



SYNTHESIS OF TWO NEW HYDRAZONE DERIVATIVES AS POTENTIAL ANTIBACTERIAL AGENTS

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ABSTRACT

Hydrazone derivatives constitute a versatile class of organic compounds with significant implications in medicinal chemistry with diverse biological and pharmacological properties. They are characterized by the presence of a functional group (-NHN=C) formed by the reaction between a hydrazine and a carbonyl compound, resulting in a carbon-nitrogen double bond. This research aims to carry out the synthesis and anti-bacterial study of two hydrazone derivatives. The hydrazones [L3 ((E)-1-(1-(2,4-dimethoxyphenyl) ethylidene)-2-(2,4-dinitrophenyl) hydrazine) and L4 ((E)-1-(2,4-dinitrophenyl)-2-(1-(2,4,5-trimethoxyphenyl) ethylidene) hydrazine)] were synthesized using the Claisen-Schmidt condensation reaction. The reaction's progress was monitored using a TLC plate, and the purity of the synthesized compounds was verified using the melting point. UV, FT-IR and 1D NMR were used to characterize the synthesized hydrazones. The two synthesized hydrazones were subjected to antibacterial assessment for in-vitro test evaluation using agar diffusion and tube dilution technique. The organisms used were standard isolates and includes two Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and two Gram-negative (*Escherichia coli* and *Salmonella Typhi*) bacteria. The synthesized hydrazones (L3 and L4) were obtained in good yields of 79.50 and 75.40% w/w respectively. The spectra data (FT-IR, UV and 1D NMR) were as expected of the synthesized compounds. Compounds L3 and L4 were found to be active against all the tested organisms with a maximum range of 18 mm zone of inhibition as compared with ciprofloxacin which has 16-39 mm zones of inhibition across the test organisms. The MIC values vary with 25 mg/mL for *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Salmonella Typhi*, and 50 mg/mL for *Escherichia coli*. Compound L3 showed MBC values that were consistently high (100 mg/mL) across all tested organisms. Compound L4 have MBC value of 50 mg/mL against *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Escherichia coli*, and 100 mg/mL against *Salmonella Typhi*. In conclusion, the two compounds demonstrated promising broad-spectrum antibacterial activity.

Keywords: Antibacterial activity, Hydrazones, Spectra property, Synthesis

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INTRODUCTION

Infectious diseases have been reported to be responsible for the largest number of premature death and disability worldwide, causing 63% of all childhood deaths and 48% of premature deaths [1]. Infectious diseases are a major threat throughout the world and are caused by microorganisms, specifically bacteria. Various antibiotics are synthesized and used against infectious diseases to minimize the death rate [2]. The major challenges posed by these infectious diseases are the increasing cases of drug resistance and microbial mutations [1].

Hydrazones are a class of azomethine with a -C=N-N- linkage, they have the general formula R1-NHN=CH-R2 [3]. These compounds possess diverse

biological and pharmacological properties including analgesic, anti-inflammatory, antihypertensive, anticonvulsant, antimicrobial, antitubercular, anticancer, antimalarial, and antiproliferative properties, which have been studied for several hydrazone derivatives [4].

Today, most classes of antibiotics are becoming ineffective at a speed faster than the rate of their development, therefore, to maintain clinical effectiveness in the face of emerging bacterial resistance, there is a need for a continuous search for promising antibacterial agents [5].

This research focused on the development of antibacterial agents from hydrazone derivatives with a broad spectrum of activity and fewer side effects or toxicity.

