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Synthesis, Characterization, and Antimicrobial Evaluation for New Azo-linked Derivative of Heterocyclic Compounds

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Abstract

Objectives: Using different activated heterocyclic amines to prepare diazonium salts to be coupled with ornidazole (heterocyclic agent) to form the azo linkage compounds (A1, A2) as possible antimicrobial agent.

Methods: The synthetic process involves the preparation of diazonium salts by diazotization reaction of sulfamethoxazole and 5-amino-1,3,4-thiadiazole-2-thiol that contain primary amino groups with (HNO_2) acid in the presence of HCl at about 0°C. Then, azo compounds (A1, A2) were synthesized, by the reaction of nitroimidazole derivative (ornidazole) with the prepared diazonium salts at 0–5°C, and the chemical structure of these compounds were characterized and confirmed by measuring its Fourier Transform Infrared (FT-IR) spectrum and Proton Nuclear Magnetic Resonance (H^1-NMR) spectrum.

Results: Thin layer chromatography and melting point measurements were used to establish the product's purity. This compound was tested for antimicrobial activity using the broth microdilution spectrometric method on five different strains of bacteria and one strain of fungi, as well as the BACTEC MGIT 960 system for mycobacterium tuberculosis bacilli.

Conclusion: Using levofloxacin and nystatin as reference drugs, the compounds (A1, A2) showed moderate to good action against tested gram-positive and gram-negative bacteria, and fungi, but no activity against mycobacterium tuberculosis.

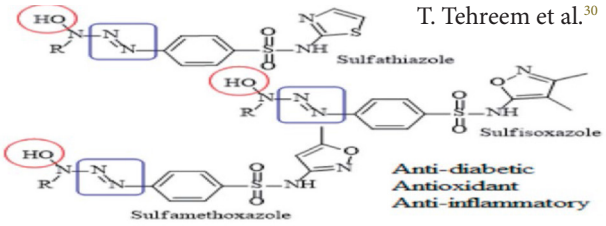
Keywords: Heterocyclic compound, azo compound, antimicrobial agents, ornidazole

Introduction

A heterocyclic compound (aromatic and non-aromatic) is a cyclic structure with at least one heteroatom in the ring. Heterocyclic compounds have five and six-membered rings and have attracted the attention of the pharmaceutical community because of their therapeutic values.[1,2]

Heterocycles bearing nitrogen, sulfur, oxygen and thiazole moieties constitute the core structure of a number of biologically interesting compounds. Some of these compounds are tetrazoles, fused thiazoles, thiadiazoles, oxadiazoles, imidazole, and triazoles, which are structural subunits of several biologically active compounds.[3]

Aromatic azo compounds have been extensively studied because of their broad-spectrum pharmaceutical applicability mainly because azo drugs act as prodrug agents as well as building blocks of various polymers and natural products.[4] Azo compounds are characterized by the presence of the azo moiety (–N=N–) in their structure conjugated with two distinct or identical, mono or polycyclic aromatic systems.[5] These compounds are medically important because of their antibiotic, antifungal and anti-human immunodeficiency virus (HIV) properties.



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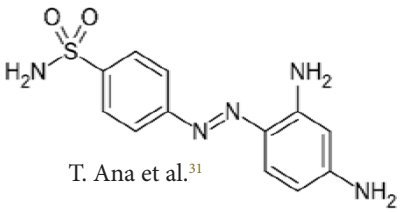
The presence of a heterocyclic ring in conjugation with azo pharmacophore enhances the bio-potency of azo compounds. The 1,3,4-thiadiazole derivative is a very important class of nitrogen-containing aromatic heterocyclic compounds which has gained increasing attention because of its diverse antibacterial, anti-inflammatory, and antifungal properties.[6]

The use of azo compounds as drugs dates back to the first commercially available antibiotic prontosil, a sulfonamide prodrug, which was identified in the 1930 and was later used to identify the first azoreductase.[7]

Azoreductases are a group of NADPH dependent flavoenzymes identified in a range of bacterial species found in the gut including *Escherichia coli* and *Pseudomonas aeruginosa*. Members of this family are also found in humans where they are known as NADPH quinone oxidoreductases.[8]

