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Synthesis and Characterization of New Β-Lactam Derived from Sulfapyridine and Study the Effect of The Synthesized Compounds on Isolated Bacteria in Basrah Government

Article Info.

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Abstract

β-lactams were prepared in two steps, including the condensation of 4-amino-N-pyridin-2-ylbenzenesulfonamide (sulfa pyridine) with 2-Hydroxy naphthaldehyde to produce a Schiff base (MW1). The synthesized compound was reacted with chloroacetyl chloride in the presence of triethylamine to produce a new beta-lactam compound (MW2) with good yield. The synthesized compounds were characterized by infrared (IR), proton, and carbon-13 nuclear magnetic resonance spectroscopy. The synthesized compounds were evaluated as antibacterial agents against pathogenic bacteria isolated and identified by conventional and biochemical tests for both Gram-positive and Gram-negative bacteria, such as Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae, and Escherichia coli. β-lactams were prepared in two steps, including the condensation of 4-amino-N-pyridin-2-ylbenzenesulfonamide (sulfa pyridine) with 2-Hydroxy naphthaldehyde to produce a Schiff base (MW1). The synthesized compound was reacted with chloroacetyl chloride in the presence of triethylamine to produce a new beta-lactam compound (MW2) with good yield. The synthesized compounds were characterized by infrared (IR), proton, and carbon-13 nuclear magnetic resonance spectroscopy. The synthesized compounds were evaluated as antibacterial agents against pathogenic bacteria isolated and identified by conventional and biochemical tests for both Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae,* and *Escherichia coli*. The results of the study proved that the synthesized compounds in this study have clear efficacy against the pathogenic organisms used compared to the standard antibiotics used (ceftriaxone, cefepime, and penicillin G). In other words, the prepared beta-lactam compound has a wide range of antibacterial activity.

Keywords: β-Lactam, Sulfa pyridine, 2-Hydroxy naphthalaldehyde, Schiff-base, Antibacterial activity

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Introduction

The overuse of antibiotics has led to the development of drug-resistant bacterial strains in many ways. This issue is becoming concerning as nearly all pathogenic bacteria have developed resistance to currently recognized and commonly utilized antibiotics (1-3).

Drug discovery has undergone a radical change in the pharmaceutical industry over the past decade due to advances in molecular biology. Designing drugs with optimal efficacy, pharmacokinetic properties, and reduced toxicity is challenging due to the conflicting requirements of absorption and metabolism (4).

Sulfonamides (SN), or sulfanilamides, constitute a significant family of synthetic antimicrobial medicines employed pharmacologically as broad-spectrum treatments for bacterial infections in humans and animals (5). Sulfonamides were the inaugural synthetic antibiotics utilized in clinical settings, demonstrating notable pharmacological characteristics, including selectivity for bacterial cells and little toxicity (6). Sulfapyridine was the preferred agent for pneumonia therapy for a period (7). Finding the optimal mix of qualities like activity, toxicity, and exposure is one of the most important parts of making and developing drugs. It is crucial to ascertain and subsequently enhance the exposure, activity, and toxicity connections of pharmaceuticals, hence determining their appropriateness for further development (8). Beta-lactam antibiotics are antibiotics that contain a beta-lactam ring in their molecular structure. This includes penicillin derivatives (Penams), cephalosporin (Cephems), monobactams, carbapenems, and carbacephems (9). Sulfa pyridine inhibits dihydropteroate synthase (DHPS), the enzyme that catalyses the displacement of pyrophosphate from a pteridine substrate, leading to a covalent connection with the amino group of PABA. The rivalry between sulfa pyridine and PABA for the enzyme leads to the binding of sulfa pyridine, since it has a higher affinity for the enzyme. This impedes enzyme activity and, as a result, the production of folic acid (10). Mammals obtain folic acid from food and are unable to synthesize it. Because of this, sulfonamides are selective agents against bacteria and well-tolerated by humans. Unfortunately, resistance to sulfonamides, mainly due to the presence of drug-resistant variants of the DHPS enzymes, limits their clinical use (11).

β–lactams are synthetic antibiotics active against Gram-positive and Gram-negative bacteria by inhibiting the synthesis of the peptidoglycan layer from the cell wall (12). The highly stained β – lactam nucleus is stabilised by means of the fusion of a variety of either 5-membered or 6-membered heterocyclic moieties to give rise to a wide spectrum of newer antibiotics (13).

β – lactam compound was prepared from 4,4-diamino diphenylsulphon (DDS) with 3-methoxy-4-hydroxybenzaldehyde (Vanillin) (14). A series of new β-lactams was synthesized by Staudinger ketene-imine cycloaddition reaction after multi-step synthesis (15). Schiff base, β- lactam and Zn (II), Cu (II) complexes derived from sulfamerazine were prepared and their antimicrobial activity (16).

