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RESEARCH ARTICLE

Synthesis, Characterization of Di-1, 3-Oxazepine and Evaluation of their Antimicrobial Activity against Molecular Diagnostic Bacterial Isolates

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Abstract

This work presents Two Schiff bases derived from 4, 4'-Diaminodiphenylmethane**,** distinguished by the para substituted halogen of benzaldehyde. These Schiff bases used to synthesis of four compounds of di-1, 3-oxazepine by direct condensation with tetrachloro phthalic anhydride and tetrabromophtalic anhydride. All structures were characterized by using spectroscopic techniques such as FT- IR, ^{1}H-NMR, ^{13}C-NMR. The synthesized compounds were screened as an anti-bacterial agent against bacterial strains (Gram positive *S. aureus* and Gram-negative *P. aeruginosa* and *K. pneumonia*) which were diagnosed using high specific method "genetic method for *S. aureus* and *P. aeruginosa* and Vitec 2 for *K. pneumonia*.

Keywords: *Schiff bases, 1, 3-Oxazepine, Tetrachlorophthalic anhydride, Antibacterial activity.*

Introduction

Since the starting of Schiff's bases preparation until now, its occupies reputability used for synthesis wide range of compounds because they contains carbon-Nitrogen double bond (C=N) which is considered the central nervous system (CNS) for these compounds and play a vital role in the expansion of heterocyclic chemistry via its reactions with other chemicals.

The first synthesis of oxazepam (benzodiazepine) was in late of 1960 for use in relief of the psychoneuroses characterized by anxiety and tension [1]. Oxazepam is non-homologous seven member ring contain two hetero atoms (oxygen and nitrogen).

The importance of 1,3-oxazepine ascribed to their application as anticonvulsant [1-3], enzyme inhibitor[4], chemotherapeutic agent in the treatment of colorectal cancer [5], antifungal [6], hypnotic muscle relaxant [7], antagonistic [8], anti-inflammatory [9] and antiepileptic [10]. *S. aureus* is the most repeatedly segregated bacterium among both hospitalized and community acquired infections. It causes the infection of skin, soft tissue, respiratory tract, bone, joint, and endovascular disorders [11]. *Pseudomonas aeruginosa* is a bacterium implicated in severe and life-threatening infections [12].

Klebsiella pneumoniae is a bacterium responsible for urinary tract infections of nosocomial patients and compromised individuals. Infections are particularly difficult to treat since most clinical isolates exhibit resistance to several antibiotics leading to treatment failure and the possibility of systemic dissemination [13].

A literature survey indicates that synthesis of 1,3-oxazepine by using substituted phthalic anhydrides are very rare, so, our

project aimed to prepared series of substituted 1,3-oxazepine based on 4,4'-Diaminodiphenylmethane as backbone for synthesized molecules and test there antibacterial activity against *S. aureus*, Pseudomonas aeruginosa and Klebsiella pneumoniae .

