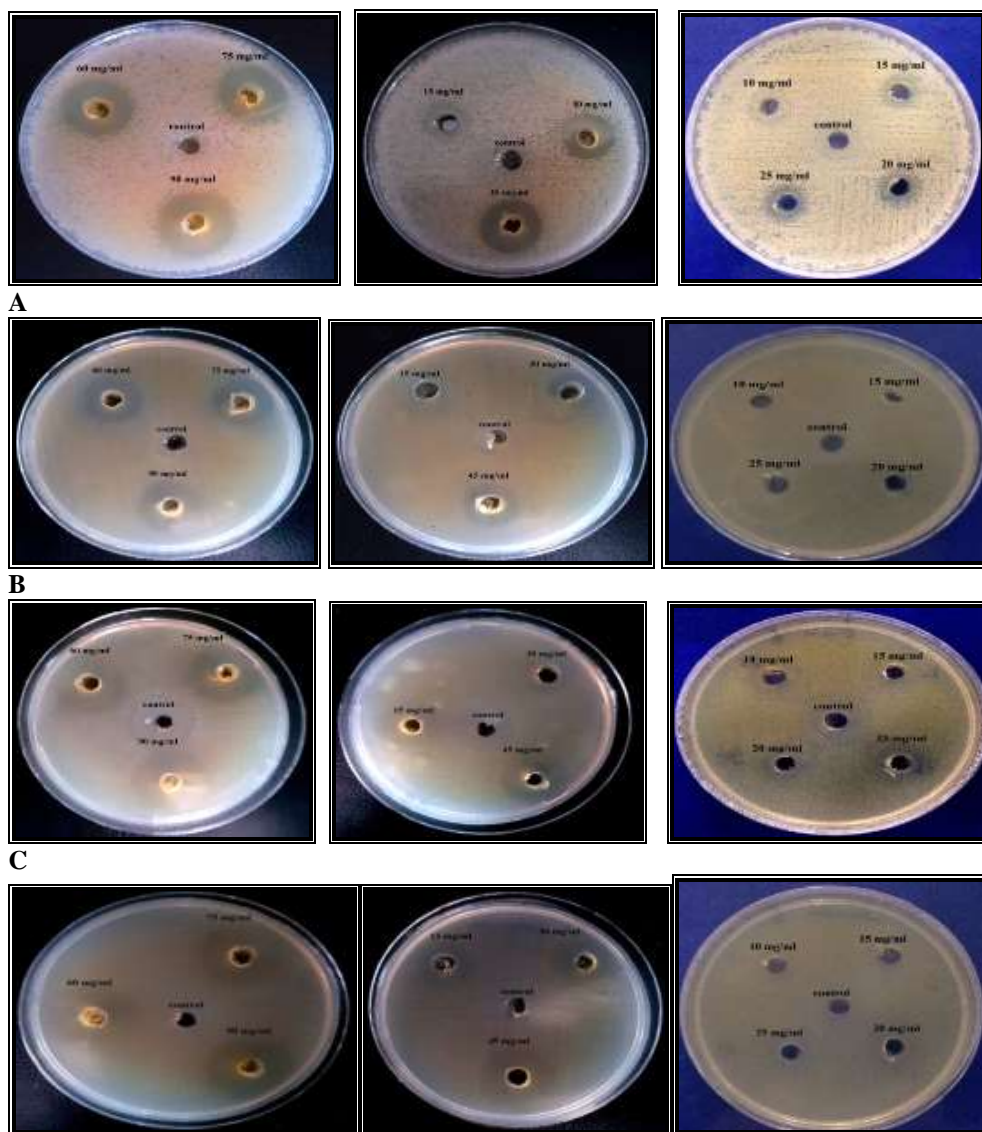
D
Figure 2: Biological activity of Schiff-base compound against different types of bacteria. A= *B. cereus* , B= *S. aureus* ,C= *E. coli* , D= *Salmonella sp.*

Minimum inhibitory concentration (MIC) of the compound: The MIC of this compound was 15 mg/ml for both *B. cereus* and *E. coli*, but it was 30 mg/ml for *Staph. aureus* and *Salmonella sp.* Table (2), Figure (6).