MATERIALS AND METHODS

Materials

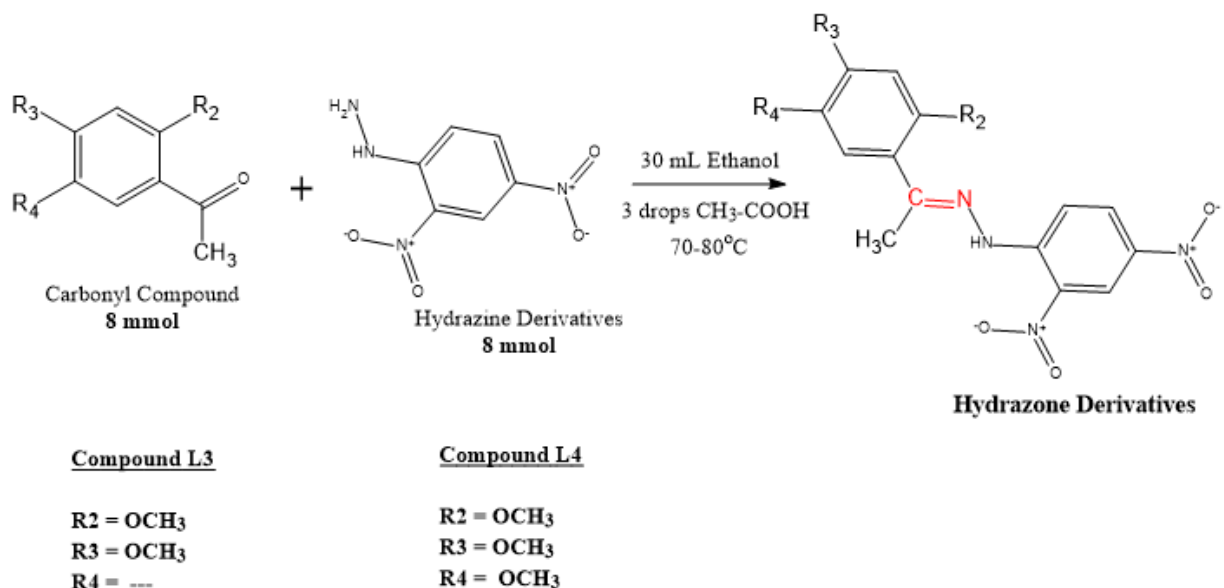
The glassware apparatus used in this research were; a conical flask, spatula, test tubes, beakers, measuring cylinder, glass rod, funnel, pipette, watch glass, transparent petri dish, and sintered glass. Equipment included syringes, capillary tubes, a TLC tank, a magnetic stirrer, a stirring bar, a refrigerator, and Gallenkamp melting point apparatus.

Methods

Eight (8) mmol of carbonyl compounds were reacted with 8 mmol of hydrazine derivative in 30 mL ethanol in a 100 mL round bottle flask followed by the addition of three drops of acetic acid. The reaction mixture was stirred vigorously and refluxed in a water bath for 2 to 3 h at 80°C and the progress of the reaction was observed via TLC (scheme 1). After the completion of the reaction, the precipitate was filtered, washed with cold distilled water and dried. The desired compound was further purified by washing it twice with 30% ethyl acetate: hexane [5]. The reactant, molar ratio and time are given in Table 1

Table 1: Reagents used for the synthesis of hydrazone derivatives

Code	Substituted hydrazine	Carbonyl compound	Time	Molar ratio
L3	2,4-dinitrophenyl hydrazine	2,4-dimethoxy acetophenone	2 hrs	8 mmol each
L4	2,4-dinitrophenyl hydrazine	2,4,5-trimethoxy acetophenone	3 hrs	8 mmol each



Scheme 1: Scheme for the Synthesis of Hydrazone Derivatives

Characterization

The thin layer chromatography (TLC) and one-way ascending technique were used. The color and appearance of synthesized hydrazone compounds were observed and recorded. The solubility of synthesized hydrazone compounds was observed in different solvent systems (dichloromethane, Chloroform and Methanol). The melting points of the synthesized hydrazone compound were determined using a Gallenkamp melting point apparatus (Table 3). Detailed structural analysis of the synthesized compounds was performed using Infrared Spectroscopy (IR), and Nuclear Magnetic Resonance (NMR) which include; proton (¹H) NMR, carbon-13 (¹³C) NMR. The IR data are reported in

terms of frequency of absorption cm⁻¹ while data for ¹H NMR and ¹³C NMR are reported as chemical shift (δ ppm), multiplicity (s= singlet, d= doublet, dd= doublet of doublet, m= multiplet), integration (J).

Antibacterial evaluation of the synthesized hydrazones

Source of test organisms

Two Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*), and two Gram-negative (*Escherichia coli*, and *Salmonella Typhi*). *S. aureus* (ATCC-29213), *S. pneumoniae* (ATCC-700303), *E. coli* (ATCC-11775), and *S. Typhi* (ATCC-021001) were

obtained from the Department of Pharmaceutical Microbiology, Ahmadu Bello University, Zaria.

