Antimicrobial activities of hydrazones with 2,4-dichloro moiety.

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Abstract

The goal of this research was to come up with novel antibacterial agents. Two hydrazones with 2,4-dichloro moiety were synthesized by conventional synthetic methods with good yields. The success of the synthesis was confirmed by structure determination techniques; FITR and NMR analyses. The synthesized hydrazones were evaluated for antimicrobial activity using strains of bacterial ad fungi. The two hydrazones demonstrated significant antibacterial and antifungal activities which were comparable to those of ciprofloxacin and fluconazole respectively. Specifically, compound 3b with a para nitro group on its aniline fragment indicated a broader spectrum of activity compared to compound 3a.

Additionally, the two hydrazones were active against bacterial strains; *Staphylococcus aureus*, *Campylobacter fetus Proteus, mirabilis*, and methicillin-resistant *Staphylococcus aureus* which were resistant to ciprofloxacin with ZI between 25-31 mm and MIC of 12.5 µg/ml for *Proteus mirabilis* and 25 µg/ml for others accordingly. Amazingly, the two hydrazones demonstrated bactericidal and fungicidal activity between 25 µg/ml to 100 µg/ml against all the sensitive bacterial and fungi strains. The two hydrazones with 2,4-dichloro moiety have been identified as leads and are recommended for further *in-vivo* efficacy studies.

Keywords: 2,4-dichloro hydrazone, antimicrobial activity, p-nitrophenyl hydrazones, Synthesis.

1. INTRODUCTION

Infectious diseases have afflicted humans from the dawn of time, wreaking havoc on communities, causing economic losses, and steadily reducing empires' workforces. Infectious diseases account for 63 percent of all pediatric mortality and 48 percent of deaths in children under the age of five [2]. Many of these deaths are the result of pandemic infectious illnesses including meningococcal disease, measles, SARS-COV2, and others [1]. Acute respiratory infections, acquired immune deficiency syndrome (AIDS), malaria, diarrheal illnesses, measles, and tuberculosis (TB) are estimated to account for more than 85 percent of infection-related fatalities globally, according to the World Health Organization (WHO) [2].

However, with the introduction of many new drugs, there has been progressed in the battle against infectious diseases. Antibiotic discovery and development have long been recognized as one of the most important medical breakthroughs of the twentieth century. Millions of lives have been saved thanks to antibiotics, which have permitted crucial medical treatments such as surgery and cancer chemotherapy [3]. Antimicrobial agents have shifted the paradigm not just in the management of infectious illnesses,

but also in humanity's existential. Antimicrobials have decreased morbidity and increased survival in patients with bacterial infections, and they are still needed to treat a variety of bacterial illnesses [2]. Despite advances in the treatment of many communicable illnesses, bacterial infections continue to be a leading source of morbidity and death, especially in the developing world.

Antimicrobial chemotherapy has advanced significantly, leading to an overly optimistic expectation that infectious diseases would be eliminated soon. In actuality, however, developing and re-emerging pathogenic diseases have left humans vulnerable to infection. Infections with drug-resistant strains remain a significant and difficult-to-solve issue in clinical research [4]. Emerging bacterial resistance is becoming a significant difficulty in the treatment of a variety of illnesses. These new illnesses, as well as the re-emergence of old ones, are on the rise. The rise of Antimicrobial Resistance (AMR), which renders antimicrobial agents less effective or useless, poses a danger to their effectiveness. These infections have limited treatment options, particularly in debilitated and immune-compromised individuals [5]. As a result, medicinal chemists will continue to have a challenging and never-ending quest in the discovery of novel antimicrobial drugs.

Hydrazones are an important family of organic compounds with the formula R1-NHN=CH-R2 that can be used in the development of novel drugs. Various biological properties of hydrazone analogs have been documented, including analgesic, anti-inflammatory, antihypertensive, anticonvulsant, antibacterial, anti-tubercular, anticancer, antimalarial, and antiproliferative [6].

