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Synthesis and In-Vitro Antibacterial Studies of Two New Hydrazone Derivatives †

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Abstract: Throughout history to the present day, infectious diseases have been a persistent global threat, causing significant harm to public health and economic stability. To address these challenges, the development of novel antimicrobial drugs is crucial. Hydrazones have gained significant attention in scientific literature as promising candidates for developing new antimicrobial drugs. Two new hydrazone (H3 and H4) incorporating moieties that are known to enhance the antimicrobial activity were synthesized. Method: The hydrazone derivatives were synthesized through a condensation reaction of substituted acetophenone and nitro phenyl hydrazine. The compounds were characterized by their melting points and spectral analyses, including FT-IR, ^{1}H NMR, ^{13}C NMR, and 2D NMR. Their antibacterial effects on *Escherichia coli* and *Staphylococcus aureus*, were assessed in-vitro using the agar diffusion and broth dilution method. Result: in-vitro testing demonstrated good activity against tested organisms, particularly gram-positive bacteria. At a concentration of 50 mg/mL, H3 produces zone of inhibition (19 mm) comparable to the standard ciprofloxacin (20 mm) at 0.05 mg/mL. P5 produces less inhibition in comparison to H3 and H4 producing minimum inhibition (12 mm) at highest concentration of 50 mg/mL. Only H3 was able to kill both *Staphylococcus aureus* and *Escherichia coli* at a concentration of 50 mg/mL. In all cases, H3 was found to be the most effective with optimum bactericidal and bacteriostatic activity against staphylococcus aureus and *Escherichia coli*. Conclusion: All the synthesized compounds were proven to possess a promising antibacterial activity *in-vitro* against tested organism.

Keywords: synthesis; antibacterial; in-vitro; hydrazones

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1. Introduction

Infectious diseases significantly impact human health, causing widespread death and suffering, particularly among vulnerable groups [4]. In 2017, infections were responsible for over 20% of global deaths, including more than 10 million deaths linked to sepsis [24]. Antimicrobials are crucial for treating infections in humans, animals, and plants [32]. Antimicrobial resistance is a growing concern, making infections harder to manage and increasing the risk of severe illness and death [6,16,25,29]. Researchers are exploring novel antimicrobial agents, such as plant and microbial extracts, essential oils, and synthetic molecules, to combat resistant strains and address healthcare system limitations [1]. Over the past two decades, organic compounds containing hydrazone have shown promise due to their antimicrobial properties and diverse biological activities [33]. Hydrazones,

formed by reacting hydrazine with carbonyl compounds [3,8], exhibit a wide range of biological effects including anticancer [5,14,17], antibacterial [2,18–21], anti-inflammatory [2,12], antiviral [5,27], antiprotozoal [7], *antitubercular* [2], and antidepressant activities [30]. Their antimicrobial activity makes them potential candidates for new antimicrobial agents against pathogenic bacteria and fungi [22]. The hydrazone function is also critical in various antibiotic drugs [22]. Our research yielded two hydrazones derivative incorporating functional moieties (NO_2 and OCH_3) that have been documented to enhance the antimicrobial activity of hydrazones [28]. Notably, the two hydrazone compounds H3 and H4 (Figure 1) exhibit distinct substitution patterns on the ring. It is noteworthy that all the synthesized compounds are entirely novel, with no prior mention in existing literature or chemical databases (www.scifinder.com, (2021).

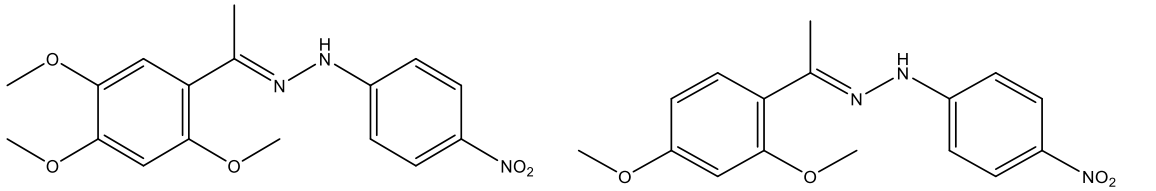

Figure 1. chemical structure of H3 and H4.