Table 2: The MIC for the compound against different types of bacteria

Conc. mg/ml	Inhibition zone (mm)			
	<i>B. cereus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>Salmonella sp.</i>
90	17	17	22	12
75	16	15	21	12
60	15	14	20	11
45	15	9	15	11
30	14	8	8	10
25	12	0	7	0
20	10	0	7	0

15	7	0	6	0
10	0	0	0	0
5	0	0	0	0



D Figure 3: The MIC of prepared compound against different types of bacteria. A= *B. cereus*, B= *S. aureus*, C= *E. coli*, D= *Salmonella* sp.

3.3 Biological activity of bacterial strain against standard antimicrobial disks

According to antimicrobial activity of chemical compounds and their comparison with standard antimicrobial disks, we used different types of antimicrobial disks, (Table 3; Figure 7). All *Bacillus cereus* isolates are susceptible to ciprofloxacin, gentamycin and chloramphenicol. Some of the isolates were susceptible and some resistance to tetracycline. All bacillus cereus isolates were resistance to pencillin, oxacillin, streptomycin, erythromycin, lincomycin, amoxillin, kanamycin and clindamycin.

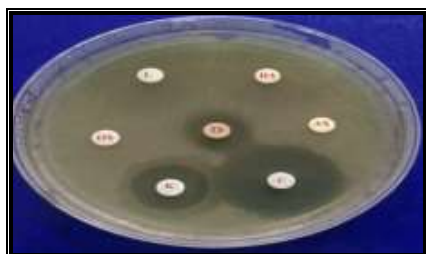
All *Staphylococcus aureus* isolates are susceptible to ciprofloxacin, gentamycin and chloramphenicol. All *Staphylococcus aureus* isolates were resistance to pencillin, oxacillin, streptomycin, erythromycin, tetracycline, lincomycin, amoxillin, kanamycin and clindamycin. All *E. coli* isolates are susceptible to ciprofloxacin, gentamycin and chloramphenicol. All *E. coli* isolates were resistance to pencillin, oxacillin, streptomycin, erythromycin, tetracycline, lincomycin, amoxillin, kanamycin and clindamycin. All *Salmonella* spp. isolates are

susceptible to ciprofloxacin, gentamycin and kanamycin. All *Salmonella* spp. isolates were resistance to penicillin, oxacillin, streptomycin, erythromycin, tetracycline, lincomycin, amoxillin and clindamycin.

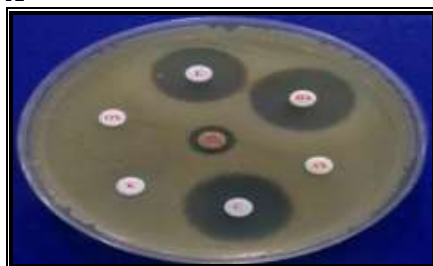
Table 3: Antimicrobial activity of selected antibiotics against different bacterial isolates.

Antimicrobial	Conc.	Bacterial isolates			
		<i>S. aureus</i> R/S	<i>E. coli</i> R/S	<i>Salmonella</i> sp. R/S	<i>B. cereus</i> R/S
Penicillin	P 10 U	R	R	R	R
Oxacillin	OX 1 mcg	R	R	R	R
Streptomycin	S 10 mcg	R	R	R	R
Erythromycin	E 15 mcg	R	R	R	R
Ciprofloxacin	Cip 10 mcg	S	S	S	S
Gentamycin	CN 10 mcg	S	S	S	S
Tetracycline	TE 30 mcg	R	R	R	S,R
Lincomycin	L 10 mcg	R	R	R	R
Amoxillin	AX 25 mcg	R	R	R	R
Chloramphenicol	C 30 mcg	S	S	R	S
Clindamycin	DA 2 mcg	R	R	R	R
Kanamycin	K 30 mcg	R	R	S	R