Using the micro broth dilution technique, Yurttaş *et al*. produced and tested a variety of thiazole hydrazones for antibacterial and antifungal activity against twelve distinct species. As standard reference medications, ketoconazole and chloramphenicol were utilized. All the compounds were effective against *Staphylococcus aureus* and *Enterococcus faecalis* [7]. The 4-Chloro-N-(2-hydrazinocarbonyl-phenyl)-

benzamide intermediate was used to prepare some new hydrazone derivatives. The synthesized hydrazones were screened against eleven standard strains of bacterial and fungi using tetracyclin as the reference drug. The synthesized hydrazones demonstrated good to excellent activities while *P. aeruginosa, Serratia, S. aureus, S. mutans, and E. feudalist* were particularly more susceptible to the compounds. *Genus Serratia* was the susceptible strain inhibited by nine of the synthesized hydrazones [8].

Using the microplate dilution method, Pham *et al*. synthesized hydrazide-hydrazones with a 1-adamantane carbonyl moiety and tested their *in-vitro* growth inhibition against standard bacteria strains such as Pseudomonas aeruginosa, *Escherichia coli*, *Enterococcus faecalis*, *Salmonella enterica*, *Bacillus cereus,* and *Staphylococcus aureus* and a fungi strain *Candida albicans*. The standard medications in the trial were the antibiotic Streptomycin and the antifungal agent Cycloheximide. The synthesized compounds have activities that were equivalent to those of established medicines [9].

Here, we report the antibacterial and antifungal activity of our previously synthesized compounds, two hydrazones with 2,4-dichloro moiety [10].

2. MATERIALS AND METHODS

2.1 Chemistry

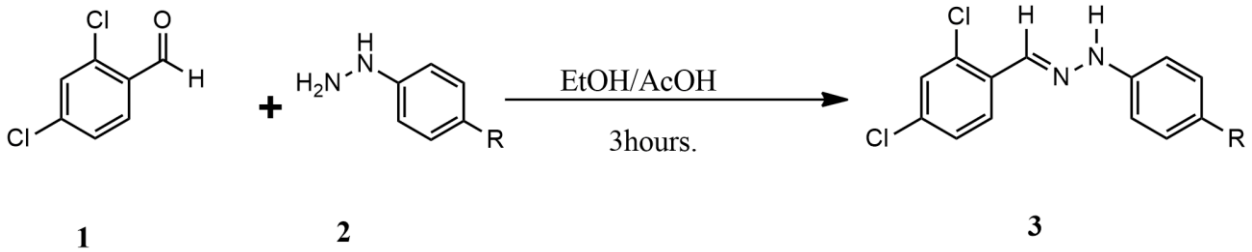
All chemicals which were were purchased from Sigma Aldrich, St. Louis, MO USA, and were utilized without additional purification. The melting points were determined using the Electrothermal Engineering LTD 9100 instrument. The ^{1}H and ^{13}C NMR spectra were collected using a Brucker AMX 400 MHz spectrometer running at 400 MHz and 100 MHz, respectively, while the FTIR spectra were recorded using Agilent technologies spectrophotometer model 543. Chemical shifts (*d*) are expressed in parts per million and are calculated using the NMR solvent peak as a reference.

Synthesis of phenyl hydrazone 3a

Equimolar quantities of 2,4-dichloro benzaldehyde **1** (20mmol) and phenylhydrazine **2a** (20mmol) were mixed in 30ml of ethanol at room temperature. The mixture was continuously stirred for 3hrs and the progress of the reaction was monitored by thin layer chromatography (TLC). The white crystalline solid formed was filtered off, dried, and then recrystallized from pet ether.

Synthesis of p-nitrophenyl hydrazone 3b

Equimolar quantities of p-nitrophenyl hydrazine **2b** (5.09 mmol) and each of the 2,4-dichloro benzaldehyde **1** (5.09 mmol) were grounded in a universal tube with the aid of a glass rod for 5 minutes. The reactions were carried out under room conditions. The progress of the reaction was monitored by TLC. On completion, the mixture product was transferred into a beaker and 20 ml of cold 2 M hydrochloric acid was added and stirred to scavenge the possible unreacted p-nitrophenyl hydrazine **2**. The product precipitate was filtered off, dried, and subsequently washed with 30 ml of cold distilled water and 20 ml of cold 95% ethanol step-wisely to afford colored powdered product **3b** in high yield[10].