Experimental Section

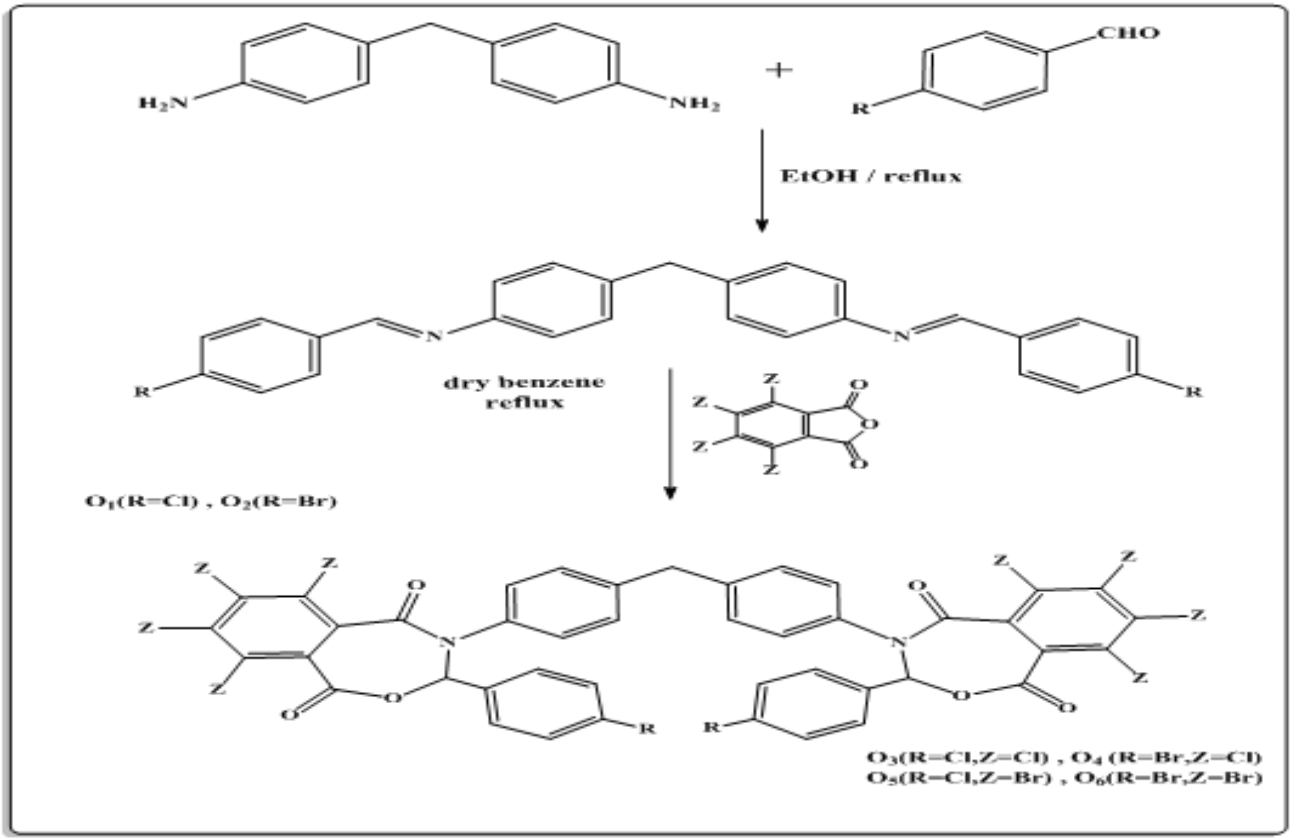
Materials

4-Chloro benzaldehyde, 4-Bromo benzaldehyde, Tetrachlorophthalic anhydride, tetrabromophthalic anhydride, 4,4'-Diaminodiphenylmethane were supplied from Sigma-Aldrich chemical Co. used directly without further purification, Solvents were supplied from Scharlau, Agarose was supplied from Bio basic, Coagulase plasma "citrated rabbit plasma was provided by Bio life, Hydrogen peroxide and N, N, N, N, -tetramethyl-P phenylenediamine Dihydrochloride were supplied from Schuchardt, Tris borate EDTA (TBE) was provided by Bio basic, Primers were synthesized by eurofins Genomics company, DNA ladder (100 bp) and PCR Pre Mix Master mix were supplied from Bioneer, Genomic DNA extraction-mini kit was provided by Geneaid "Presto" and Gram stain was supplied from Jourilabs.

Instrumentation

Infrared spectra were recorded as KBr pellets on a Bruker-Tensor 27 spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker-300 MHz spectrometer using DMSO-d_6 as a solvent and TMS (Tetra Methyl Silane $(CH_3)_4Si$).

Procedure



Scheme 1: Synthetic rout for synthesis of O_1-O_6 compounds

General Procedure for Synthesis of (Imines) 4, 4'-methylenebis *(N-(4-halobenzylidene) aniline) O_1-O_2*

A mixture *of* 4, 4'-Diaminodiphenylmethane *(0.01 mol)* and different aldehydes (0.002) mol in ethanol (30 ml) was refluxed for 2 hrs. *the mixture was refluxed for 2 hr., the obtained precipitate was filtered and washed with ethanol, recrystallized from ethanol[14].*

Characterization of 4, 4'- methylenebis (N-(4-chlorobenzylidene) aniline) [O_1]

White solid, yield 84%; mp 175 ^{o}C . ^{1}H NMR spectrum , δ, ppm : 3.99 [s,(2H),(CH_2] 7.29-7.32 (d, *J*=9 Hz , $H_{12,10}$) , 7.22-7.25 (d, *J*=8.9 Hz , $H_{13,9}$) , 7.57-7.60 (d, *J*=8.3 Hz , $H_{2,6}$) , 7.93-7.96 (d, *J*=8.7 Hz , $H_{3,5}$) and 8.64 (s, H_7) . IR (KBr) v cm^{-1}: 3033 (C-H aromatic) 2879, 2822 (C-H (-CH_2-) aliphatic (Asym. Sym.),

1620 (C=N), 1563, 1493 (C=C aromatic), 1082 (Ph-Cl). UV/visible (THF) nm: 240 π→π*(rings), 318 π→π*(C=N). TLC: R_f =0.83 (benzene: methanol) (9:1) [14].