By the end of February 2020 and the beginning of March



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2020, SARS-CoV-2 (coronavirus) infection spread worldwide and the World Health Organization (WHO) declared it a pandemic on March 11th, 2020. As a result, designing and formulating new therapeutic agents became highly important. Imidazole rings are ubiquitous in natural products and possess unique structural features with a wide spectrum of biological activities. Therefore, the incorporation of imidazole ring and

diazo (N=N) moiety in a single molecule could result in the formulation of compounds with interesting properties and diverse biological activity. The main protease (Mpro) or (3CLPro) of the coronavirus is conserved among the coronaviruses and it is mainly responsible for the viral replication. Novel azo imidazole derivatives have been made for the computational study of the inhibitory potential of these novel azo imidazole derivatives against the main protease Mpro: (6LU7) of SARS-CoV-2.[9]

The threat posed by multiple drug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) indicates the need to develop antibiotics with novel mechanisms of action. Analogues of nitroaromatic compound (PT638) shown in Figure 1 were synthesized to explore the importance of the sulfonamide linker and the impact of altering the functionalization of the phenyl ring. These structure–activity relationship studies revealed that the nitro substituent was essential for antimicrobial activity.[10,11]

This raises the possibility that bio-reduction of newly developed anti-tuberculosis (TB) drugs (delamanid and (R)-PA-824) may be mediated by nitroreductase reduction.[12]

The antimicrobial mechanism of sulfonamides involves competitive inhibition of folic acid synthesis which prevents the growth and reproduction of micro-organisms.[13]

Yet, few studies have investigated the activity of trimethoprim and sulfamethoxazole against mycobacterium TB (MTB). One study suggests that the folate biosynthesis pathway is a good MTB target for drug development.[14]

Sulfonamides are the potent agents that monitor growth and proliferation of MTB by inhibiting the activity of dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) enzymes, this could explain the mechanism of sulfonamides effect on MTB.[14] Some studies investigated 3-nitrotriazole or 3-Nitroimiazole based amides and sulfonamides as potential antitubercular agents.[15] Considering the importance of sulfonamide-based aromatic heterocycles, new research has been designed to synthesize novel azaheterocyclic sulfonamide Schiff bases synthesized as carbonic anhydrase inhibitors.[16]

Recently, carbonic anhydrases (CAs) have emerged as potential drug targets in mycobacteria. Several *in vitro* studies have shown that all the MTB-CAs could be efficiently inhibited by sulfonamides/sulfamates. To increase the inhibitory properties, new molecules were synthesized by diazotization of aminosulfonamide and by coupling with phenols or amines and prontosil was found to be the best inhibitor of this enzyme.[17]

Ornidazole has a heterocyclic structure consisting of a nitroimidazole nucleus with a 2-hydroxy-3-chloro-propyl group in position 1 and a methyl group in position 2. It is used in the treatment of susceptible protozoal infections and also in anaerobic bacterial infections.[18] It is a third-generation 5-nitroimidazole antibiotic, and compared with other antibiotics, it has a longer elimination half-life and greater capacity to penetrate into lipid tissues, which makes it a good choice in dental and gastrointestinal surgeries.[19]

We aimed to synthesize and characterize new azo-linked derivatives as antimicrobial agents and study their biological activity using the broth microdilution spectrometric method for selected bacteria and fungi and the BACTEC MGIT 960 system for Mycobacterium Tuberculosis Bacilli (MTB).

Materials and Methods

Materials

All reagents and solvents were purchased from commercial sources and utilized without any further purification. For analytical thin layer chromatography (TLC), Merck silica gel plates (Germany) covered in aluminum sheets (silica gel 60 F254) were utilized. The melting points were calculated without correction using a Barnstead/Electrothermal device (USA). AGILAN VARIAN 400 MHz spectrometer (USA) was used to record the 1 H-NMR spectra and Shimadzu FT-IR spectrometer (Japan) was used to record the FT-IR spectrum.

Chemical Synthesis

Nitroimidazole derivatives were synthesized following procedures listed below and the steps are summarized in Scheme 1.[20,21]

Synthesis of compound (A1): 1-chloro-3-(4-((5-mercapto-1,3,4-thiadiazol-2-yl) diazenyl)-2-methyl-5-nitro-1H-imidazol-1-yl) propanol

The diazotization of 5-amino-1,3,4-thiadiazole-2-thiol was accomplished by dissolving (0.399 g, 3 mmol) in 2 ml ethanol, to which 3 ml concentrated hydrochloric acid and 3 ml water were added in a suitable beaker. The resulting solution was agitated for 30 mins and cooled in an ice bath. During the reaction, the temperature of the reactants was kept below 5°C. A solution of sodium nitrite (0.28 g, 4 mmol) in 2 ml water was chilled with crushed ice before being dropped into a solution of amino-1,3,4-thiadiazole-2-thiol, which was held in an ice bath with constant stirring and kept below 10°C. In a 2M sodium hydroxide solution, the resulting product was combined with (0.658 g, 3 mmol) ornidazole. In an ice bath, the mixture was agitated for 6 hours at a temperature of 0–5°C. The precipitate was then filtered, washed thoroughly with

