RESEARCH ARTICLE

Synthesis, Characterization and Evaluation of Antimicrobial Activity for New Nitroimidazole Derivatives

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ABSTRACT

Background: The nitroimidazole ring has gained more importance in recent years because it is considered a potent biologically active nucleus.

Methodology: The nitroimidazole derivative (ornidazole) was used to prepare some new azo compounds as possible antimicrobial agents. Different activated aromatic compounds containing primary amino groups with sodium nitrite were carried out to prepare diazounium salt which was coupled with ornidazole to form the azo linkage.

Results: The reaction steps and the purity of the products were confirmed by thin-layer chromatography (TLC) and melting points measurement. The chemical structures of the final compounds were characterized and confirmed by measuring their fourier transform infrared (FTIR) and Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra. These compounds were screened for their antimicrobial activity on five different strains of bacteria and one strain of fungi by broth microdilution spectrometric method and for mycobacterium tuberculosis bacilli employing BACTEC MGIT 960 System. These compounds show moderate to good activity against tested gram-positive bacteria and fungi using levofloxacin and nystatin as standard drugs, but no activity against mycobacterium tuberculosis.

Keywords: Antimicrobial, Anti-tubercular Susceptibility Testing (AST), Nitroimidazole, Nitroreductases (NTR).

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INTRODUCTION

A pathogenic microorganism is a microorganism that causes disease in another organism (host), it is known as the infectious agent, which may be bacteria, virus, fungi, and protozoa. The diseases caused by these microorganisms in the human body are distinguished as infectious diseases.[1] The occurrence and severity of disease due to pathogen depend upon the pathogen's ability to damage the host and resist the pathogen.[2]

This condition was further worsened by increasing microbial resistance to most antibiotics today. Overuse or the improper use of antibiotics can result in drug-resistant bacteria and considerable expense to the health care system.[3] The recent ongoing global pandemic, COVID-19, was caused by novel SARS-CoV-2. It was soon identified as a highly contagious and infectious disease with an exponential increase in daily cases.[4]

There are several mechanisms by which antimicrobial resistance is mediated in bacteria.[5] Gram-positive bacterial infections particularly caused by Staphylococcus aureus and Enterococcus spp. represent a major problem affecting the therapeutic efficacy of antibacterial drugs due to their multidrug resistance. In addition, an increased incidence of multidrug resistance Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*, coupled with the lack of novel antibiotics, represents one of the biggest threats to the control of respiratory and other infections.[6]

The Candida species represent the main reason for fungal infections worldwide. Candida albicans is the most common etiological agent of candidiasis. The World Health Organization (WHO) evaluates tuberculosis as one of the deadliest global diseases associated with the occurrence of multiple drug-resistant strains of Mycobacterium tuberculosis discovered by Robert Koch in 1882.[7]

Nitroimidazole is an imidazole derivative that contains a nitro group. The imidazole derivatives are important due to a large number of drugs in use today contain this moiety.[8] Several

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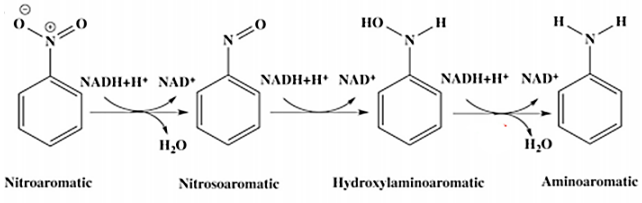
5-nitroimidazole derivatives such as metronidazole, tinidazole, ornidazole, secnidazole, and ronidazole have been used for a long time to treat the critical infections caused by protozoa and anaerobic bacteria. They have many other biological activities of therapeutic importance.[9-13]

Nitroreductases (NTR) are homodimer enzymes using flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide phosphate (NADPH) as sources of reducing power. The reduction reactions catalyzed by these enzymes include reducing nitro compounds to hydroxylamine or amino forms.[14]



Accordingly, two classes of these enzymes were identified: one to reduce the nitro group by direct two-electron transfer, and the other, by single electron transfer to produce another product. The oxygen-insensitive nitro-reductases are of the first type, while the oxygen-sensitive types are of the second type. The nitrogen oxide, NO_2, is produced from the re-oxidation of the nitric oxide (NO) by futile cycle in which reducing power is used to produce superoxide anions, not reduced substrate.[15,16]

This work aimed to synthesize and characterize new nitroimidazole derivatives, as antimicrobial agents and study their biological activity using BACTEC MGIT 960 System for mycobacterium tuberculosis bacilli and broth microdilution spectrometric methods for other selected bacteria and fungus.