Characterization of 4, 4'- Methylenebis (N-(4-Bromobenzylidene) Aniline) [O_2]

White solid, yield 84%; mp 199 °C . ^{1}H NMR spectrum , δ, ppm : 3.99 [s,(*2*H),(CH_2] 7.29-7.33 (d, *J*=8.1 Hz , $H_{12,10}$) , 7.22-7.25 (d, *J*=8.3 Hz , $H_{13,9}$) , 7.71-7.74 (d, *J*=7.9 Hz , $H_{2,6}$) , 7.85-7.88 (d, *J*=8.1 Hz , $H_{3,5}$) and 8.63 (s, H_7) . IR (KBr) ν cm^{-1}: 3036 (C-H aromatic), 2878 , 2821 (C-H(-CH_2-) aliphatic(Asym. Sym.), 1620 (C=N), 1568, 1491 (C=C aromatic), 1082 (Ph-Br). UV/visible (THF) nm: 244 π→π*(rings), 316 π→π*(C=N). TLC: R_f =0.92 (benzene: methanol) (9:1). [14]

General Procedure for Synthesis of 4,4'-(4,4'- methylenebis (4,1-phenylene))bis(6,7,8,9-tetrahalo-3-(4-halophenyl)-3,4-dihydrobenzo[1,3]oxazepine-1,5-dione) O_2-O_4

To 100-ml round-bottom flask containing 30 ml dry benzene was dissolved (0.02 mol) of appropriate anhydride then [(0.01 mol) of Schiff base was dissolved in (30 ml) dry benzene] , the reaction mixture was refluxed for 4hr. filtered and washed with 5% $NaHCO_3$ solution then distilled water , dried and recrystallization from benzene[14].

Characterization of O_3-O_6 Compounds

The yields, FT-IR, ^{1}H and ^{13}C NMR data for compounds ***O_3-O_6*** are summarized as follows:

- O_3. Pale yellow solid, yield 73%; mp 259 ° C dec.. ^{1}H NMR spectrum δ, ppm : 3.89 [s,(*2*H),(CH_2] , 10.73 (s, H_7) , 6.88-7.60 (complex , $H_{aromatic}$). ^{13}C NMR spectrum , δ, ppm : 40.24 (C_{22}) ,116.61 (C_7) , 161.53 (C_{17}) , 165.29 (C_{14}) , 120.11-159.24 ($C_{aromatic}$). IR (KBr) ν cm^{-1}: 3039 (C-$H_{aromatic}$), 2959 , 2868 (C-H(-CH_2-) aliphatic(Asym. Sym.) , 1725 (C=$O_{lactone}$) , 1676 (C=O_{lactam}) 1604, 1540 (C=$C_{aromatic}$), 1514 (CO)-N , 1316 (CO)-O. UV/visible (THF) nm : 245 π→π*(rings) ,. TLC: R_f =0.65 (benzene: methanol)(7:3).
- O_4. Pale yellow solid, yield 89%; mp 269 °C dec.. ^{1}H NMR spectrum , δ, ppm : 3.89 [s,(*2*H),(CH_2],10.70 (s, H_7) , 6.86-7.88 (complex , $H_{aromatic}$). ^{13}C NMR spectrum , δ, ppm : 40.24 (C_{22}) ,116.61 (C_7) , 161.53 (C_{17}) , 165.29 (C_{14}), 120.1-159.24 ($C_{aromatic}$). IR (KBr) ν cm^{-1}: 3035 (C-$H_{aromatic}$) , 2909 , 2864 (C-H(-CH_2-) aliphatic(Asym. Sym.) , 1724 (C=$O_{lactone}$), 1667 (C=O_{lactam}) 1602 , 1538 (C=$C_{aromatic}$), 1513 (CO)-N , 1334 (CO)-O. UV/visible (THF) nm : 252 π→π*(rings) ,. TLC: R_f =0.60(benzene: methanol)(7:3).
- O_5. Pale yellow solid, yield 64%; mp 261 °C dec.. ^{1}H NMR spectrum δ, ppm : 3.87 [s,(*2*H),(CH_2] , 10.66 (s, H_7) , 6.84-7.60 (complex , $H_{aromatic}$). ^{13}C NMR spectrum , δ, ppm : 40.25 (C_{22}) ,115.22 (C_7) , 163.02 (C_{17}) , 166.50 (C_{14}) , 120.05-159.26 ($C_{aromatic}$). IR (KBr) ν cm^{-1}: 3034 (C-$H_{aromatic}$), 2905 , 2846 (C-H(-CH_2-) aliphatic(Asym. Sym.) , 1722 (C=$O_{lactone}$) , 1669 (C=O_{lactam}) 1599, 1515 (C=$C_{aromatic}$), 1515 (CO)-N , 1312 (CO)-O. UV/visible (THF) nm : 245 π→π*(rings) ,. TLC : R_f =0.79 (benzene: methanol)(7:3).
- O_6.. Pale yellow solid, yield 66%; mp 249 °C dec.. ^{1}H NMR spectrum δ, ppm : 3.88 [s,(*2*H),(CH_2] , 10.66 (s, H_7) , 6.84-7.88 (complex , $H_{aromatic}$). ^{13}C NMR spectrum , δ, ppm : 40.25 (C_{22}) ,115.24 (C_7) , 163.01 (C_{17}) , 166.48 (C_{14}) , 120.06-159.39 ($C_{aromatic}$). IR (KBr) ν cm^{-1}: 3034 (C-$H_{aromatic}$), 2906 , 2847 (C-H(-CH_2-) aliphatic(Asym. Sym.) , 1722 (C=$O_{lactone}$), 1670 (C=O_{lactam}) 1599, 1515 (C=$C_{aromatic}$), 1515 (CO)-N , 1312 (CO)-O. UV/visible (THF) nm : 250 π→π*(rings) ,. TLC: R_f =0.64 (benzene: methanol) (7:3).