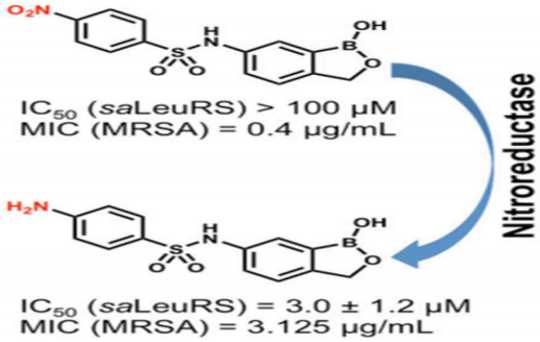
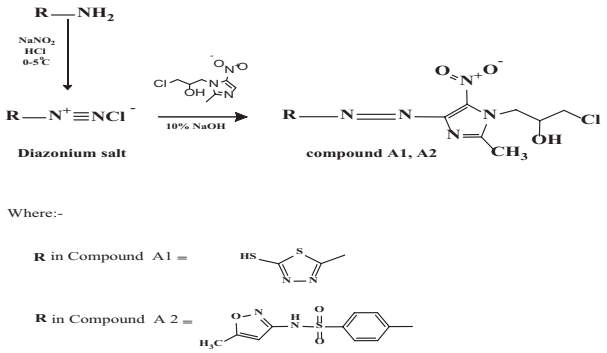


Fig. 1 **Enzymatic activation of nitroaromatic compound (PT638).**



Scheme 1. **The general synthetic pathway of compound (A1, A2).**

water, recrystallized from absolute ethanol, dried, and collected as compound A1.

Synthesis of compound (A2): 4-((1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitro-1H-imidazol-4-yl)diazenyl)-N-(5-methylisoxazol-3-yl)benzenesulfonamide

The diazotization of sulfamethoxazole was done by dissolving (0.759 g, 3 mmol) in 1 ml ethanol, to which 3 ml of concentrated HCl and 3 ml of water were added in a suitable beaker, and the resulting solution was stirred for 20 minute and cooled by immersing it in a bath of crushed ice. The same procedure was done as in the synthesis of compound (A1). The mixture was stirred for 15 hours at 0–5°C in an ice bath. Then, this mixture was filtered, washed well with water, recrystallized from absolute ethanol, dried, and collected as compound A2.

Methods of Characterization and Identification

Thin Layer Chromatography (TLC)

The TLC was done on silica gel (F-254 type 60) pre-coated aluminum sheets (MERK, Germany) to check the purity of the product and track the progress of the reaction. By interacting with iodine vapor or exposing it to UV light, the final compound was identified. Acetone:Methanol (5:5) was used as the solvent system to elute the chromatogram.

Melting Point

The melting points of the synthesized compounds were determined using an electro-thermal melting point apparatus (Barnstead/electrothermal, USA), and the uncorrected results were recorded.

Infrared Spectra

Infrared spectra were determined and recorded using Shimadzu FTIR-8400 at the chemistry department, College of Science, Mustansiriyah University.

Proton Nuclear Magnetic Resonance (H^1-NMR)

The H^1NMR spectra for each synthesized compound were determined at the College of Science, University of Tehran, Iran.

Antimicrobial Activity

Determination of Inhibition Percent

The minimum inhibitor concentration for the finally synthesized compound was compared to levofloxacin, which was used as a reference drug against five bacteria strains: gram-positive (*Methicillin resistant Staphylococcus aureus* MRSA, *Enterococcus faecalis*) and gram-negative bacteria (*Salmonella, Escherichia coli, Pseudomonas aeruginosa*). Moreover, nystatin was used against *Candida albicans* for the antifungal activity test.[22] For each one of tested compounds, a broth microdilution method was used on a 96-well microtiter plate; this approach was done according to the (CLSI.2018) references.[23]

Determination of Inhibition Percent[24,25]

A spectrophotometer set to a wavelength of 600–630 nm was used to detect the turbidity or growth (also known as optical density [OD]) of the tested substance in the well. The absorbance (A) of each specified well was recorded which is proportional to the microbe's growth (turbidity) and the percentage of inhibition was calculated using the following formula:

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

Where, OD test: microbes in culture media and tested compound; OD control: OD of control (microbes in culture media).