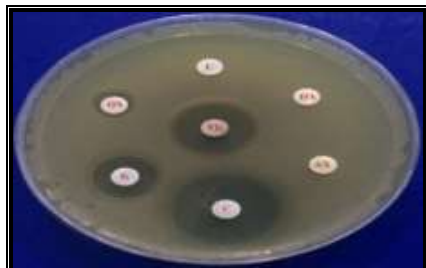
S: susceptible , R: resistance



A



B



C

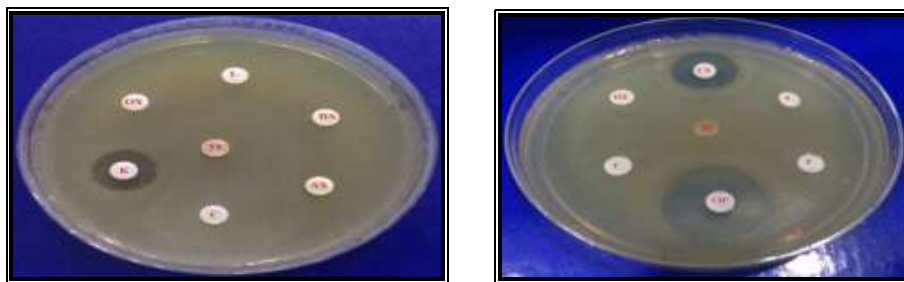
**D**

Figure 4: Antimicrobial activity of selected antibiotics against different bacterial isolates. A= *B. cereus*, B= *S. aureus*, C= *E. coli*, D= *Salmonella* sp.

3.4 Toxicity assay of the compound: The produced compound did not affect the red blood cells at the concentration 5, 10, 15, 20, 25, 30 and 45 mg/ml. The concentration 60 mg/ml had low hemolysis and the concentrations 75 and 90 mg/ml had moderate hemolysis but the concentration 100, 150, 200 and 250 mg/ml caused high hemolysis in red blood cells. Table (4), Figures (8, 9).

Table (4) Toxicity of prepared compound.

Conc.	Toxicity
5, 10, 15, 20, 25, 30, 45	-
60	+
75, 90	++
100, 150, 200, 250	+++

- = No hemolysis, += few hemolysis, ++= moderate hemolysis, +++ high hemolysis

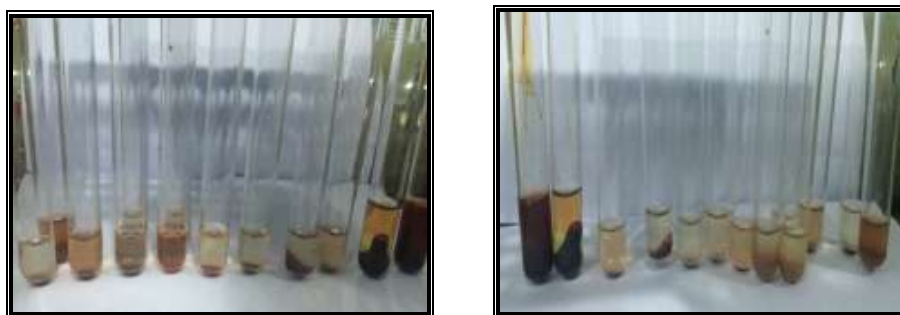


Figure 5: Toxicity of the prepared compound.

3.5 Median Lethal Dose (LD₅₀) assay: The code that found was (OXXO) for prepared compound, and the LD₅₀ was determined according to the formula described by [11]. The code that found was (XOOX) for compound B, and the LD₅₀ was 0.28278 gm/kg bw

4 Discussion

The novel compound was prepared by using microwave method, this new compound was prepared by using one mole of compound A which was prepared previously (Synthesis of 5,5'[(1,4-Phenylene) bis(1,3,4-thiadiazol-2-amine)]) and tow moles of 4-hydroxybenzaldehyde in the presence of one ml. of distilled water, the reaction was monitoring by TLC which after recrystallization by ethanol gave R_f value equal to 0.8 using (3:7, Hexane/Ethyl acetate), in very good yield percent. The new compound was orange-yellow crystals melt at 110 °C, stable in air, solid, insoluble in water, but dissolved in common organic solvents. This reaction was also carried out in absolute ethanol and refluxed for 3hrs, gave 0.904gm, 47% yield, so using microwave method gave good method with high yield% and very short time (2-3 minutes) and no organic solvents were consumed. The new compound was elucidated by Elemental, FT-IR, ¹H-NMR and ¹³C-NMR spectroscopic analysis. The elemental analysis of compound B are within ±0.5% of the theoretical values. The IR spectrum of compound B (KBr disc) showed characteristic bands at $\nu_{3209} \text{ cm}^{-1}$ and $\nu_{3172} \text{ cm}^{-1}$ due to O-H asymmetrical and symmetrical stretching, respectively. Weak band at $\nu_{\text{C-H}} 3090 \text{ cm}^{-1}$ attributed to stretching of aromatic hydrogen atoms, very strong band at $\nu_{\text{C=N}} 1666 \text{ cm}^{-1}$ may have assigned to C=N stretching, while very strong band at $\nu_{\text{OH}} 1597 \text{ cm}^{-1}$ due to