Bacterial Isolates Isolation and biochemical diagnostic

Three types of bacteria [*Staphylococcus aureus* (65 isolates), *Pseudomonas aeruginosa* (50 isolates) and *Klebsiella pneumonia* (37 isolates)] were isolated from different hospitals in Iraq including (Ibn Al Bitar, Al Wasity, Al Yarmouk, Al-Nu'man, Al-Ramadi, Baghdad and Educational laboratories in medical city).

The bacterial isolates were initially identified based on colony morphology, Gram stain, cultural methods and biochemical tests.

To diagnosis of *Staphylococcus aureus*, catalase- oxidase- mannitol fermentation on Mannitol Salt Agar (MSA) medium - slide and tube coagulase tests were used [15].

To diagnosis of *Pseudomonas aeruginosa*, catalase- oxidase- Indol-Methyl Red- Voges-Proskauer- Simmon's citrate utilization-Growth at 42°C and growth on Cetrimide agar **[16]**.For *Klebsiella*, catalase- oxidase-Indol-Methyl Red- Voges-Proskauer and capsule production [17].

Confirmation of Diagnosis

For professional diagnosis, PCR technique was used to diagnosis of Staphylococcus *aureus* and *Pseudomonas aeruginosa* by detection of *nuc* gene that is specific gene for *Staphylococcus aureus* and detection of *16SrRNA* that specific gene for *Pseudomonas*. Confirmation of *Klebsiella pneumonia* was achieved by Vitec2. [18, 19].

Table 1: Primers used in this study

Gene	Primers' Sequences (5'→3')	Tm (ºC)	Product size (bp)	Accession no.	Refer-ence
nuc	F: GCGATTGATGGTGATACGGTT	57.9	270	*DQ507382*	[17]
	R: AGCCAAGCCTTGACGAACTAAAGC	62.7			
16SrRNA	F: GGGGGATCTTCGGACCTCA	58	956	*AY268175.1*	[18]
	R: TCCTTAGAGTGCCCACCCG	58			

The bacterial genomic DNA was extracted according to a method described in the used kit (Geneaid "presto", Korea). The thermal cycling conditions for detection of *nuc* gene included an initial denaturation step (5 min at 94°C) followed by 25 cycles of amplification (denaturation for 1 min at 94 °C, annealing for 1 min at 55°C, and extension for 30 seconds at 72 °C). The reaction was terminated with a 10-min incubation step at 72 °C then incubation at 4 °C for 3 min was performed.

The amplification was performed in a Thermal Cycler (Bioneer, Korea). The protocol was used according to master-mix provide company (Bioneer "PCR premix", Korea). For detection of 16SrRNA gene , The thermal cycling conditions included an initial denaturation step (5 min at 94°C) followed by 35 cycles of amplification (denaturation for 1 min at 94 °C, annealing for 45 sec at 58°C, and extension for 45 sec at 72 °C).

The reaction was terminated with a 10-min incubation step at 72 °C. Then incubation at 4 °C for 3 min was performed. The amplification was performed in a Thermal Cycler (Bioneer, Korea). The protocol was used according to master-mix provide company (Bioneer "PCR premix", Korea). Primers were described in table1. PCR productions were electrophoresed using, agarose gel (1.5%) for 1 hour with 70 volt and the bands of genes were detected with UV light by gel documentation device.