Materials and Methods

a) **Synthesis:**

Synthesis of Schiff base:

Dissolve (2.4 g, 10 mmol) of 4-amino-N-pyridin-2-ylbenzenesulfonamide (sulfapyridine) in 15 mL of ethanol in a round-bottom glass flask then Add (1.72 g, 10 mmol) of 2-hydroxy-naphthalaldehyde to the glass flask, add 3 drops of anhydrous acetic acid, and connect the flask to a glass condenser after that we will Heat the mixture using a magnetic stirrer for 4 hours at a temperature of 70-80 C and Cool the resulting yellow solution by placing it in the refrigerator for one day at a temperature of 4 C After the cooling period, a yellow precipitate appears. Filter and wash the product with cold water, then recrystallize it with ethanol. Leave the product to dry for a full day at room temperature to obtain yellow crystals. The yield of the product is 87%, with a melting point of 159-161 ^{0}C (17).

Synthesis of β-lactam compound :

Dissolve (2 g, 5 mmol) of Schiff base (compound 1) in 10 mL of dioxane in a conical flask and place in an ice bath then add triethylamine (3.49 mL, 0.025 mol), then add chloroacetyl chloride (2 mL, 0.025 mol) dropwise until the addition is complete at a temperature of 0-5 °C after that we will mix for 4 hours and leave at room temperature for one day and then pour the reaction mixture into crushed ice. The separated solid material was dried and recrystallized from a mixture of ethanol and water. The reaction was monitored by thin-layer chromatography (TLC) to obtain yellow crystals. The yield of the product was 74% with a melting point of 138-140 °C (17).

b) **Physical measurements**

The IR spectra were recorded at the Polymer Research Centre, University of Basrah, Basrah, Iraq, in the range of (3800-3454 cm^{-1}) on a Pye-Unicam SP3-300 spectrometer using KBr disks. The IR, ^{1}H, and ^{13}C-NMR spectra were measured at 300 MHz on the Bruker, with TMS as internal reference at the University of Basrah, College of Science, Department of Chemistry. The melting point was determined at the College of Veterinary Medicine, University of Basrah, Iraq, using a Philip Harris melting point unit.

Identification of bacterial cultures

Samples were collected from local and imported frozen chickens for bacteriological isolates *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae, and Escherichia coli*. Bacterial samples were collected from local chickens and imported frozen chickens, and the bacteria were isolated using traditional methods and molecular techniques by culturing the samples in petri dishes with different media agar, such as MacConkey agar, Eosin methyl blue (EMB), mannitol salt agar, and blood media agar and using Biochemical procedures, including oxidase test, catalase test, coagulase test, indole test, urease test, and citrate utilization test, identified all probable colonies (18), the inoculated media are incubated at 37 °C for 18 -24 hrs (19).

DNA extraction

Genomic DNA was extracted using the Wizard Genomic DNA Isolation Kit (Promega, USA) according to the manufacturer's instructions. After DNA extraction, the concentration and purity of the isolated DNA were assessed utilizing a Nanodrop One spectrophotometer (Thermo Fisher Scientific, USA). Bacterial DNA was preserved at -20°C until employed for PCR assays.

Molecular characterization

Two of these bacteria were identified as the *Staphylococcus aureus* and *Escherichia coli. S*equencing PCR was done using universal primers that target the 16S rRNA gene as described in Table 1 (20).

Identification of Species- specific PCR was performed using PCR primers that target the *nuc* and *uidA* genes (21). The primer sequences and corresponding lengths of the amplicons are shown in Table 1.

Table 1: The genes, primers, and PCR programs used in this study

Gene	primers	Sequence (5'-3')	Size (bp)	Amplification(35 cycle)			Ref
				Denaturation	Annealing	Extension	
16SrRNA	Uni-16SrRNA F Uni-16SrRNA R	AGAGTTTGATCMTGGCTCAG CTACGGCTACCTTGTTACGA	1500	94°C 1min	50°C 1min	72°C 1min	19
uidA	uidA F uidA R	TGGTAATTACCGACGAAAACGGC ACGCGTGGTTACAGTCTTGCG	166	94°C 40 sec	55°C 1 min	72°C 50 sec	
nuc	nuc F nuc R	GCTTGCTATGATTGTGGTAGCC TCTCTAGCAAGTCCCTTTTCCA	423	94°C 1 min	55°C 0.5 min	72°C 2min	

Antibacterial activity

The potential antimicrobial activity of the laboratory-prepared compounds was examined to study their antibacterial activity using the paper disc-agar diffusion technique (22). The inhibition diameter was measured in millimeters. The biological activity of the manufactured compounds was tested against two types of Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two types of Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). Before using filter paper discs, they should be sterilized in an autoclave. Prepare stock solutions. Dimethyl sulfoxide was used at concentrations of 50, 100, 200, and 400 µg/ml (DMSO) to dissolve the prepared compounds. 1 filter paper disc (6mm in diameter) impregnated with the solution in DMSO of the test was placed on the Petri plates. A paper disc impregnated with dimethyl sulfoxide (DMSO) was used as a negative control. The plates were incubated for 24 h at 37°C with bacteria (23). The inhibition zone diameters were measured in millimeters.