MATERIALS & METHODS

Materials

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Merck silica gel plates coated in aluminum sheets (silica gel 60 F254) were used for analytical TLC. Melting points were determined using a barnstead/electrothermal apparatus and were uncorrected. The ^{1}H-NMR spectra were recorded on AGILAN VARIAN 400 MHz spectrometer. FTIR spectra were recorded on a Shimadzu FTIR spectrometer.

Chemical Synthetic Methods

The synthesis of the nitroimidazole derivatives was achieved following the procedures listed below, and the steps were summarized in (Scheme 1).[17,18]

Synthesis of compound M1: 4-((1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitro-1H-imidazol-4-yl) diazenyl)-N-(thiazol-2 yl) benzene sulfonamide.

The diazotization of Sulfathiazole was carried out by dissolving (0.765 g, 3 mmol) in 1-mL ethanol, 3 m: conc. hydrochloric acid and (3 mL) of water were added to a suitable beaker. The resulted solution was stirred for 30 minutes and cooled by immersing it in an iced bath. The temperature of the reactants was kept at less than 5^{o}C during the reaction. A solution of sodium nitrite (0.28 g, 4 mmole) in 2 mL water was cooled using crushed ice, then it was added dropwise into sulfathiazole solution, which was kept in the ice bath with continuous stirring. The temperature was kept below to 10°C. The obtained product was coupled with (0.658 g, 3 mmol) ornidazole in 2M sodium hydroxide solution. The mixture was stirred for 8 hours at a temperature from 0 to 5°C in an iced bath. Then, this mixture was filtered, washed well with water, recrystallized from absolute ethanol, and dried to obtain the precipitate of compound M1.

Synthesis of compound M2: 4- ((1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitro-1H-imidazol-4-yl)diazenyl)-N(diaminomethylene) benzene sulfonamide.

The diazotization of sulfaguanidine was carried out by dissolving (0.642 g, 3 mmol) in 3 mL conc. hydrochloric acid and 3 mL of water in a suitable beaker, and the resulted solution was stirred for 10 minutes and cooled by immersing it in a bath of crushed ice. The same procedure was carried out as in the synthesis of compound (M1). The mixture was stirred for 2 hours at a temperature from 0 to 5°C in an iced bath. Then, this mixture was filtered, washed well with water, recrystallized from absolute ethanol, dried, and collected as compound M2.

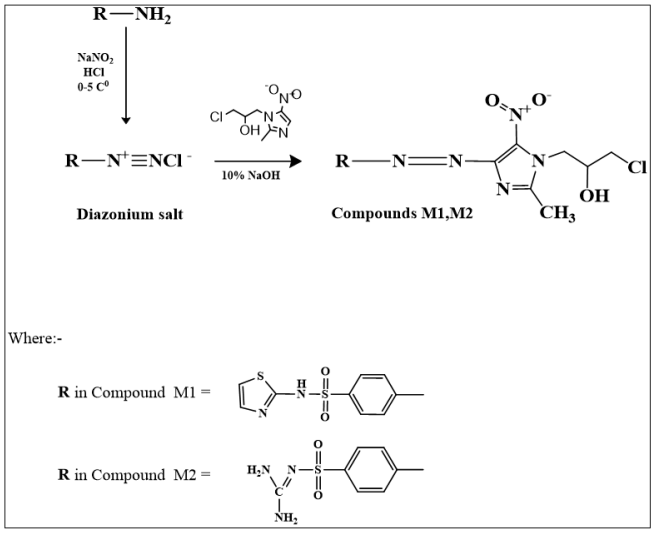
Methods of Characterization and Identification

Thin-layer Chromatography (TLC)

To check the purity of the products and monitor the progress of the reaction, the TLC was run on silica gel (F-254 type 60) pre-coated aluminum sheets. The synthesized compounds were detected by reacting with iodine vapor or irradiating with UV light. The chromatograms were eluted with Acetone: Methanol (5:5) as a solvent system.