Antibacterial Activity

The disk diffusion assay was used according to Kirby-Bauer's methods (CLSI) [19,20]. Paper discs were prepared according to Thompson, 1950 by punching of filter paper discs approximately 6mm diameter and were sterilized by autoclaving.

The stock solutions (30 mg/ml) were prepared by dissolving of a known weight of the synthesized compounds powder in a known volume of DMSO. Many working Solutions (0.5, 1, 1.5, 2, 2.5 and 5) mg/ml were prepared from stock solutions. The discs were impregnated with working Solutions (20 µl) to obtain the final concentrations (10, 20, 30, 40, 50 and 100) µg/disc respectively then the discs were allowed to dry in a clean incubator at 37°C for 4 hours.

The control discs were impregnated with 20 µl of DMSO. The overnight bacterial inoculums was adjusted to 0.5 McFarland standard (1.5×10^8 CFU.ml^{-1}), then it was spread on Muller Hinton agar. The prepared discs were placed on the inoculated agar plate. After incubation at 35 ºC for 24 hours, inhibition zone diameter in millimeters was recorded [21, 22].

Results and Discussion

Characterization of O_1-$O_2$by FTIR and ^{1}H NMR

Schiff's bases were chosen as starting materials for synthesis seven member heterocyclic rings by their reaction with cyclic anhydride, Schiff's bases were synthesized by direct condensation of primary amine and aldehyde. FTIR data for **O_1-O_4** are tabulated in Table (2).

All spectra shows appear of stretching vibration of aromatic C-H at range of 3033-3036 cm^{-1}, azomethine groups (C=N) in 1620 cm^{-1}and disappear of asymmetric and symmetric stretching vibration of NH_2 groups . ^{1}H NMR data are tabulated in Table (3). All spectrums exhibited singlet signal at range of δ 8.63-8.64 ppm which attributed to protons for azomethine group and set of signals at aromatic region 7.22-7.96ppm [14].

Characterization of O_3-O_6 by FTIR and ^1H, ^{13}C NMR

FTIR data are tabulated in Table (4). All spectrums shows appear of stretching vibration of aromatic C-H in range of 3034-3039 cm^{-1}, and disappear of azomethine bands in 1620, the stretching vibrations of the $C=O_{lactone}$, $C=O_{lactam}$ groups substantiated by strong absorption band observed at the frequency range of 1722-1725, 1667-1676 cm^{-1} respectively, stretching vibrations (CO)-N and (CO)-O observed at the frequency range of 1513-1515, 1312-1334 cm^{-1} respectively ^1H NMR data are tabulated in Table (5).

All spectrums exhibited singlet signal in range of δ = 10.66-10.73 ppm which attributed to H_7 and disappear signal for azomethine proton, While the aromatic protons appear complex signal pattern in range of δ 6.84-7. 88 ppm . The structures of the O_3-O_6 are further substantiated by ^{13}C NMR spectroscopic data, all spectrums indicate the presence of aromatic carbons with peaks within the frequency range of δ = 120.05-159.39 ppm, C_{22} at range of 40.24 - 40.25 ppm, C_7 at range of 115.22-116.61 ppm, C_{14} at the range of δ = 165.27-166.50 ppm and C_{17} at the range of δ = 161.53-163.03 ppm, Table (6). [14]

Diagnosis of Bacterial Isolates

All isolates were positive for oxidase which is considered an important diagnostic test for P. aeruginosa because this enzyme activates an electron transport among donor bacteria and pigment which is reduced to dark purple color, and also positive for catalase. IMViC tests results showed that all isolates were negative for indol, methyl red (MR) and voges-Proskauer (VP) and were positive for citrate as a sole carbon source. All isolates were able to grow at 42°C while no growth at 4°C.

These results agree with Todar 0228( ) 0224. All *S. aureus* isolates (100%) that were diagnosis by conventional method harboured *nuc* gene "that is a specific gene for *S. aureus* " with end product size equal to 270 bp, see figure (1) . The *16SrRNA* gene was found in all *Pseudomonas* isolates (100%), see figure (2). Most based molecular identification of *S. aureus* is PCR; this required to amplify of species-specific targets [23]. Therefore, the detection of *nuc* gene in all S. *aureus* isolates and *16SrRNA* gene in all *Pseudomonas* isolates provide high accuracy in diagnosis of bacterial isolates in this study.