O-H bending, two strong bands at ν 1517 cm^{-1} and ν 1425 cm^{-1} may be attributed to stretching of asymmetrical and symmetrical of C=C bond, respectively, ν strong band at 1315 cm^{-1} due to stretching of C-N bond, while very strong band appears at ν 1161 cm^{-1} may be attributed to stretching of N-N bonds, medium band appears at ν 705 cm^{-1} attributed to cyclic C-S-C bonds. The $^1\text{H-NMR}$ spectra of compound B were recorded on Bruker Ultra shield spectrophotometer (300 MHz) using (DMSO- d_6 as a solvent, δ ppm), In general, $^1\text{H-NMR}$ spectra shows band at 6.94ppm(d) attributed to 4H (C31, C29 and C24, C26), ($J=8.49\text{Hz}$), band at 7.77ppm(d) may assigned to 4H (C28, C23 and C27, C32), ($J=8.56\text{Hz}$), band at 8.03ppm(s) may assigned to 4H (C3, C6 and C2, C5), the band at 9.79ppm(s) may attributed to 2H (C19, C20) and broad band 10.4ppm(s) may assigned to 2OH (C33, C34). The $^{13}\text{C-NMR}$ spectrum for new (B) compound was recorded using DMSO - d_6 as a solvent. Band at 115.80ppm may attributed to the aromatic (C29, C31, C26, C24), band at 124.06ppm may assigned to aromatic (C2, C3, C5, C6), while band at 128.39ppm may attributed to aromatic (C22, C21), band at 129.31ppm due to aromatic (C32, C27, C28, C23), band at 131.71ppm may assigned to aromatic (C4, C1), another band at 155.39ppm due to aromatic carbon atoms attached directly to hydroxyl groups (C25, C30), band at 163.29ppm belong to aromatic carbon atoms within heterocyclic rings (C12, C7), while band at 169.59ppm may assigned to (C19, C20) and the last band at 173.46ppm belong to another aromatic carbon atoms within heterocyclic rings occurs between two electronegative nitrogen atoms (C10, C14).

Specific toxic action on pathogenic organisms by antimicrobial agents [12]. The compound had biological activity against four bacterial strains, two of which were gram-positive bacteria, i.e. *Bacillus cereus*, *Staphylococcus aureus* and the other two are gram-negative bacteria that is to say. *Escherichia coli* and the spp of *Salmonella*. Results have been found leading to increased concentration in the compounds' inhibition zone. This results because of the compounds' chemical structure.

At the lowest concentrations. The best biological activity of the compound (100 mg / ml) was against *B. cereus*, you know, *E. coli* and the spp of *Salmonella*. But at conc, it was more affected. 250 mg / ml with *B. cereus* and *Staph. aureus*. Sulfur compounds have a wide range of bio-active properties and characteristics due to their sulfur atom content and significant pharmacological activity (alfatlawi, 2016). The reason for this is the difference in the nature of the general structure of the cell wall between the two types of the bacteria (gram positive and gram negative) that used in this study.

The Gram-positive cell envelope differs first and foremost from its Gram-negative counterpart, the outer membrane is absent. By excluding toxic molecules and providing an additional stabilizing layer around the cell, the outer membrane plays a major role in protecting Gram-negative organisms from the environment. Since the outer membrane helps to stabilize the inner membrane indirectly, the peptidoglycan mesh around Gram-negative cells is relatively thin [13].