Preparation of compounds

This was carried out as described by [6]. The stock solution of the synthesized compounds was prepared by adding 1 g of each compound into 10 mL of 10% dimethylsulphuroxide (DMSO) as the reconstituting solvent to make a 100 mg/mL solution. From the stock solution, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL concentrations were prepared using the Two-fold serial dilution method. These concentrations were labeled and kept in bijoux bottles for subsequent use.

Preparation of turbidity standard

McFarland standards was used as the reference standard to adjust the turbidity of bacterial suspension so that the number of bacteria will be within a given range. First, (1% w/v) BaCl₂ and (1% v/v) H₂SO₄ were prepared by dissolving 1 g of BaCl₂ in 100 mL of sterile distilled water and 1 mL of concentrated H₂SO₄ in 99 mL of sterile distilled water respectively to serve as stock solutions for the preparation of the McFarland standard. From the stock solutions, 0.5 McFarland scale was prepared by adding 9.95 mL of (1% v/v) H₂SO₄ to 0.05 mL of (1% w/v) BaCl₂ with constant stirring to maintain a suspension of (1% w/v) BaSO₄ whose density is equivalent to 1.5 × 10⁸ CFU / mL or 150 million/mL approximate cell density of bacteria. The barium sulphate suspension in 4 to 6 mL aliquots was transferred to screw-cap tubes, tightly sealed, and stored in the dark at room temperature to prevent loss by evaporation. This was subsequently used for comparison with the turbidity of the bacterial inoculum.

Standardization of bacterial inoculum

For inoculum standardization, the density of isolated cultures was adjusted equal to that of 0.5 McFarland standards (1.5 × 10⁸ CFU/mL) by suspending some quantity of the bacterial culture into 2 mL of sterile physiological saline as suspending medium. The physiological saline was prepared by dissolving and sterilizing 8.5g of NaCl in 1 L of distilled water. To aid comparison, the test organisms and standards were compared against a white background with contrasting black lines.

Determination of anti-bacterial activity

The antibacterial activity of the compounds against the test organisms was evaluated by using the agar well diffusion method of sensitivity test as earlier described by Alm and Lahiri [5]. Mueller Hinton agar plates were inoculated with 100 µl of standardized inoculum of the bacterium (in triplicates) using a micropipette of 100 µl size and spread uniformly with sterile glass spreader. Wells of 8 mm size were made with sterile cork borer

into the agar plates containing the bacterial inoculum. Using the micropipette, 100 µl volume of the various concentrations; 100 mg/mL, 50mg/mL, 25mg/mL, and 12.5 mg/mL each of the compounds were poured into wells of the inoculated plates with separate pipette tips. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the compounds into the agar and then incubated for 24 hours at 37°C. The diameters of the inhibition zones (DIZ) were measured and expressed in millimeters. The mean values of the diameters of zones of inhibition were calculated to the nearest whole number. To check the activity of the compounds, the reconstituting solvent (DMSO) was used as a negative control. Commercially available standard antibiotic; Ciprofloxacin (20 ug/ml) was used as positive control parallel with the compounds. For this antibiotic, zone of inhibition was interpreted by the CLSI (Clinical Laboratory Standards Institute) interpretation guideline [7].

Determination of minimum inhibitory concentration (MIC)

The compounds were further assayed for minimum inhibitory concentrations (MIC). The broth dilution technique was employed using Mueller Hinton broth as described by Elhady *et al.* [6]. Two-fold serial dilutions of the reconstituted compounds were made to obtain the following concentrations; 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL and 1.56 mg/mL. From the Mueller Hinton broth prepared, 9 mL broth was added to each of the test tubes labeled as containing 1 mL of 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL and 1.56 mg/mL concentrations of each compound resulting in four sets of five different test tubes for the four organisms which were then inoculated with 100 µl inoculum size of the test organisms. Mueller Hinton broth samples with 100 µL of active inoculum of standardized bacterial isolates in tubes were incubated for 24 hours at 37°C. The MIC was determined as the lowest concentration of the compound which inhibited the organism and results were observed in the form of turbidity. Negative controls were set up to contain Mueller Hinton broth only and Mueller Hinton broth with compounds only. Positive control was set up to contain Mueller Hinton broth and the test organism only [8].