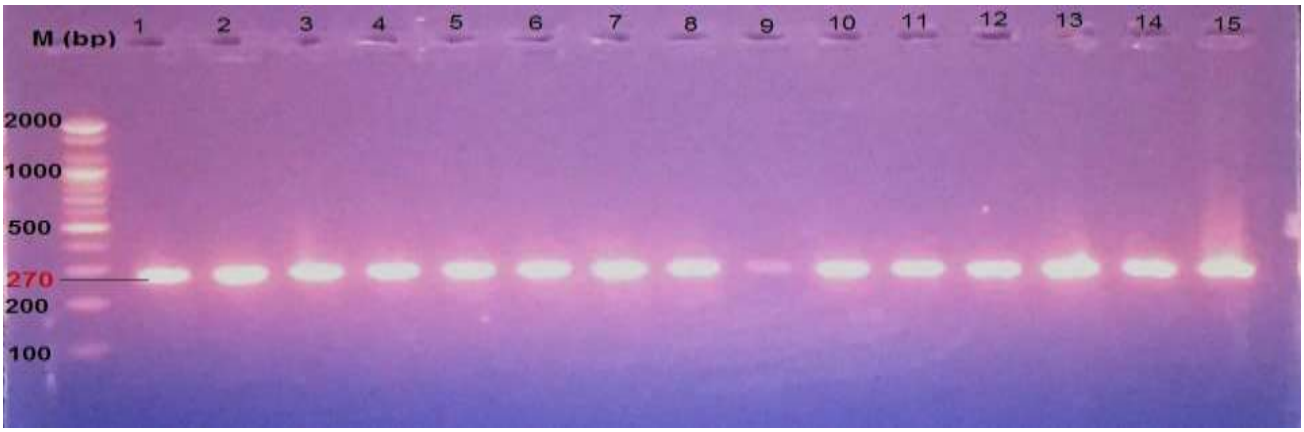


Figure 1: 1.5 % agarose gel electrophoresis for *nuc* gene, M: DNA marker

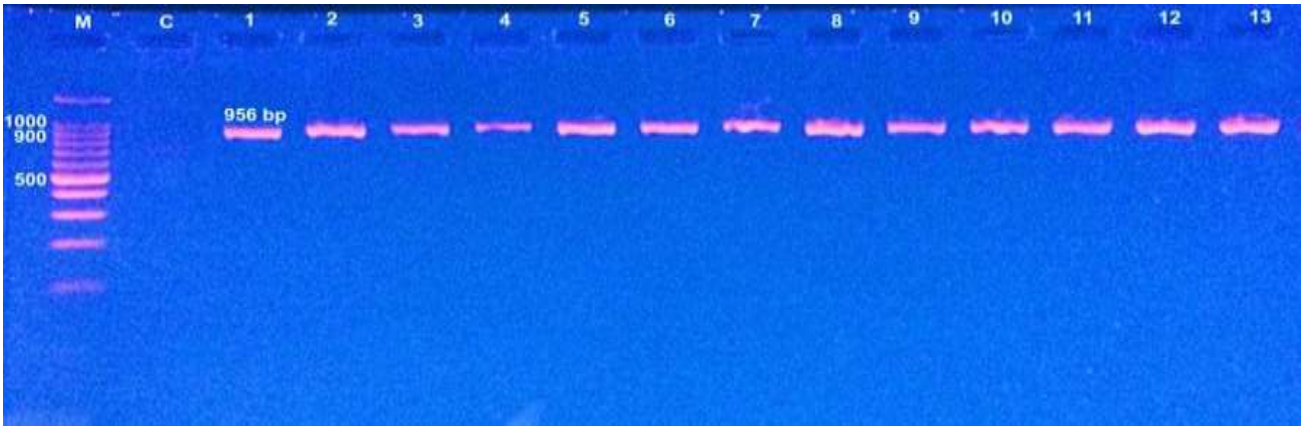


Figure 2: 1.5 % agarose gel electrophoresis for *16SrRNA gene,* M: DNA marker.

Anti-bacterial Activity

Compounds O3 and O4 shoed low activity against tested bacterial isolates where no recorded zone of the concentrations (10, 20, 30 and 40) µg/disc. While in concentrations (50 and 100) µg/disc slight zones were observed. Compounds O5 and O6 also didn't affect against all tested bacterial types in the concentrations (10, 20, 30 and 40) µg/disc but they have larger anti-bacterial activity in high concentration (50 and 100) µg/disc than compounds O3 and O4 (see table 7 and figure 3). On the other hand, *S. aureus* and *Pseudomonas aeruginosa* were more sensitive than Klebsiella *pneumonia*. That may be occurring due to production of capsule by *Klebsiella pneumonia* which can prevent antimicrobials agents to reach to their targets.