1 **2** **3**

Scheme 1: Synthesis of dichloro hydrazones.

R: 3a = H, 3b = NO_2

2.2 Anti-microbial activity

The antimicrobial property of the compounds was tested using pathogenic microorganisms acquired from the Ahmadu Bello University teaching hospital in Zaria's department of medical microbiology.

2.2.1 The antimicrobial screening.

0.001mg and 0.002mg of compounds 3a and 3b were prepared by dissolving 10mg in 10mml of DMSO, respectively, to obtain concentrations of 100g/ml and 200g/ml for each compound. The method for screening the chemical was the diffusion method. The microorganisms were grown on Mueller Hinton agar as the growth medium. The medium was sterilized at 121°C for 15 minutes, put onto sterile Petri plates, and allowed to cool and solidify per the manufacturer's instructions.

The sterilized medium was seeded with 0.1ml of the test microbe's standard inoculum, which was dispersed evenly across the medium's surface using a sterile brush. Using a standard cork borer of 6mm in diameter as well as cut at the center of each inoculated medium. Separately, 0.1ml of compound solution with a concentration of 100g/ml for 3a and 200g/ml for 3b was added to the well on the infected medium. After a 2-hour incubation period at 37°C, the plates of media were examined for zones of inhibition of growth, which were determined with a transparent ruler, and the result was recorded in millimeters.

2.2.2 Minimum Inhibitory Concentration (MIC).

The minimum inhibition concentration of the compound was determined using the broth dilution method.

The Mueller Hinton broth was prepared, 10ml was dispensed into test tubes, and the broth was sterilized at 121°C for 15 minutes before cooling. The solution was calculated using MC-turbidity Farland's

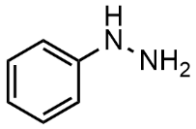
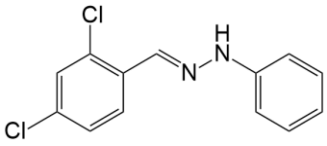
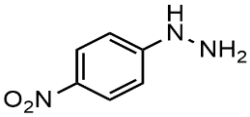
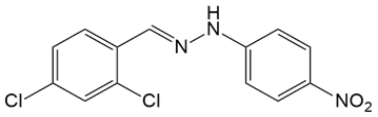
standard scale number of 0.5. The test microbe was inoculated and incubated at 37°C for 6 hours after 10ml of normal saline was dispensed into the sterile test tube. The microbe was diluted in normal saline until the turbidity matched the MC-scale Farland's by visual comparison; at this point, the test microbe had a concentration of 1.510 8cfu/ml. The compounds were serially diluted twice in sterile broth to generate concentrations of 100g/ml, 50g/ml, 25g/ml, 12.5g/ml, and 6.25g/ml. To obtain the starting concentration, 0.001 mg of the compound was dissolved in 10 mL of sterile broth. After obtaining the various concentrations of the compounds in the sterile broth, 0.1ml of the test microbe was added to normal saline and inoculated into the various concentrations, incubation was carried out at 37°C for 24 hours, and the test tubes of the broth were examined for turbidity (growth), and the lowest concentration of the compounds in the sterile broth that showed no turbidity was recorded as the minimum inhibition concentration.

2.2.3 Minimum Bactericidal/fungicidal Concentration (MBC/MFC).

MBC and MFC were carried out to determine whether the test microbes were killed or only their growth was inhibited. Mueller Hinton agar was prepared and sterilized at 121°C for 15 minutes before being put into a sterile petri dish to cool and solidify. The contents of the MIC in serial dilutions were then subcultured onto the prepared medium, incubated at 37°C for 24 hours, and then colony growth was evaluated on the plates of the medium. MBC and MFC were the plates with the lowest concentration of the drug without colony growth.

3. RESULTS

Table 1: Synthesis of compounds **3a-b**.