Melting Points

The synthesized compounds' melting points were determined using an electrothermal melting point apparatus, and the uncorrected results were recorded.



Scheme 1: The general synthetic pathway of compounds (M1, M2).

Infrared Spectra

The infrared spectra were determined and recorded using Shimadzu FTIR-8400 at the Chemistry Department, College of Science, Mustansiriyah University, Iraq.

Proton Nuclear Magnetic Resonance (^{1}H-NMR).

^{1}H-NMR spectra for each synthesized compound were performed at College of Science, University of Tehran, Iran.

Antimicrobial Activity.

Determination of Minimum Inhibitor Concentration (MIC)[19]

The minimum inhibitor concentrations for the finally synthesized compounds were investigated in comparison with levofloxacin which was used as a reference antibacterial activity against five bacteria strains: gram positive bacteria (Methicillin-resistance *S. aureus* (MRSA)*, Enterococcus faecalis*) and gram-negative bacteria (*Salmonella, E. coli*, *P. aeruginosa*) as well as using nystatin against *Candida albicans* for the antifungal activity test.[20] Broth microdilution method in a 96 wells microtiter plate was performed for each one of the tested compounds, this method was carried out according to the (CLSI.2018) references.[21]

Determination of Inhibition Percentage[22,23]

The turbidity or growth (absorbance known as optical density) for each tested compound in well was measured using spectrophotometer at (600–630 nm) wavelength. The absorbance (A) of each specified well was recorded which is proportional to the microbe's growth (turbidity), and the %inhibition was calculated using the following formula:

$$\%\text{Inhibition} = 1 - (\text{OD test}/\text{OD control}) \times 100$$

Where:-

OD test; optical density (microbes in culture media and tested compound).

OD control; optical density of control (microbes in culture media).

Determination of Anti-tubercular Susceptibility Testing (AST)[24]

In vitro anti-tuberculosis activity of the synthesized compounds was studied at the reference center of the National TB program in Iraq -ministry of health. Their effects have been evaluated by the MGIT960 method by using mycobacteria growth indicator tube (MGIT), to determine the sensitivity of the *Mycobacterium tuberculosis* to the synthesized compounds in liquid media.[24]

Anti-tubercular susceptibility test is interpretive in two different categories, e.g., the *in-vitro* results are categorized by either "critical concentration" or "minimal inhibitory concentration"; however, the first is used internationally. The critical concentration indicates the lower concentration of the anti-tubercle drug that inhibits 99% of growth. In the wild strain of MTB, the inhibition of 90% induced by pyrazinamide is considered the critical concentration.[114] So, the stock solution in this study was prepared accordingly.[24]

Liquid and solid media were used for the preparation of the Isolate. The liquid medium (7 mL of modified Middlebrook 7H9 broth) for BACTEC MGIT 960 system and the solid medium of Lowenstein-Jensen (LJ) were obtained from the reference center of the National TB program in Baghdad, Iraq.

Ornidazole (10 mg/2.5 mL DMSO) was prepared like the critical concentration (84 µg/mL) of the second line anti-TB delamanid drug because both were nitroimidazole derivatives.[24,25] The doses of synthesized compounds which are (22.105, 20.239 mg) for compounds M1 and M2, respectively, are calculated according to the following equation.[26]

Dose of tested compound = [Dose of ornidazole * Molecular weight of compound] / Molecular weight of ornidazole

RESULTS AND DISCUSSION

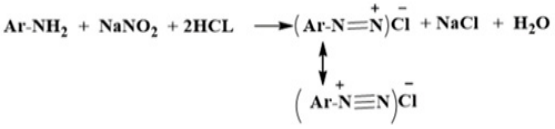
Results of Chemical Syntheses

he nitroimidazole derivatives were successfully synthesized and the physical appearance, molecular weight (M.WT.), percentage of yield, melting points, and retardation factor (R_f) values of final compounds were summarized in Table 1.