Table 2: FTIR spectral data (cm^{-1}) for synthesized O_1-O_2 compounds

Comp.	v C-H arom.	v C=N	v C=C ring		Ph-X
O_1	3033	1620	1563	1493	1082
O_2	3036	1520	1568	1491	1062

Table 3: ^{1}H NMR chemical shift (ppm) for synthesized O_1-O_2 compounds

	Compounds Symb.	
	O_1	O_2
-Z-	-CH_2-	-CH_2-
-Y-	-Cl-	-Br-
CH_2	3.99(s)	3.99(s)
H_1	——	——
$H_{2,6}$	7.60-7.57 (d,*J*=9 Hz)	7.74-7.71(d,*J*=9 Hz)
$H_{3,5}$	7.96-7.93 (d,*J*=9 Hz)	7.88-7.85 (d,*J*=9Hz)
$H_{9,13}$	7.25-7.22 (d,*J*=9 Hz)	7.25-7.22 (d,*J*=9 Hz)
$H_{10,12}$	7.32-7.29 (d,*J*=9Hz)	7.32-7.29 (d,*J*=9 Hz)
H_7	8.64(s)	8.63(s)

Table 4: FTIR spectral data (cm^{-1}) for synthesized O_3-O_6 compounds

		Compounds Symb.			
		O_3	O_4	O_5	O_6
-Z-		-CH_2-	-CH_2-	-CH_2-	-CH_2-
-Y		-Cl	-Br	-Cl	-Br
-X		-Cl	-Cl	-Br	-Br
v C-H arom		3039	3035	3034	3034
v C-H (-CH_2-) aliph	Asym.	2959	2909	2905	2906
	sym	2868	2864	2846	2847
v C=O lactone		1725	1724	1722	1722
v C=O lactam		1676	1667	1669	1670
v C=C ring		1604	1602	1599	1559
		1540	1538	1515	1515
v (CO)-N		1514	1513	1515	1515
v (CO)-O		1316	1334	1312	1312

Table 5: ^{1}H NMR chemical shift (ppm) for synthesized O_3-O_6 compounds

Comp.	H_7	$H_{aromatic\ rings}$	-CH_2-
O_3	10.73 s	6.88 – 7.60 complex	3.89 s
O_4	10.70 s	6.86 – 7.88 complex	3.89 s
O_5	10.66 s	6.84 – 7.60 complex	3.87 s
O_6	10.67 s	6.84 – 7.88 complex	3.88 s

Table 6: ^{13}C NMR chemical shift (ppm) for selected synthesized compounds

Comp. Symb.	-Y	-Z-	-X	C_7	C_{14}	C_{17}	C_{22}	$C_{aromatic\ rings}$
O_3	-Cl-	-CH_2-	-Cl-	116.61	165.29	161.53	40.24	159.24-120.11
O_4	-Br-	-CH_2-	-Cl-	115.82	165.35	161.54	40.24	159.38-120.07
O_5	-Cl	-CH_2-	-Br	115.22	166.50	163.02	40.25	159.26-120.05
O_6	-Br	-CH_2-	-Br	115.24	166.48	163.01	40.25	159.39-120.06

Table 7: Anti-bacterial activity

Compound	Con. µg/disc	Inhibition zone in mm against					
		S. aureus (65)		*Pseudomonas aeruginosa* (50)		*Klebsiella pneumonia* (37)	
		No.	Zone~(mm)	No.	Zone~(mm)	No.	Zone~(mm)
O3	10	65	0	50	0	37	0
	20	65	0	50	0	37	0
	30	65	0	50	0	37	0
	40	65	0	50	0	37	0

	50	65	8	7	0	37	8
				43	10		
	100	50	10	5	8	17	8
		15	12	45	11	20	10
O4	10	65	0	50	0	37	0
	20	65	0	50	0	37	0
	30	65	0	50	0	37	0
	40	65	0	50	0	37	0
	50	65	10	50	8	37	8
	100	65	11	50	8	37	10
O5	10	65	0	50	0	37	0
	20	65	0	50	0	37	0
	30	65	0	50	0	37	0
	40	65	0	50	0	37	0
	50	23	8	22	9	31	0
		42	**12**	**28**	**11**	**6**	**10**
	100	14	11	9	11	25	10
		51	**15**	**41**	**13**	**12**	**12**
O6	10	65	0	50	0	37	0
	20	65	0	50	0	37	0
	30	65	0	50	0	37	0
	40	65	0	50	0	37	0
	50	20	8	16	8	18	0
		45	**13**	**34**	**12**	**19**	**12**
	100	17	8	12	8	29	10
		48	**14**	**38**	**13**	**8**	**13**