Determination of minimum inhibitory concentration (MIC)

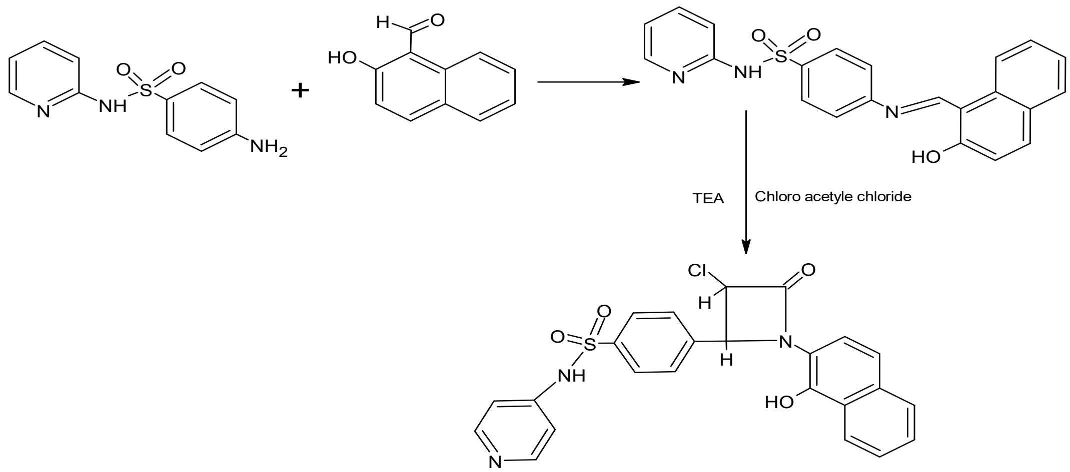
The minimum inhibitory concentration for compounds MW1 and MW2 was determined using the Macrodilution Broth Method. Various concentrations (800.0 to 1.5 µg/ml) were prepared and combined with a bacterial suspension of 1 × 10^8 cfu/ml. Each combination was incubated in Muller-Hinton broth at 37°C for 18 hours. Growth was assessed by comparing the tubes with antimicrobial agents to control tubes without them, identifying the MIC as the lowest concentration inhibiting visible bacterial growth, expressed in µg/ml.

Cytotoxicity test of a Schiff base and β-lactam compound

involved preparing a blood solution by mixing 1ml of human blood with 20ml of physiological solution. Different concentrations (100, 500, 1000 µg/ml) of the compounds were created using DMSO. Sterile tubes were used to establish negative (physiological solution only) and positive (physiological solution with tap water) controls. Each tube received 2ml of the blood solution, followed by 0.1ml of the prepared concentrations. The tubes were allowed to sit at room temperature for 15, 30, and 60 minutes, with turbidity observed to assess the compounds' toxicity against the controls.

Results

The reaction of 4-amino-N-pyridine-2-ylbenzene sulfonamide (sulfapyridine) with 2-hydroxy naphthalaldehyde, in equal molar ratios and in the presence of glacial acetic acid, to produce Schiff base 1. In the second step, the Schiff base (compound 1) was reacted with chloroacetyl chloride in the presence of triethylamine at a temperature of 0-5 °C to produce the beta-lactam derivative (compound 2) with a good yield by using thin-layer chromatography to monitor the reaction and achieve the best conditions for good yield. As shown in the reaction scheme below (Scheme 1).



Scheme 1: Preparation of a beta-lactam compound derived from sulfa pyridine

The IR analysis of synthesized compound MW2 (beta-lactam derivative) shows the most important absorption band, which is 3800-3454 cm^{-1}, corresponding to hydroxyl and amine groups (NH, OH). Absorption bands also appear at 3060 cm-1, attributed to aromatic C-H groups, while aliphatic C-H groups appear as absorption bands in the 2938 cm^{-1} region. The infrared spectrum also shows a strong band at 1700 cm-1 attributed to the (C=O) group, which did not appear in compound MW1 (Schiff base), confirming the formation of the beta-lactam ring, as well as bands at 1920-1596 cm-1 attributed to the C=C and (C=N) groups (Figures 1 and 2).