Mechanism for Synthesis of Compounds (M1, M2)

1. Formation of Diazonium Salt

The reaction of primary aromatic amines with nitrous acid at about 0°C in the presence of HCl, was produced diazonium salts as an intermediate.[27]



2. Coupling Reaction[28]

It involves the reaction of diazonium salts with nitroimdazole derivative (ornidazole) in the presence of sodium hydroxide to form an azo compound. Both nitrogen atoms (N=N) are retained in the products of coupling reaction.[28]

Results of Characterization and Identification of the Synthesized Compounds

FTIR Characterization

The Fourier Transform Infrared (FTIR) spectra of the final compounds (M1, M2), showed specific bands of absorption by which they were identified. IR data were helpful not only to identify the final compounds but also, they are advantageous to follow up the reactions depending on the appearance or disappearance of specific group frequencies.

Table 1: The physical appearance, M.WT., percentage of yield, melting points, and retardation factor (R_f) values of final compounds

Compound No.	*Physical appearance*	*M.WT. (gm /mole)*	*%yield*	*Observed melting point (˚C)*	*R_f values*
M1	Deep red powder	485.5	68.9	193 (decom.)	0.787
M2	Reddish orange powder	444.5	22.97	234 (decom.)	0.762

FTIR Characterization of Compound (M1)

3294.53 cm^{-1} (N-H stretching vibration of sulfonamide), 3200-3600 cm^{-1} (O-H Stretching vibration broad band of alcohol), 1593.25 cm^{-1} (C=N Stretching vibration of heterocyclic ring), 1529.60 cm^{-1}(N-O Stretching vibration of NO_2), 1431.23 cm^{-1} (N=N Stretching vibration), 1365.65 cm^{-1} (S=O Stretching vibration of sulfonamide), 1136.11&1082.10 cm^{-1} (C-O Stretching vibration of alcohol), 740.69 cm^{-1} (C-Cl Stretching vibration).

FT-IR Characterization of Compound (M2)

3443.05 & 3317.67cm^{-1} (NH stretching vibration of primary amine), 3200-3600 cm^{-1} (O-H Stretching vibration broad band of alcohol), 1626.05 cm^{-1} (C=N Stretching vibration of heterocyclic ring), 1535.39 cm^{-1}(N-O Stretching vibration of NO_2), 1467.88cm^{-1} (N=N Stretching vibration), 1336.71 cm^{-1} (S=O Stretching vibration of sulfonamide), 1259.56 cm^{-1} (C-N Stretching Vibration of primary amines), 1134.18, 1091.75cm^{-1} (C-O Stretching vibration of alcohol), 823.63cm^{-1} (C-Cl Stretching vibration).

^{1}H-NMR Characterization

The ^{1}H NMR spectroscopy allows the identification of hydrogen atoms (proton) in an organic molecule. This spectrum was performed for the synthesized compounds (M1, M2) and tabulated in Tables 2 and 3, respectively, Showing a characteristic signal that complies with their structures.

Antimicrobial Activity Result

Minimum Inhibitor Concentration (MIC)

The Broth Dilution method is a simple method used in this study for determining minimum inhibitory concentration (MIC) for gram positive (*E. faecalis*, Methicillin Resistance *S. aureus* MRSA), gram-negative bacteria (*E. coli*, *P. aeruginosa, Salmonella typhi*) and fungi (*C. albicans*). This was performed by putting in wells, serial dilution of tested compounds (M1, M2), and a known amount of bacterial suspension was added except for the negative control (DMSO and bacterial suspension). After incubation at 37^{o}C for 24 hours, MIC was recorded which is the lowest dilution that led to lack of turbidity (inhibited the microbe growth) in the tube and are presented in Table 4.