These group of compounds derived from thiosemicarbazide and their metal combinations are of great importance for their pharmacological properties such as antibacterial, antimicrobial, antitumoral, antiviral and anticancer [14, 15, 16, 17, 18, 19].

Any drug that has a toxic effect on cells; commonly used in chemotherapy to inhibit the proliferation of cancerous cells [20]. The compound was used in a different concentrations ranging from (5 - 250) mg/ml and caused red blood cell hemolysis at 60 mg/ml. The mechanical stability of the membrane of red blood cells (RBCs) is a good indicator to evaluate *in vitro* the effects of various compounds when screening for cytotoxicity [22-21]. Treating cells with a cytotoxic compound can cause different problems to human beings because the cells may undergo a loss of membrane integrity and die rapidly as a result of cell lysis [23].

In the present study, the standard antimicrobial disks were used. The *B. cereus*, *Staph aureus* and *E. coli* isolates are sensitive to ciprofloxacin, gentamycin, chloramphenicol and some *B. cereus* isolates were sensitive to tetracycline. The *Salmonella* spp. isolates are sensitive to ciprofloxacin, gentamycin and kanamycin. The increased incidence of drug resistant strains observed in our study may be because of strain variation. Also before attending the hospital, most of the patients get different antibiotics from general practitioners or due to over-the-counter sell of antibiotics often in improper dose [24]. In many countries, excessive use of antibiotics are due to the easy availability of antimicrobial drugs that can be purchased without prescription of a physician or other qualified health professional [25].

The compounds is a novel compounds and no data were available regarding its toxicity; therefore, the experiment focused to determine its acute toxicity by measuring its LD_{50} in adult's male and rats. There are a number of methods for LD_{50} determinations. Acute toxicity study was carried out by measuring the median lethal dose (LD_{50}) by using [11]. It was soluble in DMSO and used intraperitoneal injection (IP) as suspension to abdominal cavity of the rats for the determination of the LD_{50} . For compound B, the mortality recorded when rats are exposed to 0.28278 gm/kg body weight. The mortality was increased when the doses of the compounds were increased so when the compound is injected IP in the rat with the lethal or nearest to the lethal dose, the rat lost his feeling with its extremities will paralysed, start crawling, fatigue and then lice the site of injection.

5 Conclusion

Schiff bases compound was a novel compound which was synthesized and characterized by analytical and spectral techniques. It had significant activity against *B. cereus*, *Staph. aureus*, *E. coli* and *Salmonella* spp.