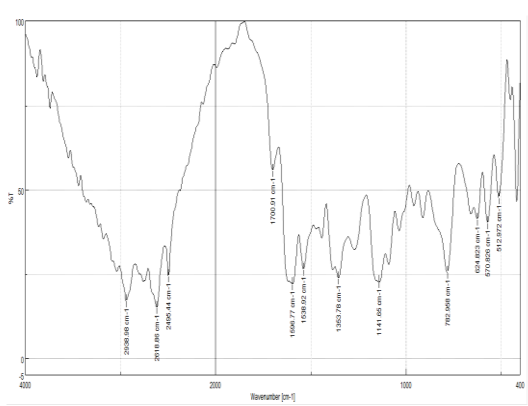


Figure 1: IR of Schiff base (MW1)

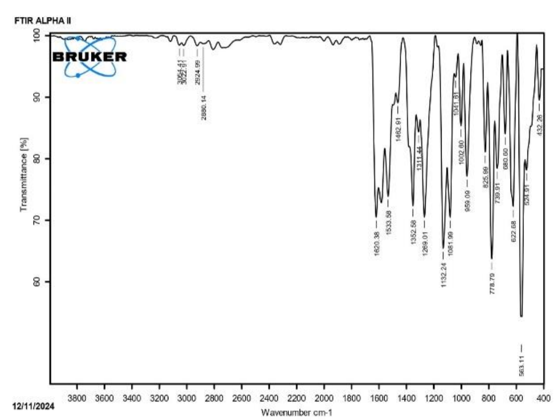


Figure 2: IR of new beta lactam (MW2)

The proton NMR spectrum of the prepared compounds MW1 and MW2 (Figures 3 and 4) showed the presence of signals attributed to protons in aliphatic, aromatic, and other groups, as follows. In compound 1, a single signal is observed at a shift of 8.5 ppm, attributed to the protons of the isomethine group.CH=N single signal at a shift of 9.65 ppm attributed to the protons of the amine group (NH). Multiple signals at shifts of 6.56 -8.43 ppm are attributed to the protons of the aromatic rings of compound 1 (Schiff base) single signal at 12.01 ppm is attributed to the phenolic hydroxyl group (Ar-OH). A single signal at a shift of 3.55 ppm attributed to the protons of the CH-N group of the beta-lactam ring a single signal at a shift of 4.35 ppm attributed to the protons of the CH-Cl group in the beta-lactam ring and multiple signals at 6.88-8.49 ppm attributed to the protons of the aromatic rings of compound 2 (beta-lactam) A single signal at 10.52 ppm is attributed to the proton of the amine group in sulfonamide (NH) A single signal at 11.10 ppm is attributed to the proton of the phenolic hydroxyl group (Ar-OH).

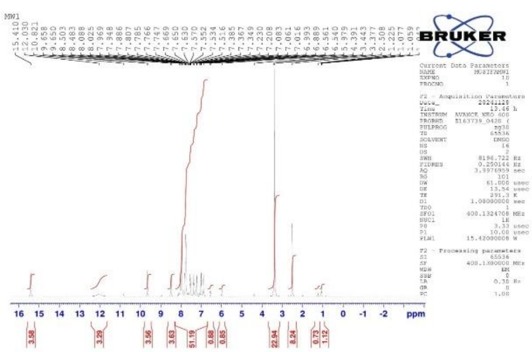


Figure 3: [1]HNMR of Schiff base

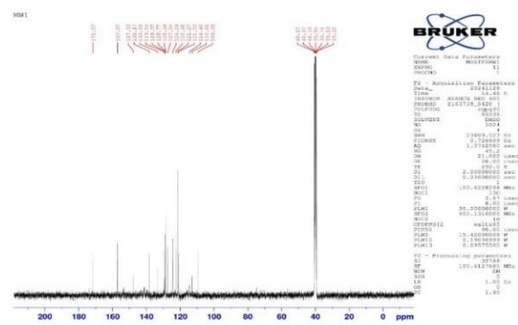
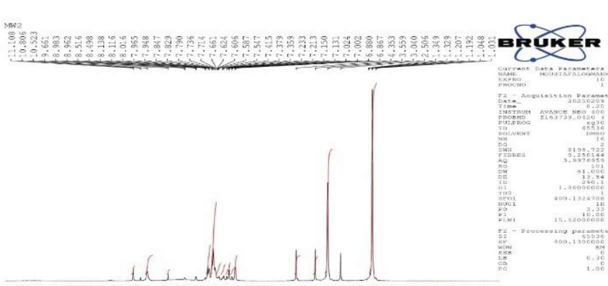


Figure 4: [1]HNMR of β-lactam

The carbon-13 NMR spectrum of the prepared compounds 1-2 (Figures 5 and 6) showed the presence of signals attributed to carbon-13 in the aliphatic, aromatic, and other groups, as follows. In compound 1 (Sheff base), a signal appears at a shift of 157 ppm, attributed to the carbon of the isomethine group CH=N. A signal at a shift of 138 ppm is attributed to the carbon of the =N C group. Multiple signals at 109-133 ppm are attributed to the protons of the aromatic rings of compound 1 (Schiff base).Signal at offset 171 ppm attributed to carbon associated with the phenolic hydroxyl group (Ar) and Signal at offset 45 ppm attributed to carbon-13 of the CH-N group of the beta-lactam ring, signal at offset 66 ppm attributed to carbon-13 of the CH-Cl group in the beta-lactam ring , multiple signals at shifts of 109-153 ppm are attributed to carbon-13 of the aromatic rings of compound 2 (beta-lactam). Signal at 165 ppm attributed to carbon-13 phenol group Ar-OH , signal at 193 ppm attributed to carbon carbonyl group C=O