2. Methods

2.1. Synthesis and Characterization

2.1.1. Reagents and Equipments Used for the Synthesis

All reagents were purchased from sigma-Aldrich, Germany. All the starting reagents and solvents used for the experiments were of analytical grade and were used without further purification. IR spectra were recorded on 400 MHz Agilent FT-IR spectrometer. ^{1}H NMR spectra were recorded in chloroform on 500 MHz Bruker NMR spectrometer using TMS as reference.

2.1.2. General Procedure for the Synthesis of Hydrazone Derivatives (H3 and H4)

The synthetic pathways adopted to prepare the new hydrazone compounds are depicted in Figure 2. Compounds H3 and H4 Were obtained via the reaction of an equimolar amount of 2,4,5-trimethoxyacetophenone/2,4-dimethoxyacetophenone and 4-nitrophenylhydrazine dissolved in 30 mL of ethanol in a round bottom flask to which 5 mL of 40% NaOH was added. The reaction mixture was refluxed for 4hour, while being continuously stirred using magnetic stirrer. The product was filtered and air-dried. The resulting residue was then purified by recrystallization in an ethanol to give the pure product.

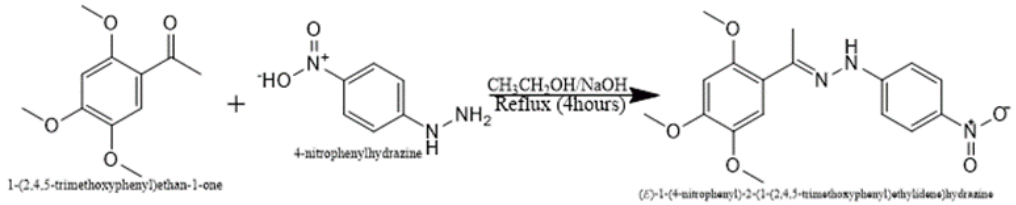

Figure 2. Reaction Scheme for Hydrazones.

2.2. Evaluation of Antibacterial Activity

The in vitro antibacterial activity of the test compounds H3 and H4 was assessed against 24 h cultures of clinical isolates of *Escherichia coli* and *Staphylococcus aureus* obtained from the department of microbiology, Ahmadu Bello University Teaching Hospital, Shika, Nigeria. The antibiotic ciprofloxacin was used as standard. Minimum inhibitory

concentration (MIC) and minimum bactericidal concentration (MBC) of the synthesized compounds were determined by Employing Agar Diffusion Method broth dilution method.

2.2.1. Procedure for Agar Diffusion Method

To determine the MICs of the test compounds against *S. aureus* and *E. coli*, agar diffusion method as described by Ahmad et al., 2016 was employed. Mueller Hinton agar (MHA) was used as the growth medium and was prepared according to the manufacture's instruction. The freshly prepared liquid agar medium (20 mL) was poured into each petri dish. About 0.1 mL of standard inoculum of test microorganisms were evenly spread unto the surface of the solid medium using a sterile swab. The seeded Petri dishes were dried in an incubator at 37 °C for duration of 1 h. Cups of approximately 6 mm diameter were made in the centre of the petri dishes using sterile cork borer and were labelled. A prepared solutions (0.1 mL) of the test compounds (in a mixture of dimethylsulfoxide and methanol) were added to each cup in petri dishes and were kept aside in an aseptic area for 1 h to allow diffusion of the drug/sample, followed by incubation at 37 °C for 18 h. Each plate of the medium was observed for the zone of inhibition.