Determination of Antitubercular Susceptibility Testing (AST)[26]

The synthesized compound's anti-TB activity *in vitro* was investigated at the National TB Program's reference center in Iraq's Ministry of Health. The MGIT960 method was used to test the sensitivity of MTB to the produced compound in liquid media using the mycobacteria Growth Indicator Tube (MGIT).

AST was interpreted in two different categories; the *in vitro* results are categorized by either "critical concentration" or "minimal inhibitory concentration", however, the first is used internationally. The critical concentration of an anti-TB medication is the lowest concentration that prevents 99% of growth. The critical concentration for the wild strain of MTB is the 90% inhibition produced by pyrazinamide. As a result, in this study, the standard solution was prepared accordingly.[26]

The Isolate was prepared using both liquid and solid medium. The BACTEC MGIT 960 liquid medium (7 mL modified Middlebrook 7H9 broth) and Lowenstein-Jensen (LJ) solid medium were procured from the National TB Program's reference facility in Baghdad, Iraq. Because both were nitroimidazole derivatives, ornidazole (10 mg/2.5 ml DMSO) was manufactured in the same way as the critical concentration (84 g/ml) of the second-line anti-TB delamanid medication.[27] The doses of synthesized compounds were 16.564 mg and 22.014 mg for compounds A1 and A2, respectively, and were calculated according to the following equation.[28]

$$\text{Dose of tested compound} = \frac{\text{Dose of ornidazole} * \text{Molecular weight of compound}}{\text{Molecular weight of ornidazole (219.6 g/mol)}}$$

Results and Discussion

Results of Chemical Syntheses

The compound (M) was successfully synthesized, and Table 1 summarizes its physical appearance, molecular weight (M.WT.), percentage yield, melting point, and retardation factor (R_f) value.

Results of Characterization and Identification of the Synthesized Compounds

FT-IR Characterization: With respect to the final compounds (A1, A2), Fourier Transform Infrared (FT-IR) spectra revealed distinct bands of absorption that allowed recognition. Not only were IR data useful in identifying the final chemical, they were also useful in following up on reactions based on the emergence or absence of specific group frequencies.

FT-IR Characterization of Compound (M): 3200–3600cm^{-1} (O-H stretching vibration broad band of alcohol), 3066.92 cm^{-1} (C-H stretching vibration of heterocyclic ring),

Table 1. **The physical appearance, molecular weight, percentage of yield, melting points, and retardation factor (R_f) values of final compounds**

Compound No.	Physical appearance	Molecular weight (g/mol)	% yield	Observed melting point (°C)	R_f values
A1	Yellow powder	363.80	18.40	218-220	0.75
A2	Orange powder	483.5	13.66	183	0.781

2630.99 cm^{-1} (S-H stretching vibration of thiol), 1614.47 cm^{-1} (C=N Stretching vibration of heterocyclic ring), 1500.67 cm^{-1} (N-O Stretching vibration of NO_2), 1388.79 cm^{-1} (N=N Stretching vibration), 1134.18, 1033.88 cm^{-1} (C-O Stretching vibration of alcohol), 750.33 cm^{-1} (C-Cl Stretching vibration).

FT-IR Characterization of Compound (A2): 3300 cm^{-1} (N-H stretching vibration of sulfonamide), 3200–3600 cm^{-1} (O-H Stretching vibration broad band of alcohol), 1645.33 cm^{-1} (C=N stretching vibration of heterocyclic ring), 1467.88 cm^{-1} (N-O stretching vibration of NO_2), 1427.37 cm^{-1} (N=N stretching vibration), 1365.00 cm^{-1} (S=O stretching vibration of sulfonamide), 1166.97, 1138.04 cm^{-1} (C-O stretching vibration of alcohol), 742.62 cm^{-1} (C-Cl stretching vibration).

H^1-NMR Characterization: H^1NMR spectroscopy can be used to identify hydrogen atoms (protons) in organic molecules. These spectra were created for the synthesized compounds (A1, A2) and listed in Tables 2 and 3, showing a distinct signal corresponding to their structures.