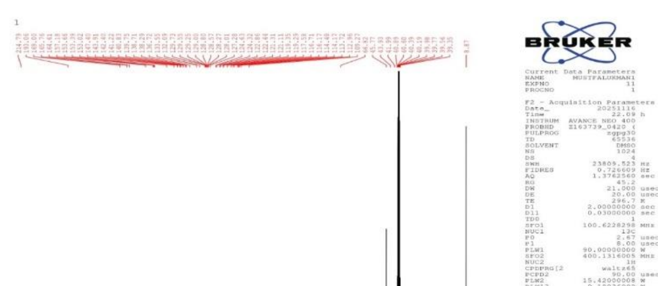


Figure 5: ^{13}CNMR of Schiff base

Figure 6: ^{13}CNMR of B-lactam

Identification of Bacteria

The growth on media shows a positive result of bacteria prevalent in local and imported frozen chicken, as indicated in Table 2, using the conventional method and biochemical test.

Table 2: bacterial isolation from chicken

	Number of samples	Number of Positive samples	Prevalence of bacteria (%)
E.coli	8	6	75%
K.pneumoniae	4	2	50%
E.faecalis	3	1	33.33%
S.aureus	4	3	75%
Total	18	12	66.67%

The molecular diagnosis of *Escherichia coli* and *Staphylococcus aureus* using the 16S rRNA:

The positive samples on the plates were identified using conventional PCR and the 16S rRNA gene for *Staphylococcus aureus* and *Escherichia coli*. All tested samples, 6 *Escherichia coli* (100%) and 3 *Staphylococcus aureus (*100%), showed a positive 16S rRNA PCR band of 1500 bps (Figure 7). A positive 166 bps band by targeting the uidA gene (Figure 8), and a positive 423 bps band by targeting the nuc gene (Figure 9).

Figure 7 : Gel electrophoresis for 16S RNA gene (1500 bp) . lane M=100-1500 DNA ladder , lane 1=Negative control, lane 2-11= positive result

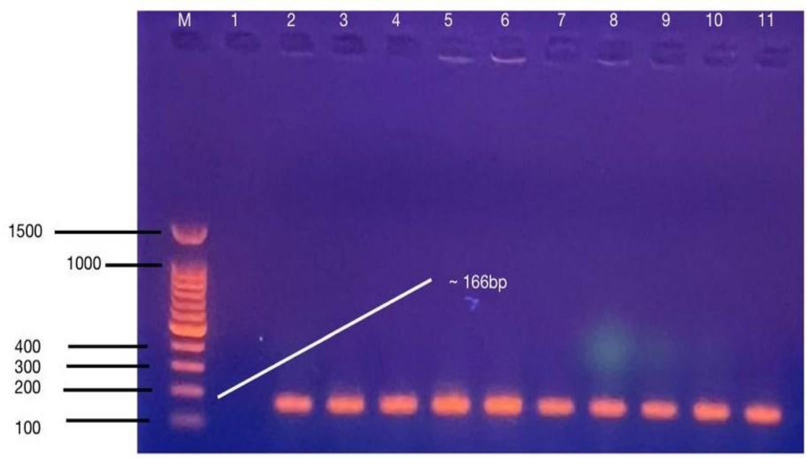


Figure 8 : Gel electrophoresis for the uidA gene (166 bp) , Lane M=100-1500 DNA ladder Lane 1 =Negative control, Lane 2-11= positive result.

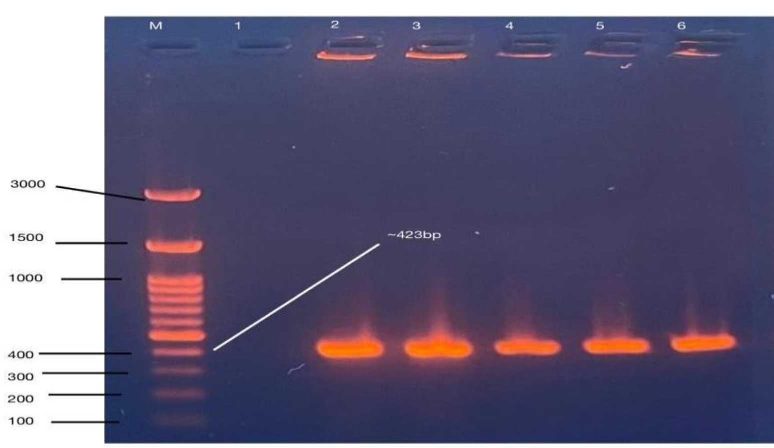


Figure 9 : Gel electrophoresis for the nuc gene-specific PCR (423 bp) of the local isolates.