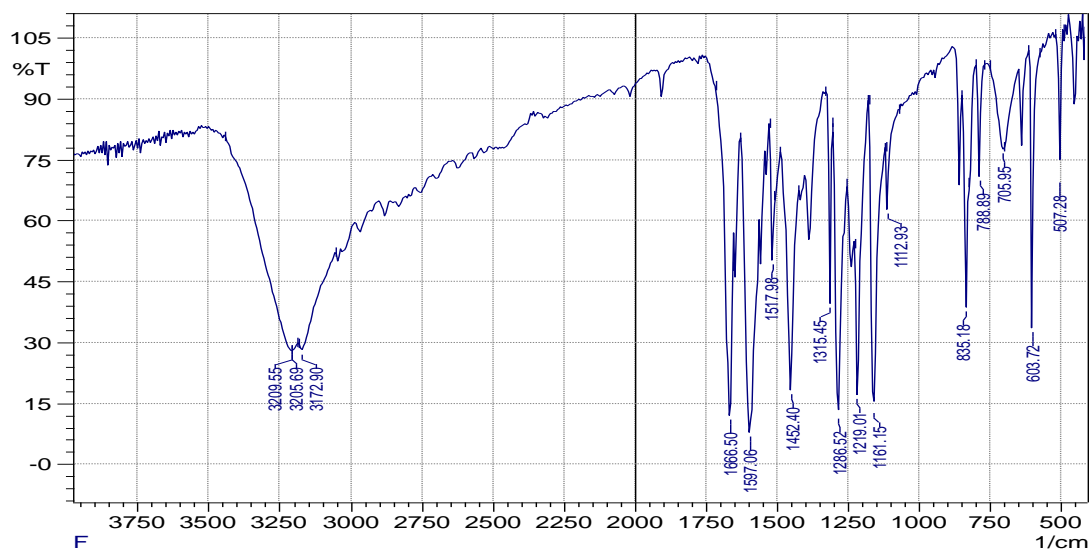


Figure 6: The IR spectrum of compound B

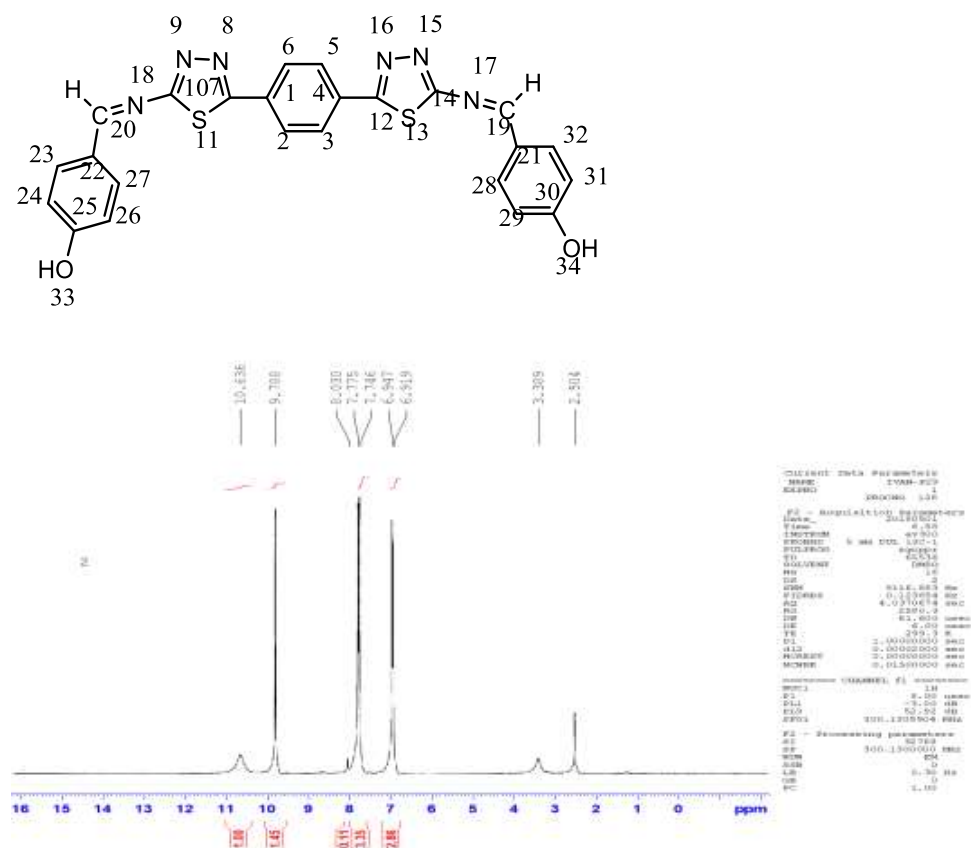


Figure 7: 1H-NMR spectrum for compound B (DMSO-d₆).