Antimicrobial Activity Result

Determining percent of growth inhibition:[24,29] The broth dilution method was used in this work to determine the percent of growth inhibition for gram-positive bacteria (*Enterococcus faecalis, Methicillin Resistance Staphylococcus aureus MRSA*), gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi*), and fungi (Candida albicans) except for the negative control (DMSO and bacterial suspension). This was done by filling wells with serial dilutions of the tested compounds (A1, A2) and a known amount of bacterial suspension. After a 24-hour incubation period at 37°C, the OD was determined depending on the absorbance in a spectrophotometer at 620 nm and the resulting value showed a negative inhibition value (growth promotion); this was recorded as stimulation using the following formula:

$$\text{Percentage of negative inhibition} = (\text{OD test}/\text{OD control}) \times 100$$

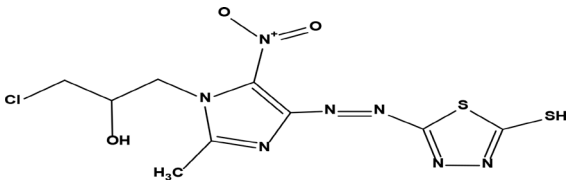
While the growth inhibition for the test was determined using the formula

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

The effects on % of inhibition of the selected bacteria and fungi for the tested synthesized compound (A1, A2) was calculated according to the mentioned formula (Figure 2).

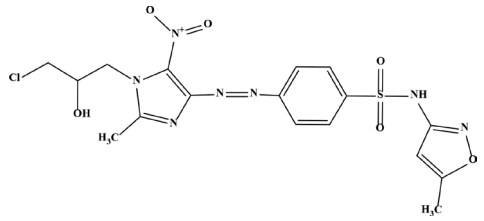
The formula (A = 2– log %) was used to find the relationship and regression between the percent of inhibition logarithm and absorbance of each tested compound (Figures 3 and 4) for the selected six microbes which gave a linear regression. We found acceptable values since the absorbance decreased as the % of inhibition increased indicating a decrease in the number of microbes absorbing light so the amount of the cell reduction (% inhibition) was calculated (Tables 4 and 5). The effects on the microbial colony count for

Table 2. **H^1NMR data and their interpretations of compound A1**



Compound	Chemical Shift (ppm)	Group	No. of Protons	Interpretations
A1	2.46	Imidazole $-CH_3$	3	Singlet
	3.43	–CH of propanol	1	Multipalate
	7.05	-NH of thiadiazole ring	1	Singlet (tautomerism of thiol to thione)
M 4*	4.01	$-CH_2Cl$	2	Doublet
	4.21	Imidazole $-CH_2$	2	Doublet
	13.16	– SH	1	Singlet

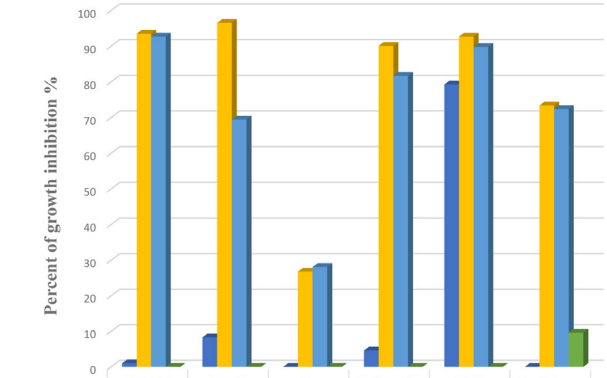
Table 3. **H^1NMR data and their interpretations of compound A2**



Compound	Chemical Shift (ppm)	Group	No. of Protons	Interpretations
A2	2.76	Oxazole-CH_3	3	Singlet
	2.84	Imidazole $-CH_3$	3	Singlet
	3.21	–CH of propanol	1	Multipalate
	3.36	–OH of propanol	1	Doublet
	4.06	$-CH_2Cl$	2	Doublet
	4.23	Imidazole $-CH_2$	2	Doublet
	4.30	–NH	1	Singlet
	6.11	Oxazole-CH	1	Singlet
	7.62-7.77	Aromatic-H	4	Multipalate signals result from overlapping of nonequivalent aromatic protons

each one of tested synthesized compounds were recorded and shown in (Figures 5 and 6).