2.2.2. Procedure for Broth Dilution Method

To determine the MBCs of the test compounds against *S.aureus* and *E.coli*, Mueller Hinton broth (MHB) was prepared according to manufacturer's instructions and was sterilized at 121 °C for 15 min and the broth was allowed to cool. The broth was inoculated with a standardized inoculum of the test microorganisms. Dilution of the test microorganism was done using normal saline until the turbidity matched that of McFarland standard number 0.5 by visual comparison; at this point the test microbe has a concentration of about 5×10^5 cfu/mL. Two-fold serial dilution of the test compounds each, was made to obtain the concentration of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.56 mg/mL, 0.78 mg/mL, 0.39 mg/mL, 0.2 mg/mL, 0.1 mg/mL, for the test compounds. Each concentration of the test compounds was inoculated with a standardized inoculum of the test microorganisms and then incubated at 37 °C for 20 h after which each test tube was observed for turbidity (growth). The lowest concentration in the sterile broth which shows no turbidity was recorded as the minimum inhibitory concentration. The minimum bactericidal concentrations (MBCs) were determined by sub culturing broth dilutions that inhibited the growth of tested organisms. The broth dilutions were streaked onto agar and incubated for 48hours. The lowest concentration in the sterile broth which prevented the growth of the organism was recorded as the minimum bactericidal concentration**.**

3. Results and Discussions

3.1. Synthesis and Characterization

3.1.1. (E)-1-(4-nitrophenyl)-2-(1-(2,4,5-trimethoxyphenyl)ethylidene) Hydrazine (H3).

Yield: 90% obtained as deep red powder; MP 148.5–149.8 °C; FT-IR (KBr) vMax/cm_1 3300 1591(C=N), 3108(C-H atom), 2840 (C-H Aliph), 3317(N-H); ^{1}H δ(ppm) 2.29 (s) (**C2**), 6.55(s) (**C3′**), 7.09(s) (**C6′**), 7.14(d) (**C2′′**), 8.17(d) (**C3′′**), 81.5(d) (**C5′′**), 7.12(s) (**C6′′**); ^{13}C NMR (CCl_4) δ (ppm) 150 ppm–164 pm (**1C**=N 16.36(**C2**), 120.66 (**C1′**), 152.44(**C2′**), 98.22(**C3′**), 151.16(**C4′**), 150.49 (**C5′**), 113.24 (**C6′**) 144.08 (**C1′′**), 112.33 (**C2′′**), 126.36 (**C3′′**), 139.79 (**C4′′**), 126.36 (**C5′′**), 112.33 (**C6′′**)

3.1.2. (E)-1-(4-nitrophenyl)-2-(1-(2,4,-dimethoxyphenyl)ethylidene) Hydrazine (H3)

Yield: 92% obtained as orange crystals; MP 149.5–151.2 °C; FT-IR (KBr) vMax/cm_1 3300 1599(C=N), 3004(C-H atom), 2832 (C-H Aliph), 3309(N-H); ^{1}H δ(ppm) 2.26(s) (**C2**), 6.49(s) (**C3′**), 6.54(d) (**C5′**), 6.74(d) (**C6′**), 7.14(d) (**C2′′**), 8.17(d) (**C3′′**), 81.5(d) (**C5′′**), 7.12(d) (**C6′′**); ^{13}C NMR (CCl_4) δ (ppm) 164.78 (**1C**=N 16.38 (**C2**), 104.97 (**C1′**), 157.39 (**C2′**), 99.62

(**C3′**), 162.29 (**C4′**), 105.77 (**C5′**), 129.46 (**C6′**), 150.25 (**C1″**), 111.54 (**C2″**), 139.77 (**C3″**), 139.79 (**C4″**), 126.38 (**C5″**), 111.54 (**C6″**).

The synthesis of hydrazones produced pure compounds in excellent yield. The use of strong base (NAOH) as the catalyst for hydrazones synthesis promotes the deprotonation of the hydrazine derivative, creating a more reactive nucleophile. This enhances the attack on carbonyl carbon, facilitating hydrazone formation. The increased nucleophilicity in basic conditions might have contributed to the high reaction yield of the hydrazones