Entry	Hydrazine	Product	Time (hrs)	Yield (%)	Mp (ºC)
3a			3	68.80	123-124
3b [10]			3	67.90	226-228

(*E)*-1-(2,4-dichlorobenzylidene)-2-phenylhydrazine 3a. Yield 68.80%, Crystalline white solid, mp 123-124 ºC. FTIR (KBr, cm-1): 3302 (N-H), 3030 (C-H_{imine}), 1572 (C=N), 1517 (C=$C_{aromatic}$), 1252 (C-N), 1047 (C-Cl). H^1 NMR spectrum (400 MHz, DMSO-*d*6) δ, ppm: H^1 NMR spectrum (400 MHz, DMSO-*d*6) δ, ppm: 7.01 d (1H_{arom}, *J* = 7.1 Hz), 7.16 d (2H_{arom}, 8.2 Hz), 7.22 d (2H_{arom}, 7.8 Hz), 7.47 (H_{arom}, 8.5 Hz), 7.63 (H_{arom}, 1.7 Hz), 8.09 (H_{arom}, 8.4), 8.19 (H_{imine}), 11.23 (1H, NH). ^{13}C NMR spectrum (101 MHz, DMSO-*d*6), δ, ppm: 112.30, 126.11, 126.54, 127.94, 128.62, 129.56, 131.89, 133.18, 134.34, 136.91, 139.89.

(*E)*-1-(2,4-dichlorobenzylidene)-2-(4-nitrophenyl)hydrazine 3b [10]. Yield 67.90%, chrome yellow powder, mp 226-228 °C. IR (KBr, cm^{-1}): 3265 (N-H), 3078 (C-H_{imine}), 1587 (C=N), 1498 (NO_2), 1461 (C=$C_{aromatic}$), 1300 (C-$N_{aniline}$), 1043 (C-Cl). H^1 NMR spectrum (400 MHz, DMSO-*d*6) δ, ppm: 7.18 d (2H_{arom}, *J* = 8.2 Hz), 7.47 d (1H_{arom}, *J* = 8.5 Hz), 7.65 d (1H_{arom}, *J* = 1.8 Hz), 8.05 d (1H_{arom}, *J* = 8.6 Hz), 8.13 d (2H_{arom}, *J* = 9.0 Hz), 8.29 s (1H_{imine}), 11.61 s (1H, NH). ^{13}C NMR spectrum (101 MHz, DMSO-*d*6), δ, ppm: 112.10, 126.54, 127.98, 128.29, 129.71, 131.39, 133.16, 134.36, 136.58, 139.43, 150.43.

Table 2: Antimicrobial activities of the synthesized dichloro hydrazones.

Test organism	3a	3b	Ciprofluxacin	Fluconazole
Methicilin resistant Staph. aureus	R	S	R	R
Escherichia coli	S	S	S	R
Vamcomycin resistant enterococci	S	S	S	R
Staphylococcus aureus	S	R	R	R
Klebsiella pneumoniea	---	R	S	R
Helicobacter pylori	S	---	S	R
Salmonella typhi	---	R	S	R
Proteus mirabilis	---	S	R	R
Listeria monocytogenes	R	---	S	R
Streptococcus pyogenes	---	S	S	R
Campylobacter fetus	S	---	R	R
Proteus vulgaris	R	---	R	R
Pseudomonas	R	---	R	R

fluorescence				
Candida stellatoidea	R	S	R	S
Candida albican	---	S	R	S
Candida tropicalis	S	---	R	S
Candida krusai	---	R	R	S

Keywords: S= Susceptible, R= Resistant.

Table 3: Zones of inhibition (mm) of the synthesized dichloro hydrazones against the test micro-organism.

Test organism	3a	3b	Ciprofluxacin	Fluconazole
Methicilin resistant Staph. aureus	0	27	0	0
Vamcomycin resistant enterococci	27	30	35	0
Staphylococcus aureus	25	0	0	0
Escherichia coli	29	29	37	0
Streptococcus pyogenes	---	26	32	0
Klebsiella pneumoniea	---	0	34	0

Salmonella typhi	---	0	40	0
Proteus mirabilis	---	31	0	0
Listeria monocytogenes	0	---	32	0
Helicobacter pylori	25	---	34	0
Campylobacter fetus	26	---	0	0
Proteus vulgaris	0	---	0	0
Pseudomonas fluorescence	0	---	0	0
Candida albican	---	25	0	34
Candida krusei	---	0	0	32
Candida stellatoidea	0	28	0	30
Candida tropicalis	27	---	0	32