Anti-TB drug susceptibility testing:[26] The anti-TB activity of the final synthesized compounds was determined by comparing growth in the agent-containing tube (compound A1, A2) to growth in the MGIT tube without the agents (the control tube). A fluorescent chemical is inserted in a silicone round-bottom tube in the MGIT tube. The fluorescent chemical becomes sensitive in the presence of oxygen dissolved in the broth. During bacterial growth in the MGIT tube, free oxygen is consumed and replenished by CO_2. This decrease in free O_2 causes the sensor within this tube to glow. As a result, O_2 depletion is exactly proportional to fluorescence intensity, which is detected automatically by the BACTEC MGIT 960 equipment (Table 6). If the test agent is successful against mycobacteria, it will prevent the bacteria from developing, resulting in reduced fluorescence, but growth in the control tube will be unaffected, resulting in increased fluorescence. AST results are indicated as resistant when the control's growth unit (GU) value is 400 or higher and the GU value of the tube containing the agent being tested is 100 or higher.



	S.Typhi	E.coli	P. aeruginosa	E. faecalis	MRS. aureous MRSA	C. albicans
levofloxacin	1.02	8.3	0	4.6	79.28	0
A1	93.52	96.54	26.8	90.1	92.7	73.4
A2	92.72	69.4	28.1	81.71	89.8	72.4
Nystatin	0	0	0	0	0	9.6

Fig. 2 **Effect of tested synthesized compounds (A1, A2) on growth of selected bacteria and fungi compared with levofloxacin and nystatin.**

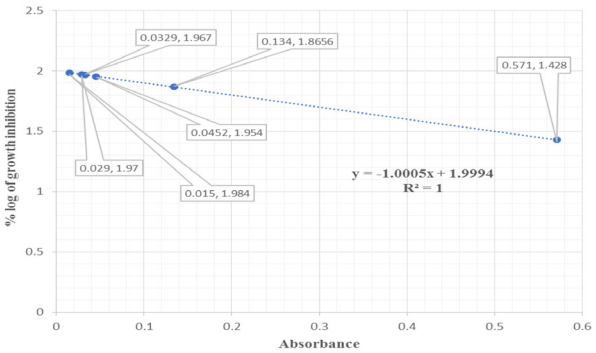


Fig. 3 **The regression (R) and linear plot of absorbance (light absorbed by microbes) versus log % of growth inhibition of compound (A1) for selected bacteria and fungi.**

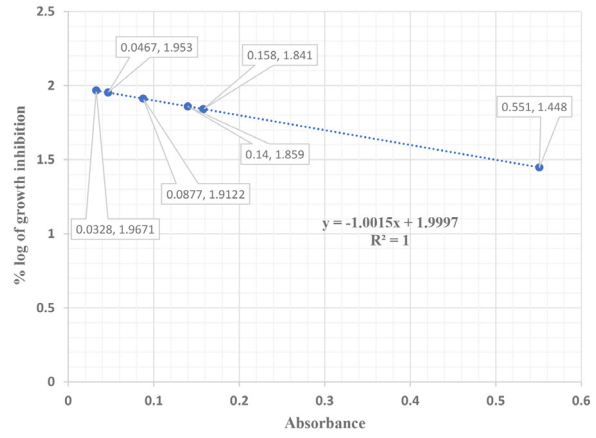


Fig. 4 **The regression (R) and linear plot of absorbance (light absorbed by microbes) versus log % of growth inhibition of compound (A2) for selected bacteria and fungi.**

Table 4. **The antimicrobial effect of compound (A1) on selected bacteria and fungi**

Microbes	Absorbance = (2–log % G.I.)	log % of growth inhibition (G.I.)
S. typhi	0.029	1.97
E. coli	0.015	1.984
P. aeruginosa	0.571	1.428
E. faecalis	0.0452	1.954
S. aureus MRSA	0.0329	1.967
C. albicans	0.134	1.8656

Table 5. **The antimicrobial effect of compound A2 on selected bacteria and fungi**

Microbes	Absorbance = (2–log % G.I.)	log % of growth inhibition (G.I.)
S. typhi	0.0328	1.9671
E. coli	0.158	1.841
P. aeruginosa	0.551	1.448
E. faecalis	0.0877	1.9122
S. aureus MRSA	0.0467	1.953
C. albicans	0.14	1.859

Table 6. **AST Report for INH, ornidazole (reference) and the synthesized compounds (A1, A2) on mycobacterium growth**

	Patient no.	G. Unit	INH	Ornidazole	A1	A2
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.[26]