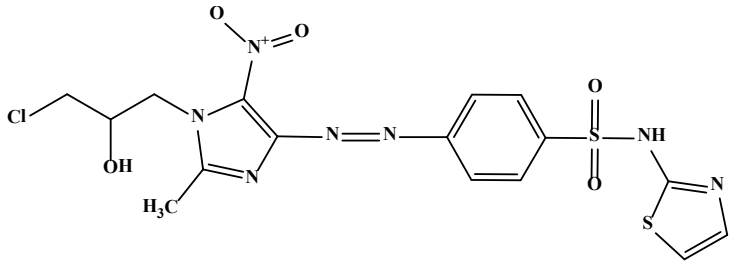
Percent of Growth Inhibition

The percent of inhibition of tested synthesized compounds (M1, M2) on selected bacteria and fungi growth in this study as shown in Figure 1 were calculated and represented in Table 5.

Anti-TB Drug Susceptibility Testing

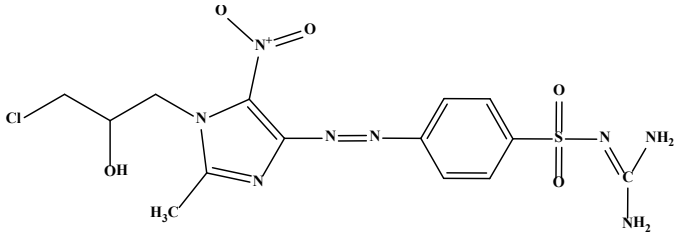
The anti-tuberculosis activity of the investigated drugs was assessed by comparing growth in the agent-containing tube (M1,M2) to that in the MGIT tube without the agents (the control tube) and the results were presented in Table 6. The MGIT tube contains a fluorescent compound embedded in silicone round-bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. The free oxygen is utilized during bacterial growth in the MGIT tube and is replaced by CO_2. This depletion of free O_2 results in fluorescence of the sensor within this tube. So the O_2 depletion is directly proportional to the intensity of fluorescence, which is automatically detected by BACTEC MGIT 960 instrument and If the test agent is effective against mycobacteria, it will inhibit the bacteria from growing as a result, fluorescence will be suppressed, whereas growth in the control tube will be unaffected, and fluorescence will rise. The results of Antimicrobial Susceptibility Testing (AST) are

Table 2: ^{1}H NMR data and their interpretations of compound M1.



Compound	*Chemical Shift (ppm)*	*Group*	*No. of Protons*	*Interpretations*
M 1	2.85	Imidazole –C<u>H</u>$_3$	3	Singlet
	3.7	-C<u>H</u> of propanol	1	Multipalate
	3.99	-O<u>H</u> of propanol	1	Doublet
	4.12	-C<u>H</u>$_2$Cl	2	Doublet
	4.36	Imidazole –C<u>H</u>$_2$	2	Doublet
	4.47	-N<u>H</u>	1	Singlet
	6.85	-S-C<u>H</u> of thiazole ring	1	Doublet
	7.11	-N-C<u>H</u> of thiazole ring	1	Doublet
	7.74 - 8.31	Aromatic- <u>H</u>	4	Multipalate signals result from overlapping of nonequivalent aromatic protons

Table 3: ^{1}H NMR data and their interpretations of compound M2**.**



Compound	*Chemical Shift (ppm)*	*Group*	*No. of Protons*	*Interpretations*
M 2	2.85	Imidazole –C<u>H</u>$_3$	3	Singlet
	3.46	-C<u>H</u> of propanol	1	Multipalate
	3.74	-O<u>H</u> of propanol	1	Doublet
	4.29	-C<u>H</u>$_2$Cl	2	Doublet
	4.52	Imidazole –C<u>H</u>$_2$	2	Doublet
	7.74 - 8.84	Aromatic- <u>H</u>	4	Multipalate signals result from overlapping of nonequivalent aromatic protons
	8.02 8.05	- N<u>H</u>$_2$ - N<u>H</u>$_2$	2 2	Singlet Singlet

Table 4: Effect of tested synthesized compounds (M1, M2) on bacteria and fungi growth represented by minimum inhibitor concentration.