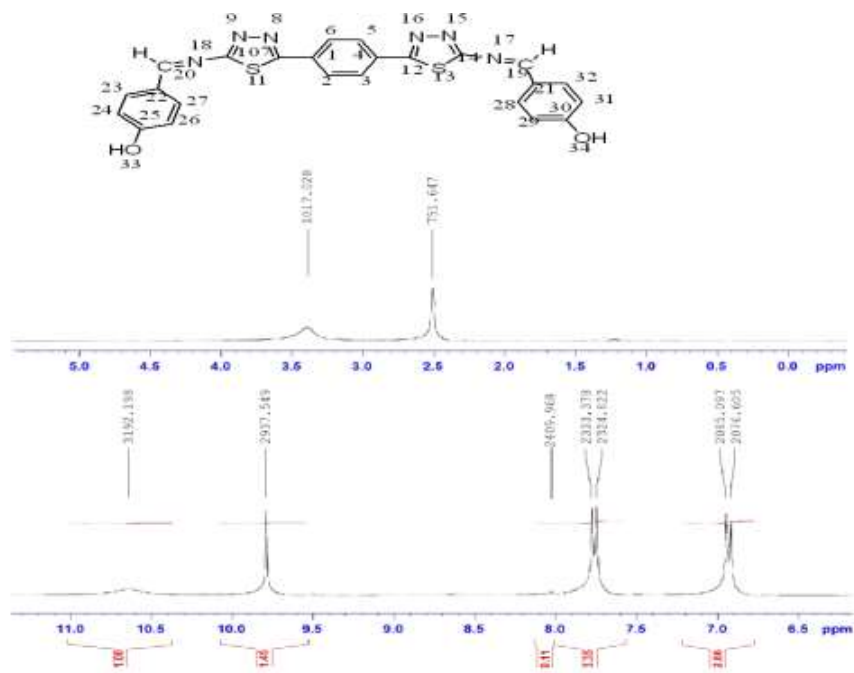


Figure 8: Expanded ¹H-NMR spectrum for compound B (DMSO-d₆)

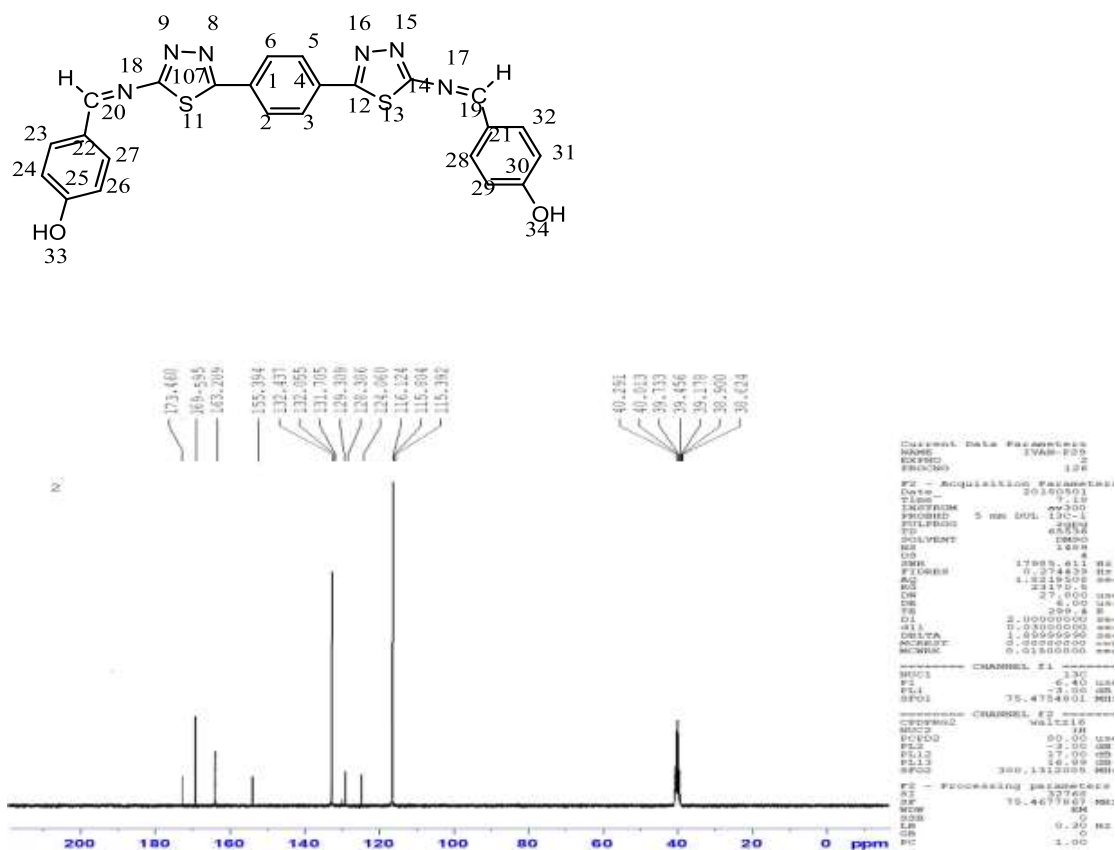


Figure 9: ¹³C-NMR for compound B (DMSO-d₆)

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