Lane M =Ladder 100-3000 base pairs, 1= Negative control, lane 2,6 = positive Samples

Antimicrobial activity of synthesized compounds

The antimicrobial activity of Schiff base 1 (MW1) and beta-lactam 2 (MW2) was tested against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*. The results showed that these compounds had a higher inhibitory effect against all tested bacteria, with statistically significant differences at a significance level of $P \leq 0.05$ between MW1 and MW2. The results were also compared with the positive control compounds cefepime, ceftriaxone, and penicillin G as antibacterial compounds, as shown in Table 3 and Figure 14.

The effect of the prepared compounds MW1 and MW2 on Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*) was greater than their effect on Gram-positive bacteria (*E.faecalis* and *S.aureus*), as shown in Table 3 and Figure (10-13). We observe that the compounds prepared in this study have high efficacy against both the positive and negative bacteria used in this study.

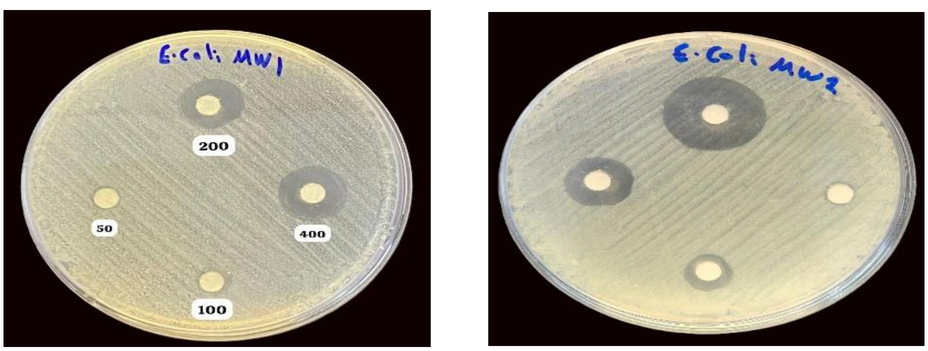


Figure 10:activity of MW1 & MW2 on *E.coli*

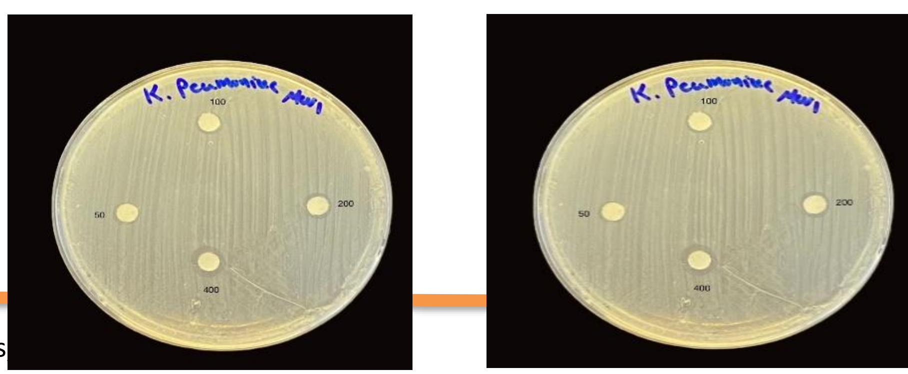


Figure 8: activity MW1 & MW2 on *K.pneumoniae*

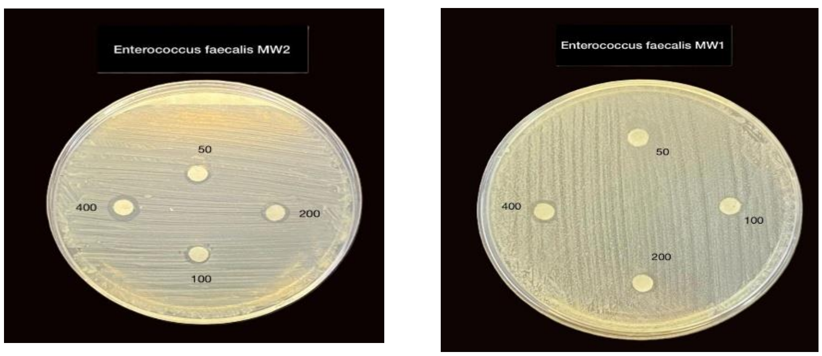


Figure 12: activity of MW1 & MW2 on *E. faecalis*

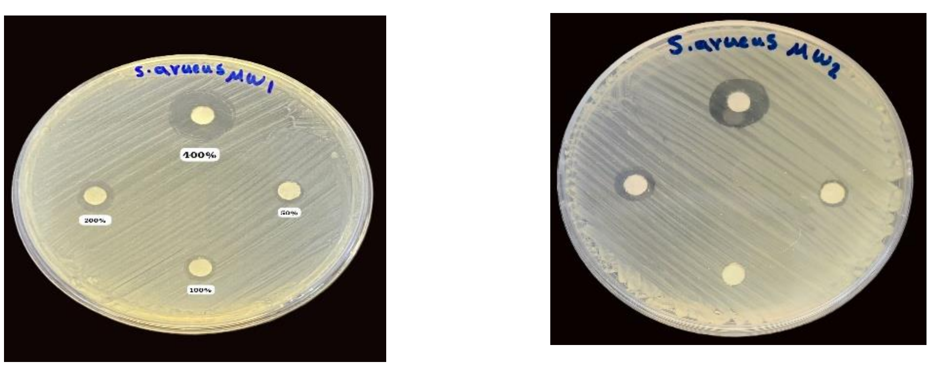


Figure 13 : activity of MW1 & MW2 on *S.aureus*

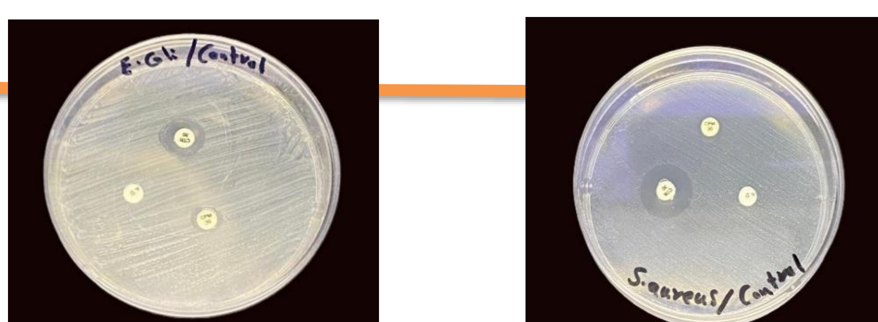


Figure 14: control (CTR, CPM and P) on *E.coli* and *S.aureus*

Determination of the minimum inhibitory concentration of the compounds :