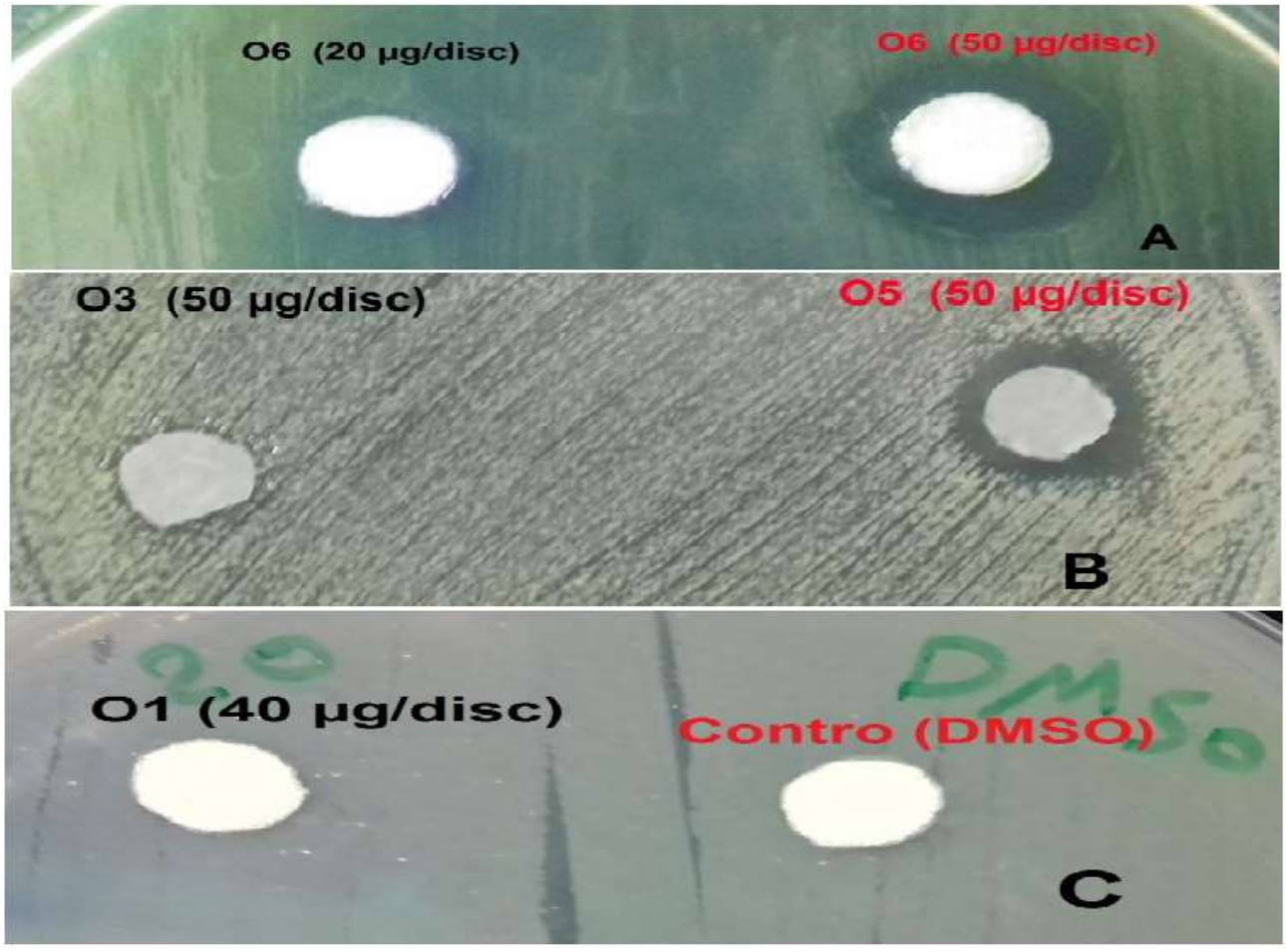


Figure 3: antibacterial activity test A: *Pseudomonas aeruginosa*, B: *Staphylococcus aureus*, C: *Klebsiella pneumonia*.

Conclusion

In this study, four compounds of di-1, 3-oxazepine (O_1, O_2, O_3 and O_4) were synthesized using Schiff bases. O_3 and O_4 had antibacterial activity with high concentration only; while compounds O_1 and O_2 appeared poor activity against the tested bacterial isolates.

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