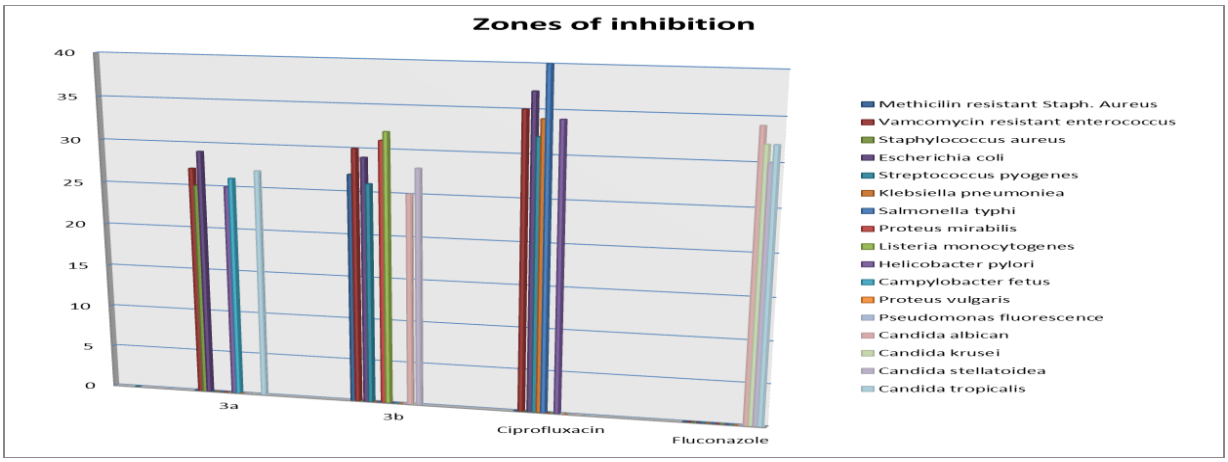


Figure 1: Visual representation of zones of inhibition (ZI).

Table 4: Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration of dichloro hydrazones.

	MIC		MBC/MFC	
Test organism	3a	3b	3a	3b
Methicilin resistant Staph. aureus	---	25µg/ml	---	100µg/ml
Vamcomycin resistant enterococci	12.5µg/ml	25µg/ml	25µg/ml	50µg/ml
Staphylococcus aureus	25µg/ml	---	50µg/ml	---
Escherichia coli	12.5 µg/ml	25µg/ml	25µg/ml	50µg/ml
Streptococcus pyogenes	---	50µg/ml	---	100µg/ml
Klebsiella pneumoniea	---	---	---	---
Salmonella typhi	---	---	---	---
Proteus mirabilis	---	12.5µg/ml	25µg/ml	---
Listeria monocytogenes	---	----	---	---
Helicobacter pylori	25µg/ml	---	50µg/ml	---
Campylobacter fetus	25µg/ml	---	50µg/ml	---

245	Proteus vulgaris	---	---	---	---
246	Pseudomonas	---	---	---	---
247	fluorescence				
248	Candida albican	---	50µg/ml	---	100µg/ml
249	Candida krusei	---	---	---	---
250	Candida stellatoidea	---	25µg/ml	---	50µg/ml
251	Candida tropicalis	25µg/ml	---	25µg/ml	---