Determination of minimum bactericidal concentration (MBC)

For MBC determination, sample wells with no visible growth were transferred to fresh agar plates without the antibacterial agent. The sub-cultured plates were incubated at 37°C for 24 h. The MBC is the lowest concentration that showed no bacterial growth on the sub-cultured plates.

RESULTS AND DISCUSSION

Thin layer chromatographic analysis

The thin layer chromatographic analysis of the synthesized hydrazones was visualized under ultra-violet light (254-366 nm) range. The R_f values of the compounds are shown in Table 2.

Physical properties of the hydrazones

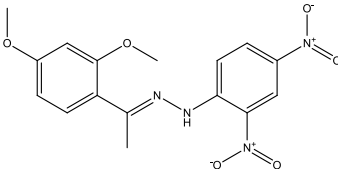
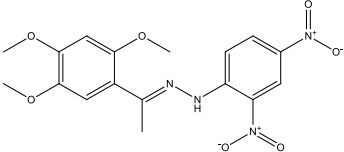
The physical characteristics of compound L3 showed orange crystal, melting point range of 178-180°C with a molecular weight of 360.32 g/mol and percent yield of 79.50% while compound L4 was obtained as maroon crystal with a melting point range of 196-197°C, molecular weight of 390.325 g/mol and percent yield of 75.40% as shown in Table 3.

Table 2: R_f values of compound L3 and compound L4

Code of compounds	Solvent System	No. of spots	R_f Value	Solubility
L3	H: EA (3-7)	1	0.698	Ethyl acetate
L4	H: EA (3-7)	1	0.667	Ethyl acetate

Key: H: EA (Hexane & Ethyl acetate)

Table 3: Physical properties of synthesized hydrazones

Code	Mol. Formula	Structure	Mol. Wt. (g/mol)	Color	M.P. (°C)	Yield (%)
L3	C ₁₆ H ₁₆ N ₄ O ₆		360.32	Orange	178-180	79.50
L4	C ₁₇ H ₁₈ N ₄ O ₇		390.35	Maroon	196-197	75.40

Spectroscopic analysis of compound L3

The UV spectrum for compound L3 showed a maximum absorption at 378.00 nm. The IR spectrum of L3 showed the main absorption peaks for N-H and C=N stretching at 3302 cm⁻¹ and 1595 cm⁻¹ respectively.

The ¹H NMR (400 MHz, CDCl₃) spectrum revealed proton signals at δ 11.35 (s, 1H), 9.18 (s, 1H), 8.32 (d, J = 9.0 Hz, 1H), 8.07 (d, J = 9.4 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 6.71 – 6.44 (m, 2H), 3.89 (s, 6H), 2.45 (s, 3H). The ¹³C NMR (101 MHz, CDCl₃) spectrum reveals carbon signals at δ 162.37, 158.92, 154.89, 145.08, 137.85, 130.61, 129.97, 129.31, 123.59, 121.01, 116.78, 104.80, 98.81, 77.36, 77.04, 76.72, 55.57, 55.52, 17.34.

Spectroscopic analysis of compound L4

Compound L4 showed a maximum absorption at 380.00 nm in the UV spectrum. The IR spectrum for L4 showed the main absorption peaks for N-H and C=N stretching at 3302 cm⁻¹ and 1595 cm⁻¹ respectively.

The ¹H NMR (400 MHz, CDCl₃) spectrum revealed proton signals at δ 11.36 (s, 1H), 9.17 (s, 1H), 8.33 (dd, J = 9.6, 2.5 Hz, 1H), 8.06 (d, J = 9.6 Hz, 1H), 7.12 (s, 1H), 6.59 (s, 1H), 4.18 – 3.57 (m, 9H), 2.47 (s, 3H). The ¹³C NMR (101 MHz, CDCl₃) spectrum reveals carbon signals at δ 154.75, 152.71, 151.44, 144.99, 143.11, 137.88, 130.01, 129.35, 123.59, 119.27, 116.70, 112.99, 97.32, 77.37, 77.06, 76.74, 56.68, 56.37, 56.20, 17.39.