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that inhibits the visible growth of microorganisms after overnight incubation. The MIC results are shown in Table 4 and Figure 15. The MIC values of the two compounds on Gram-negative bacteria *E. coli* and *Klebsiella pneumoniae* are higher than their values on Gram-positive bacteria *Enterococcus faecalis* and *Staphylococcus aureus*.

Table (3): Antibacterial activity of the studied compounds

Cytotoxicity test of the two compounds, Schiff base (MW1) and β-lactam (MW2)

The results showed that two compound Schiff bases (MW1) and β-lactam (MW2) had no toxicity against the human red blood cells with the concentrations 1000, 500, 250, and 125 µg /ml by using positive control tap water and negative control phosphate buffer saline (PBS), as shown in Table 5 and Figure 16. $P \leq 0.05$

Bacteria	Inhibition zone in mm of antibacterial activity test to compounds MW1 and MW2.									Inhibition zone of control		
	50 µg /ml		100 µg/ml		200 µg/ml		400 µg/ml		Mean 200 µg/ml	200 µg/ml	200 µg/ml	200 µg/ml
	MW1	MW2	MW1	MW2	MW1	MW2	MW1	MW2	MW1 +MW2	Cefepime (CEF)	Ceftrxion (CTR)	Penecilline G (P)
E .coli	8	9	9	11	13	16	15	22	12.87	8	13	7
K. pneumonia	7	8	9	10	10	12	12	15	11	8	10	6
Enterococcus faecalis	8	6	11	9	12	10	14	12	10.25	9	11	7
S. aureus	8	7	10	8	12	11	16	14	10.5	9	12	7
Mean									11.12	7.7	8.75	6.75
Mean										8.58		

Bacteria	MIC		
	MW1	**MW2**	**Means**
E.coli	1.5	1.5	1.9
K.pneumonia	1.5	1.5	2.27
Entero.faecalis	3.01	6.2	5.4
S. aureus	3.01	1.5	2.25
Mean	2.25	2.67	2.95
Mean		2.46	

Table (4): Determination of minimum inhibitory concentration (MIC)P ≤ 0.05

Table 5: The results revealed Schiff base (MW1) and β-lactam (MW2) were non-hemolytic and nontoxic on human blood

Toxicity against RBC

Compound	Concentration				Control Compound	
	1000 µg /ml	500 µg /ml	250 µg /ml	125 µg /ml	Tap water	PBS
Schiffbase MW1	-ve	-ve	-ve	-ve	-ve	-ve
β-lactam MW2	-ve	-ve	-ve	-ve		