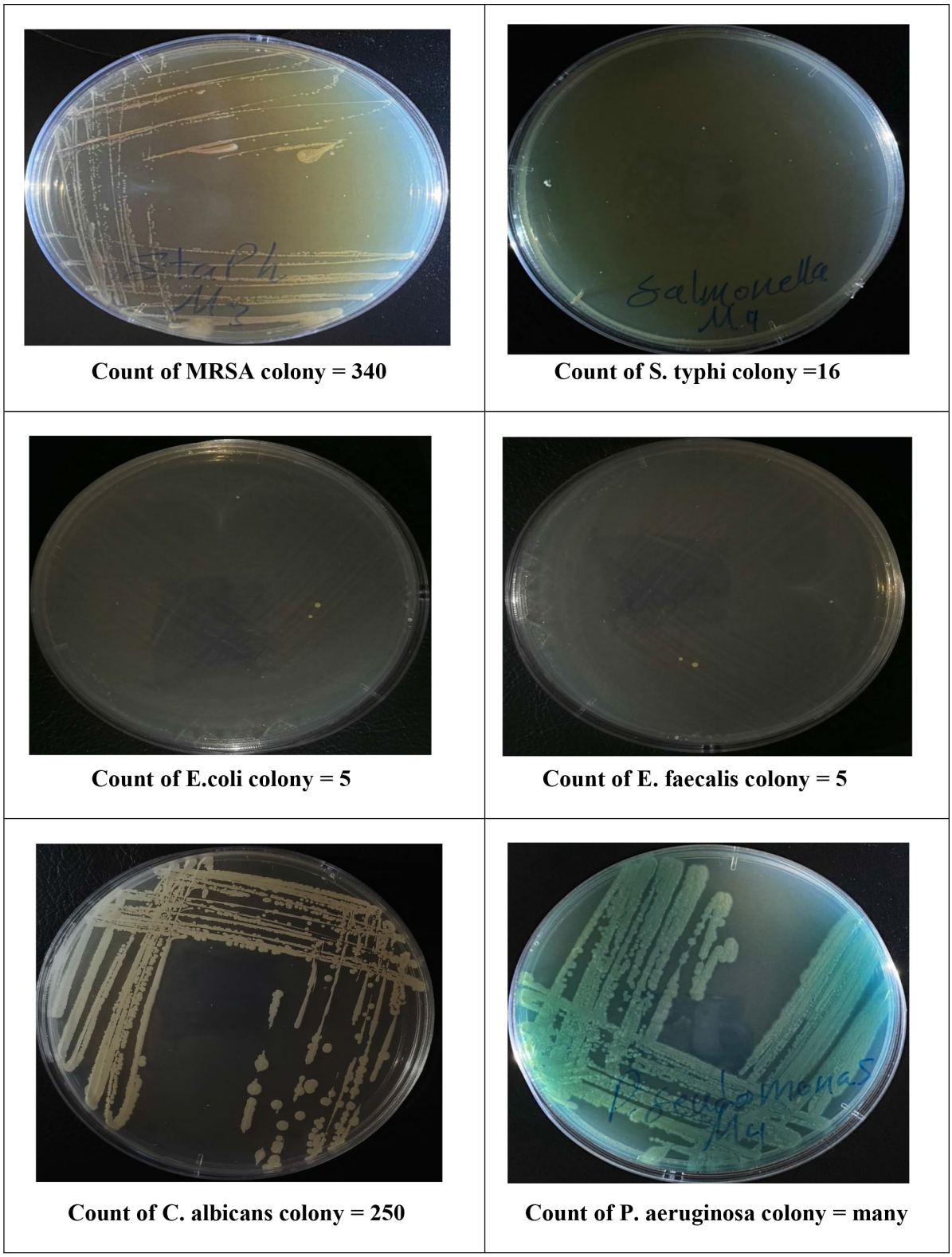


Fig. 5 **The effects on the microbial colony count for compound A1.**

Conclusion

The results of this study indicate that the diazotization and coupling reaction between nitroimidazole (ornidazole) and sulfa drugs (sulfamethoxazole) in addition to 5-amino-1,3,4-thiadiazole -5-thiol will give superior antimicrobial activity compared with levofloxacin and nystatin, when they are linked through an azo bond which has biological activity and/or through incorporation of different biologically active heterocycle rings such as thiadiazole. They have important applications in many pharmaceutical, biological and analytical fields, affecting the orientation of the tested compounds in binding to the active site of the target enzymes, leading to different affinities and biological activities. Also, the synthesized compound did not have any anti-TB effect. Although there was an error in reading the AST report in patient