Antibacterial activity of the hydrazone derivatives

The mean zones of inhibition of the hydrazones against the test microorganisms are as presented in Table 4. The hydrazone derivatives exhibited a zone of inhibition ranging from 11 – 18 mm against the test organisms with compound L4 as the most active hydrazone with 18 mm. The minimum inhibitory concentration of the hydrazone derivatives ranges from 25 - 100 mg/mL as shown in Table 5 while the MBC is shown in Table 6.

Table 4: Zones of inhibition of compounds L3 and L4 against the test organisms

Compounds	Organisms	The concentration of compounds (mg/mL)				Cipro 20ug/ml
		100	50	25	12.5	
L3	EC	16	12	0	0	16
	ST	16	13	0	0	30
	SA	15	12	0	0	34
	SP	14	11	0	0	39
L4	EC	18	14	11	0	16
	ST	17	14	12	0	30
	SA	18	15	12	0	35
	SP	18	15	12	0	38

Keys: SA=*Staphylococcus aureus*, SP=*Streptococcus pneumoniae*, EC=*Escherichia coli* and ST= *Salmonella Typhi*.

Table 5: Minimum inhibitory concentration (MIC) of compounds L3 and L4 against the test organisms

Compounds	Organisms	The concentration of compounds in mg/mL						
		100	50	25	12.5	6.25	3.125	1.56
L3	EC	-	-	+	+	+	+	+
	ST	-	-	-	+	+	+	+
	SA	-	-	-	+	+	+	+
	SP	-	-	-	+	+	+	+
L4	EC	-	-	+	+	+	+	+
	ST	-	-	-	+	+	+	+
	SA	-	-	-	+	+	+	+
	SP	-	-	-	+	+	+	+

Keys: SA=*Staphylococcus aureus*, SP=*Streptococcus pneumoniae*, EC=*Escherichia coli* and ST= *Salmonella Typhi*.
(-) No visible bacterial growth and (+) Presence of bacteria growth

Table 6: Minimum bactericidal concentration (MBC) of compounds L3 and L4 against the test organisms

Compounds	Organisms	The concentration of compounds in mg/mL				
		100	50	25	12.5	6.25
L3	EC	-	+	+	+	+
	ST	-	+	+	+	+
	SA	-	+	+	+	+
	SP	-	+	+	+	+
L4	EC	-	-	+	+	+
	ST	-	+	+	+	+
	SA	-	-	+	+	+
	SP	-	-	+	+	+

Keys: SA=*Staphylococcus aureus*, SP=*Streptococcus pneumoniae*, EC=*Escherichia coli* and ST= *Salmonella Typhi*. (-) = No bacterial growth and (+) = Presence of bacteria growth

DISCUSSION

The hydrazones were synthesized through a Claisen-Schmidt condensation reaction using the corresponding hydrazine (2,4-dinitrophenyl hydrazine) and aldehyde or ketone in ethanol catalyzed medium [9]. The reaction of 2,4,5 trimethoxy acetophenone with 2,4-dinitrophenyl hydrazine gave compound L3 within 3 hours while the reaction of 2,4 dimethoxy acetophenone and 2,4-dinitrophenyl hydrazine gave compound L4 within 2 hours (Table 1). The difference in rate of reaction might be due to 2,4,5 trimethoxy acetophenone having an additional methoxy group at the 5-position,

which may cause steric hindrance. This could lead to a slightly slower reaction rate compared to 2,4 dimethoxy acetophenone, which has fewer substituents [10]. More also, the trimethoxy substituent may withdraw more electron density from the acetophenone ring making it less reactive. In contrast, the dimethoxy substituent may have a lesser effect on electron density, allowing the reaction to proceed faster [11].

Compound L4 gave maroon color crystals with a percent yield of 75.40% while compound L3 gave an orange colour crystal with percent yield of 79.50% as shown in Table 3. All the compounds were found to be soluble in ethyl acetate. The Rf values of the compounds

were 0.7 using hexane: ethyl acetate as the solvent system (Table 2). The difference in percent yield could be due to the solubility of the reactants in the reaction solvent and trimethoxy substituent may participate in competing side reactions, reducing the yield of the desired product [12].