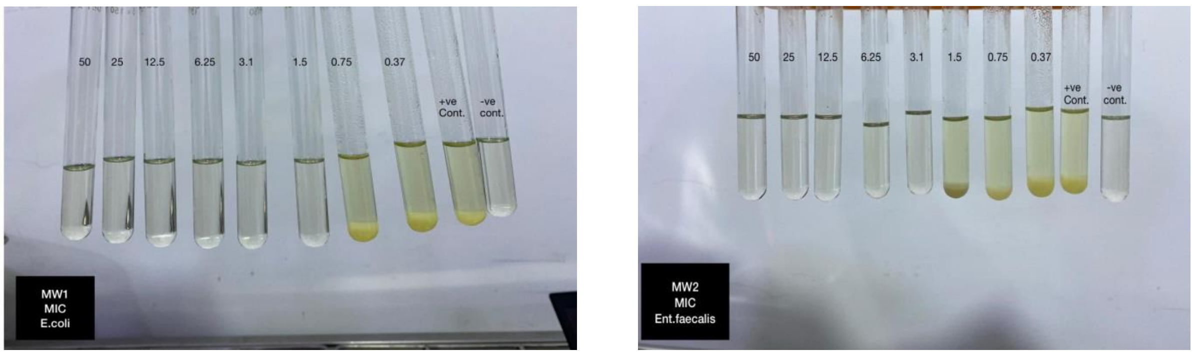


Figure 15: Determination of the minimum inhibitory concentration (MIC) of G-ve (*E.coli*) and G+ve (*Enterococcus faecalis*) bacteria.

Figure 16: The cytotoxicity test of compound Schiff base1 and β-lactam 2 ,PBS: phosphate buffer saline (negative control) , TW: Tap water (positive control), 1: Schiff base MW1, 2: β-lactam MW2

Therefore, compared to Gram-negative bacteria, Gram-positive bacteria are not easily affected by beta-lactam antibiotics (24). As shown in the bioactivity test of the prepared compounds, they have high or moderate efficacy against both Gram-positive and Gram-negative bacteria. G+ve and G-ve bacteria, i.e., the compounds have a broad spectrum of activity. Compared to the results of the control antibiotics Ceftriaxone, cefepime, and penicillin G, as shown in Table 3, Figure 14, we found that the effect of the compounds MW1 and MW2 was greater on both Gram-positive and Gram-negative bacteria in the study. During this study, cytotoxicity tests demonstrated that two novel compounds did not induce hemolysis in blood cells, unlike certain beta-lactam drugs. Previous research. (25) indicated that β-lactam antibiotics can cause hemolysis due to non-specific protein adsorption on red blood cells. Additionally, (26) discussed the effects of Cephalothin and other cephalosporin drugs, which modified the RBC membrane by inducing an autoantibody that reacted with normal RBCs and led to lysis (26).

Conclusion

In conclusion, the synthesized β-lactam compound demonstrates substantial efficacy against four tested pathogenic bacterial strains, surpassing that of conventional antibiotics (ceftriaxone, cefepime, and penicillin G) and the Schiff base. The synthesized beta-lactam compound has significant antibacterial efficacy.

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Conflicts of interest

The authors declare that there is no conflict of interest**.**

Ethical Clearance

This work is approved by The Research Ethical Committee.

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تحضير وتشخيص مركب بيتا لاكتام جديد مشتق من السلفابريدين ودراسة تأثير المركبات المحضرة على البكتيريا المعزولة في محافظة البصرة

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الخلاصة

تم تكثيف 2-Hdroxy naphthaldehyde مع - (سلفابيريدين) amino-N-pyridin-2-ylbenzenesulfonamide لإنتاج قاعدة شيف(MW1) وتمت مفاعلة المركب المحضر مع كلوريد كلورو أسيتيل بوجود ثلاثي أثيل أمين لإنتاج مركب جديد من نوع البيتا-لاكتام (MW2) بحصيلة انتاجية جيدة. شخصت المركبات المحضرة بواسطة التحليل العنصري الدقيق ومطيافية الاشعة تحت الحمراء وطيف الرنين النووي المغناطيسي للبروتون والكاربون-13 .اختبرت المركبات المحضرة كمواد مضادة للبكتريا باستخدام أنواع من البكتريا المرضية المعزولة والمشخصة بواسطة تفاعل البلمرة المتسلسل PCR للبكتريا الموجبة والسالبة الجرام مثل *Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae,* : *Escherichia coli* . اثبتت نتائج الدراسة بان المركبات المحضرة في هذه الدراسة تمتلك فعالية واضحة ضد الكائنات الحية و الممرضة المستخدمة مقارنة مع تركيز المضادات الحيوية القياسية المستخدمة)السفترازيون والسيفبيم والبنسلين ج(.أي ان للمركب البيتا لاكتام المحضر مدى واسع كمضاد بكتيري.

الكلمات المفتاحية: بيتا-لاكتام، سلفا بيريدين، 2-هيدروكسي نفثالالدهيد، قاعدة شيف، نشاط مضاد للبكتيريا.