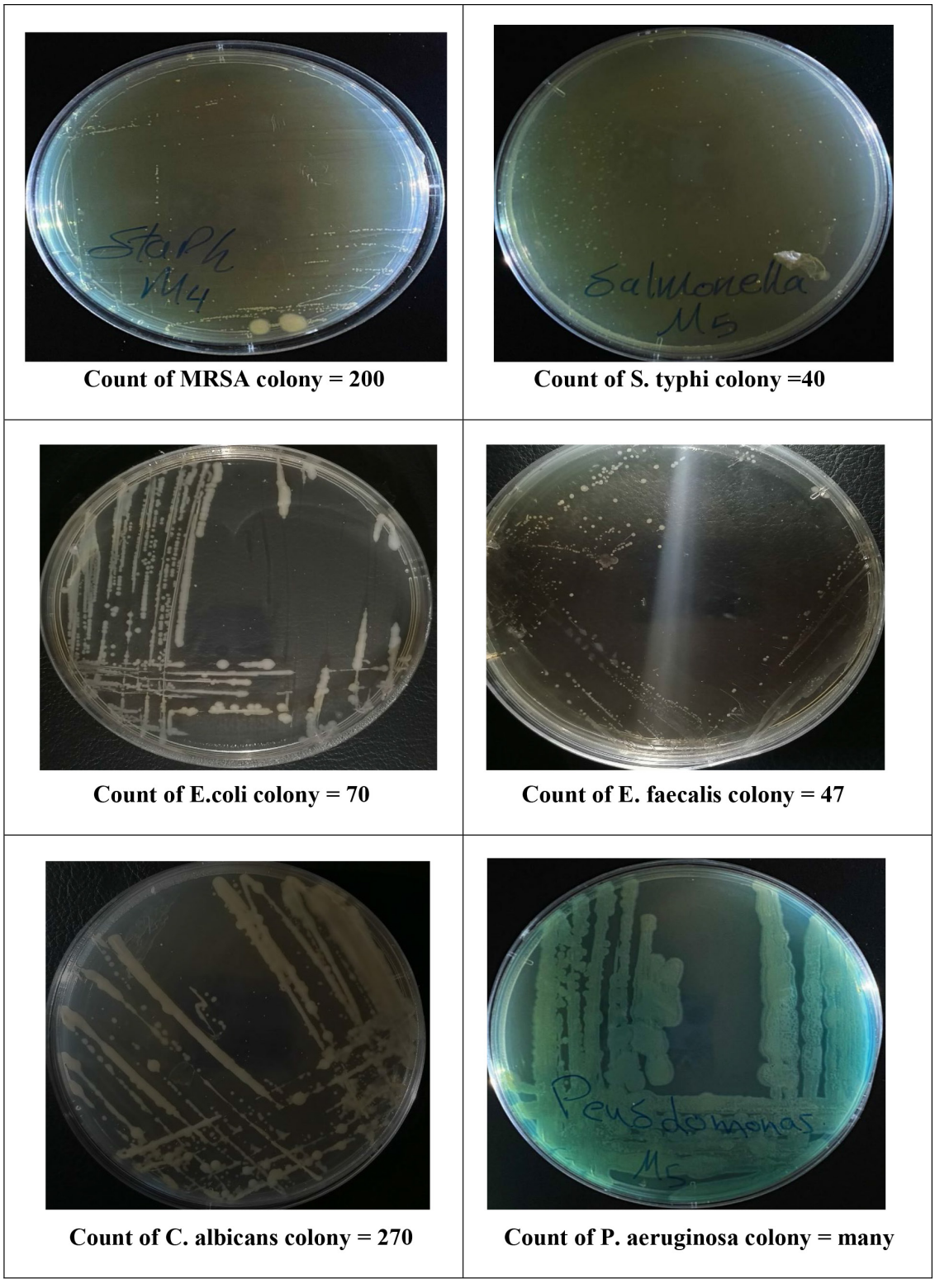


Fig. 6 **The effects on the microbial colony count for compound A2.**

number 5, still all of them were clinically classified as resistant.

The resistance to nitroimidazole (ornidazole) may be attributed to the effect of various strains of mutation in this bacteria, as a result of one or more of the mechanisms of nitroimidazole, or as alterations in the drug target, alterations in the permeation of the drug to reach its target by changing the affinity of the drug to its receptor and also the effect on enzyme deazaflavin-dependent nitroreductase (Ddn) which is the only identified enzyme within MTB that activates nitroimidazole prodrugs such as pretomanid and delamanid.

Conflicts of Interest

The authors confirm that there is no conflict of interest regarding the publication of this research paper.

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