	Minimum inhibitor concentration (MIC) µg/mL					
Compound no.	*S. typhi*	*Methicillin resistance S. aureus (MRSA)*	*E. faecalis*	*P. aeruginosa*	*E. coli*	*C.albicans*
M1	125	62.5	500	125	125	62.5
M2	500	250	500	250	500	250
Levofloxacin	1000	1000	1000	1000	1000	NT
Nystatin	NT*	NT	NT	NT	NT	1000

*NT = not tested.

Table 5: The effect of tested synthesized compounds (M1, M2) on selected bactria and fungi growth as compared with references (levofloxacin and nystatin)**.**

	*Percent of growth inhibition % = [1-(*OD Test / OD Control) x100]*					
Compound no.	*S. typhi*	*E. coli*	*P. aeruginosa*	*E. faecalis*	*S. aureus MRSA*	*C. albicans*
Levofloxacin	1.02	8.3	0	4.6	79.28	NT
M1	90.1	90.25	44.1	82.1	95.2	80.4
M2	85.7	87.74	17.3	92.22	94.2	70.7
Nystatin	**NT	NT	NT	NT	NT	9.6

*OD= Optical Density,**NT =not tested

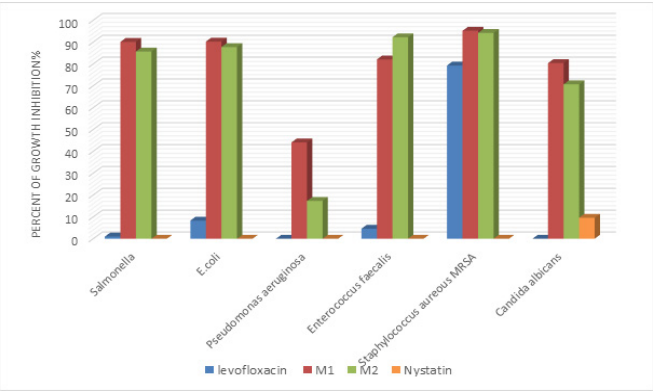


Figure 1: Effect of tested synthesized compounds (M1, M2) on selected bacteria and fungi growth as compared with references (levofloxacin and nystatin).

Table 6: Antimicrobial susceptibility testing (AST) report for INH, ornidazole (reference) and the synthesized compounds (M1- M4) on Mycobacterium growth.

	Patient no.	*G. Unit*	*INH*	*Ornidazole*	*M1*	*M2*
AST	1	400	R*	R	R	R
	2	400	R	R	R	R
	3	400	R	R	R	R
	4	400	R	R	R	R
	5	X 200*	X 200*	X 200*	X 200*	X 200*
	G.C.	400	C	C	C	C

*R = Resistance of mycobacterium.
C*= Control of Growth control Tube (G.C.)
G. Unit = growth unit.
X 200*= error occurs if the inoculum contains only a low number of viable organisms.[24]

interpreted as resistant when the growth unit (GU) value of the control reaches 400 or more and the GU value of the tube containing the agent being tested is 100 or more, the strain is said to be resistant.[24]

CONCLUSION

In this work, the authors conclude that the newly synthesized compounds (M1 and M2) show significantly different antimicrobial activity compared to levofloxacin and nystatin, and with each other, But no activity on mycobacterium. The differences in the activities of the synthesized compounds are attributed to the variable structures involved during the synthesis of azo final compounds, which affect the orientation of the tested compounds in binding to the active site of the target enzymes, leading to different affinities, selectivities and biological activities. Also, the effect on enzyme Deazaflvin-dependent nitroreductase (Ddn) is the only identified enzyme within Mycobacterium tuberculosis that activates nitoimidazole prodrug.

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