The UV of compound L3 showed a maximum wavelength of absorbance at 378.00 nm indicating the presence of chromophores. The FTIR spectra showed absorption bands at 3302 cm^{-1} , 1595 cm^{-1} , and 1121 cm^{-1} indicating the presence of N-H, imine group ($\text{C}=\text{N}$), and N-N bonds, respectively. In addition, the absorption bands around 3116-2844 cm^{-1} , indicated the presence of an aromatic ring while the absorption band at 1430 cm^{-1} , 1263 cm^{-1} , 1595 cm^{-1} and 1498 cm^{-1} indicated the presence of C-C, C-O-C, and nitro group substituent ($\text{N}=\text{O}$ and N-O) respectively [13]. The ^1H NMR spectrum reveal proton signals of NH, CH_3 and methoxy proton at chemical shift of 11.35 (s, 1H), 3.89 (s, 6H) and 2.45 (s, 3H) and ^{13}C NMR spectrum showed 15 signals indicating 15 carbon atoms. The absence of ketonic functional group on FT-IR and NH_2 frequency peak on the FT-IR and ^1H NMR spectrum confirms the formation of the hydrazone compound.

Compound L4 showed a maximum wavelength of absorbance at 380.00 nm. The FTIR spectra of the compound showed absorption bands at 3298 cm^{-1} , 1613 cm^{-1} , and 1129 cm^{-1} which indicated the presence of N-H, imine group ($\text{C}=\text{N}$), and N-N bonds, respectively. In addition, the absorption bands at 3119-2840 cm^{-1} , indicated the presence of an aromatic ring and the absorption band at 1420 cm^{-1} , 1207 cm^{-1} , 1584 cm^{-1} and 1494 cm^{-1} indicated the presence of C-C, C-O-C, and nitro group substituent ($\text{N}=\text{O}$ and N-O) respectively. The ^1H NMR spectrum reveal proton signals of NH, CH_3 and methoxy proton at chemical shift of 11.36 (s, 1H), 4.18 – 3.57 (m, 9H) and 2.47 (s, 3H).

The novel hydrazone derivatives (L3 and L4) were tested for their antibacterial activity against gram-positive bacterial strains: *Staphylococcus aureus* and *Streptococcus pneumoniae*, and two gram-negative bacterial strains: *Escherichia coli*, and *Salmonella* Typhi. Ciprofloxacin was used as the positive standard. The values for zones of inhibition were reported in Table 4.

According to the results of the antibacterial screening (Table 4), compounds L3 and L4 were found to be active against all the tested organisms with a maximum range of 18 mm zone of inhibition. The compounds showed moderate activity compared with ciprofloxacin which 16-39 mm zones of inhibition across the test organisms (Table 4). The minimal inhibitory concentrations of L3 and L4 were in the range of 100–25 mg/mL (Table 5).

Compound L3 exhibited broad-spectrum activity, demonstrating efficacy against both Gram-positive (*Staphylococcus aureus*, *Streptococcus pneumoniae*) and Gram-negative (*Escherichia coli*, *Salmonella* Typhi) bacteria. Notably, its MBC values were consistently high (100 mg/mL) across all tested bacteria. In contrast, MIC values varied; 25 mg/mL for *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Salmonella* Typhi, and 50 mg/mL for *Escherichia coli*. The MBC/MIC ratio ranged from 2 to 4 for all the test organisms, indicating potential bactericidal activity [14].

Compound L4 also displayed broad-spectrum activity, with MBC values of 50 mg/mL against *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Escherichia coli*, and 100 mg/mL against *Salmonella* Typhi while the MIC values were similar to those recorded for L3. The MBC/MIC ratio was 2 for most bacteria, suggesting improved efficacy or reduced toxicity [14].

Comparing L3 and L4 reveals distinct differences. L4 has lower MBC values (50 mg/mL) for three bacteria indicating superior bactericidal activity, particularly against resilient infections caused by these microorganisms. Conversely, L3 consistently had higher MBC values (100 mg/mL).

CONCLUSION

The emergence of antimicrobial resistance necessitates the discovery of novel antimicrobial agents. The hydrazone compounds were successfully synthesized. Compounds L3 and L4 have shown promising antibacterial activity against infections caused by the tested organisms. This study shows low minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli* and *Salmonella* Typhi.

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