The ^{1}H NMR showed the resonances due to aromatic protons appeared in the region of 6.49–8.2 ppm for H3 and H4 respectively. For H3, two doublets at 7.14/7.18 ppm and 8.14/8.17 ppm were assigned to protons on carbon 2″/6″ and carbon 3″/5″ respectively. These protons (2″/6 and 3″/5″) appeared on the same/or almost same chemical shift because they belong to the same chemical environment. A distinguishing feature from the ^{1}H NMR spectra of H3 and H4 is the appearance of proton on carbon 6“as singlet and doublet in H3 and H4 respectively. Other noticeable resonances are the methoxy proton as singlet at the region of 3.83–4.25 ppm integrating into nine and six protons for H3 and H4 respectively. The rest of the protons appear in their expected regions. Additional support for the structures comes from the ^{13}C NMR spectra of H3 and H4 which showed resonance in the range 150–164 ppm due to the carbons (C1) of the azomethine(C=N) are evidence that the hydrazones have been formed [11,14]. The two-dimensional (COSY, HSQC and HMBC) NMR spectroscopic data confirm the tentative structural assignment that was made using ^{1}H and ^{13}C NMR for H3 and H4. The correlation of each carbon atom and the attached hydrogen atom (s) was unambiguously established using HSQC and long-range correlation between protons and carbon was established using HMBC, which led to linking of sub-structural fragments. Protons at C3″–C5″, C2″–C6′ and C5″–C6″ showed COSY correlation for both H3 and H4.

3.2. Antibacterial Studies

The antibacterial studies revealed that the H3 and H4 were able to hinder the growth of *Staphylococcus aureus* and *Escherichia coli.* At a concentration of 50 mg/mL, H3 produces zone of inhibition comparable to the standard Ciprofloxacin at 0.02 mg/mL (Table 1), however only H3 was able to kill both *Staphylococcus aureu s*and *Escherichia coli* at a concentration of 50 mg/mL. (Table 3) In all cases, H3 was found to be the most effective antibacterial agent synthesized with optimum bactericidal and bacteriostatic activity against *Staphylococcusaureus* and *Escherichia coli.* The optimum antimicrobial activity produces by hydrazones may be attributed to the presence of electron withdrawing group like NO_2, OCH_3 on the aromatic ring (Kamboj, 2014; Khattab et al., 2019; Sharma et al., 2020) and the superior activity of H3 over H4 could be due to the presence of three OCH_3 substitution as compared to two of H4.

Table 1. Zone of Inhibition of Hydrazones.

Organism	Compound	Concentrations of Compounds (mg/mL)				Cipro (mg/mL)
		50	**25**	**12.5**	**6.25**	**0.05**
S. aureus	H3	19	15	12	11	20
	H4	17	14	11	0	20
E. coli	H3	18	13	11	0	24
	H4	16	14	12	0	24

Table 2. Minimum inhibitory concentration of H3 and H4 against *E. coli* and *S. aureus.*

Organism	Compound	Concentrations (mg/mL)									
		50	**25**	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**	**0.39**	**0.2**	**0.1**
S. aureus	H3	−	−	+	+	+	+	+	+	+	+

	H4	−	−	+	+	+	+	+	+	+	+
E. coli	H3	−	−	+	+	+	+	+	+	+	+
	H4	−	−	+	+	+	+	+	+	+	+

+ = growth of the test organism observed and − = no growth of the test organism observed.

Table 3. Minimum inhibitory concentration of synthesized compounds against *E. coli* and *S. aureus*.

Organism	Compound	Concentrations (mg/mL)									
		50	**25**	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**	**0.39**	**0.2**	**0.1**
S. aureus	H3	−	+	+	+	+	+	+	+	+	+
	H4	+	+	+	+	+	+	+	+	+	+
E. coli	H3	−	+	+	+	+	+	+	+	+	+
	H4	+	+	+	+	+	+	+	+	+	+

4. Conclusions

The phenylhydrazones [(E)-1-(1-(2,4,5-trimethoxyphenyl)ethylidene)-2-(4-nitrophenyl) hydrazine] and [(E)-1-(1-(2,4-dimethoxyphenyl)ethylidene)-2-(4-nitrophenyl)hydrazine] synthesized are promising candidates as antimicrobial agents.

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Conflicts of Interest:

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