252 **3. DISCUSSION**

253 **3.1 Chemistry**

254 Detailed synthesis and spectroscopic study of compound **3b** have previously been reported by Babalola
255 *et al*, [10]. The synthesis of hydrazones with 2,4-dichloro moiety was performed by the condensation of
256 2,4-dichlorobenzaldehyde with aromatic hydrazine as illustrated in scheme 1 above. Although, both
257 reactions were allowed for three hours. The reaction of the aldehyde with phenylhydrazine was the
258 fastest. This reaction afforded white crystalline solids after 20 minutes. However, the reaction with 4-
259 nitrophylhydrazine took an hour to form the chrome yellow powder product. The difference in the rate
260 of reaction and yield in table 1 may be due to the basic character of the hydrazines. The presence of the
261 nitro group also confers a high melting point in table 1 in addition to the reduced basic character of the
262 corresponding hydrazine. The FTIR, ^{1}H-NMR, ^{13}C-NMR analyses were employed in the structure
263 determination of the synthesized dichloro hydrazones. FTIR absorption signals at 3302 cm^{-1}, 3030 cm^{-1},
264 and 1572 cm^{-1} are characteristics of N-H, imine C-H, and C=N stretching. The singlet proton peaks at
265 8.19 ppm and 11.23 ppm, and the C-13 peak at 136.91 ppm confirmed the synthesis of the hydrazone

functional group in compound 3a. Al so, for compound 3b absorption signals 3265 cm^{-1}, 3078 cm^{-1}, 1587 $cm^{-1,}$ and 1498 cm^{-1} correspond with N-H, imine C-H, C=N, and NO_2 stretchings. The ^{1}H-NMR singlet peaks at 8.29 ppm and 11.61 ppm, and the ^{13}C-NMR signal at 136.58 ppm confirmed the synthesis of compound 3b. ^{13}C-NMR peak at 150.43 ppm also confirmed the presence of the para NO_2 group.

3.2 Antimicrobial activity

Compounds 3a and 3b demonstrated remarkable antimicrobial activity as indicated in table 2. Vancomycin-resistant *enterococci* and *Escherichia coli* are the only strains that are susceptible to both compounds. *Staphylococcus aureus* is resistant to compound 3b while sensitive to compound 3a. Likewise, Methicilin resistant *Staphylococcus aureus* and *Candida stellatoidea* are both susceptible to compound 3b but resistant to compound 3a as illustrated in table 2. Compounds 3a and 3b demonstrated comparable antibacterial activity as ciprofloxacin having activity against five bacterial strains according to figure 1. However, both compounds demonstrated inferior antifungal activity compared to fluconazole. Generally, compound 3b indicated a wider spectrum of action compared to compound 3a as illustrated in figure 1. Overall, compounds 3a and 3b had significant zones of inhibitions against all the susceptible micro-organisms which are comparable to those of ciprofloxacin and fluconazole as illustrated in table 3 and figure 1 respectively.

Compound 3a has shown significant activity against *Staphylococcus aureus* and *Campylobacter fetus* which are resistant to ciprofloxacin. This compound gave zones of inhibition of 25 mm and 26 mm against the said bacteria in table 3 with MIC 25 μg/ml. On the other hand, compound 3b indicated significant activity against ciprofloxacin-resistant bacteria strains methicillin-resistant *Staphylococcus aureus* and *Proteus mirabilis* with ZI of 27 mm and 31 mm respectively in table 3 with MIC of 25 μg/ml and 12.5 μg/ml accordingly in table 3 and figure 1. The lowest MIC of compound 3a was observed

against Vancomycin-resistant *enterococci*, *Escherichia coli*, and *Candida tropicalis* at 12.5 μg/ml. For compound 3b, the lowest MIC was recorded against *Proteus mirabilis* at 12.5 μg/ml.

Interestingly, both compounds demonstrated bactericidal and fungicidal activity against the tested micro-organisms. Compound 3a has its lowest MBC/MFC against Vancomycin-resistant *enterococci*, *Escherichia coli*, and *Candida tropicalis* at 25 μg/ml while compound 3b demonstrated its lowest MBC against *Proteus mirabilis* at 25 μg/ml according to results in table 4. However, compound 3b demonstrated better antifungal activity compared to compound 3a.

Conclusion

Taken together the two hydrazones have shown promising antimicrobial activities which are comparable to those of ciprofloxacin and fluconazole. The wider spectrum of activity of compound 3b compared to 3a may be due to the presence of the para nitro group of compound 3b. Both compounds have demonstrated comparable antibacterial activity with ciprofloxacin. Therefore, these compounds have demonstrated leadlike properties and may undergo further screening against the susceptible bacterial and fungi strains *in-vivo* and preclinical trials.

Acknowledgments

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