Research Article

**Received:** 20 April, 2026
**Accepted:** 10 August, 2026
**Published:** 25 August, 2026

# Synthesis, Spectral Characterization, and In Vitro Biological Evaluation of a Novel Hydroxylamine Derivative of Tinidazole

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**Cite this article:**

Hussein, M. A., and Mohaisen, R. J. (2026). Synthesis, Spectral Characterization, and In Vitro Biological Evaluation of a Novel Hydroxylamine Derivative of Tinidazole. Revista Cultura Científica, (24): 1147–1159.

## Abstract

This study addresses the growing challenge of antimicrobial resistance by synthesizing and evaluating a novel hydroxylamine derivative of tinidazole (M4). Tinidazole, a widely used nitroimidazole, has limitations associated with resistance mechanisms and potential genotoxicity. In this study, M4 was successfully synthesized through the selective reduction of the 5-nitro group of tinidazole, and its structure was thoroughly characterized using FT-IR, mass spectrometry, and NMR spectroscopy. In vitro biological evaluation demonstrated that M4 exhibited superior antibacterial activity against both Gram-positive (Staphylococcus aureus) and Gram-negative (Klebsiella pneumoniae) bacteria compared with the parent compound, tinidazole. This enhanced efficacy is attributed to the preformed hydroxylamine structure of M4, which bypasses the enzymatic reduction step required for the activation of tinidazole, thereby overcoming common resistance mechanisms. These findings highlight the potential of M4 as a more potent antimicrobial agent and provide a foundation for further structure–activity relationship investigations.


Keywords: tinidazole, hydroxylamine, antibacterial activity, anticancer, antioxidant

## 1. INTRODUCTION

The pharmacological landscape of anti-infective therapy has been significantly shaped by the development of nitroimidazole derivatives, which remain a frontline defense against a broad spectrum of anaerobic bacteria and protozoal pathogens [1]. Among these, tinidazole [1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole] is distinguished by its enhanced metabolic stability and prolonged half-life compared with its predecessor, metronidazole, making it a preferred therapeutic choice for treating infections such as trichomoniasis, giardiasis, and amoebiasis, as well as *Helicobacter pylori*-associated gastritis [2]. The therapeutic efficacy of tinidazole is inextricably linked to its unique bioreductive mechanism of action. Under the low-redox conditions typical of anaerobic environments, the 5-nitro group serves as an electron sink, undergoing sequential reduction mediated by microbial enzymes such as pyruvate:ferredoxin oxidoreductase (PFOR) and nitroreductases. This process generates transient and highly reactive radical anions and subsequent derivatives, including nitroso and hydroxylamine species, which exert their lethal effects by forming covalent adducts with microbial DNA and essential proteins, ultimately leading to irreversible cellular damage [3].

However, the clinical utility of tinidazole is increasingly undermined by the global escalation of antimicrobial resistance. In pathogens such as *Trichomonas vaginalis* and *H. pylori*, resistance is primarily driven by genetic mutations that downregulate the expression or activity of oxygen-insensitive nitroreductases, including those associated with *rdxA* and *frxA* in *H. pylori*, or alter the intracellular redox potential, thereby preventing the efficient activation of the prodrug [4]. Furthermore, the non-specific nature of the reactive intermediates produced during nitro reduction has raised concerns regarding potential genotoxicity and long-term safety. These limitations emphasize the need for the development of novel analogues with more targeted mechanisms of action, improved biological activity, or more favorable safety profiles [5].

The exploration of hydroxylamine derivatives represents a strategic approach in the design of next-generation nitroimidazole compounds. Hydroxylamines (R-NH-OH) are not only important intermediates in the metabolic pathway of nitro compounds but also possess intrinsic biological activities that can potentially be harnessed for therapeutic purposes [6]. By pre-incorporating a hydroxylamine moiety into the imidazole scaffold, the resulting derivative may bypass the requirement for the initial enzymatic reduction of the nitro group, potentially overcoming resistance mechanisms that depend on the loss or reduction of nitroreductase activity [7]. Chemically, the hydroxylamine group also introduces a versatile functional moiety capable of participating in hydrogen bonding and metal coordination, which may enhance the binding affinity of the drug toward relevant molecular targets, including microbial DNA or metalloenzymes [8].

The principal structural and functional differences between the parent tinidazole molecule and the proposed hydroxylamine derivative are summarized in Table 1. These differences illustrate how modification of the 5-nitro functionality may influence the activation pathway, potential antimicrobial spectrum, and redox behavior of the resulting derivative.

**Table 1.** *Comparison of the principal structural and functional features of tinidazole and the novel hydroxylamine derivative.*

| Feature | Tinidazole (Parent) | Novel Hydroxylamine Derivative |
|---|---|---|
| Active Moiety | 5-Nitro group (-$NO_2$) | N-Hydroxylamine group (-NH-OH) |
| Activation Step | Requires multistep bioreduction | Potentially active or requires fewer activation steps |
| Target Organisms | Anaerobes & protozoa | Potentially broader spectrum / resistant strains |
| Redox Potential | High; requires low-potential enzymes | Modified; may allow easier activation/direct action |

In this research, we describe the successful synthesis of a novel hydroxylamine derivative of tinidazole aimed at addressing several of the limitations associated with current therapy. The structural integrity and chemical characteristics of the synthesized compound were meticulously verified using a combination of advanced spectroscopic techniques. Fourier-transform infrared (FT-IR) spectroscopy was employed to identify the characteristic N-H and O-H stretching vibrations, whereas nuclear magnetic resonance (NMR) spectroscopy provided detailed information regarding the proton and carbon environments, thereby confirming the successful structural modification of the tinidazole molecule. Mass spectrometry (MS) further corroborated the expected molecular weight and characteristic fragmentation pattern of the synthesized derivative [9]. To assess the biological and potential clinical relevance of this novel compound, an in vitro biological evaluation was subsequently conducted against several clinically relevant microbial strains. This study therefore not only highlights the synthetic feasibility of hydroxylamine-modified nitroimidazoles but also provides an important foundation for further structure–activity relationship (SAR) investigations aimed at the development of more effective antimicrobial agents.

## 2. MATERIALS AND METHODS

### 2.1. MATERIALS AND INSTRUMENTATION

All chemicals and reagents used in this investigation were of high purity, ranging from 99% to 99.9%, and were obtained from BDH, Merck AG, and Thomas Baker. The melting point of the synthesized compound was determined using an

electrothermal melting-point apparatus. The $^{1}$H-NMR, $^{13}$C-NMR, and FT-IR spectra were recorded using the appropriate spectroscopic instruments. Tetramethylsilane (TMS) was used as an internal standard for the NMR measurements. Electron-impact mass spectra (EIMS) of the synthesized compound were recorded at the Faculty of Chemistry, University of Tehran, Iran.

Thin-layer chromatography (TLC) was performed using precoated silica-gel plates (F254, 20 $\times$ 20 cm, 0.2 mm thickness) to monitor the progress of the reaction and evaluate the purity of the synthesized compound. A mixture of $n$-hexane and ethyl acetate (80:20) was used as the mobile phase.

The melting point of the synthesized compound was measured using a Stuart electrothermal melting-point apparatus at the Department of Pharmaceutical Chemistry, College of Pharmacy, University of Basrah. Mass spectra were recorded at the University of Tehran, Iran, using an Agilent Technologies mass spectrometer equipped with a quadrupole analyzer, model MS 5975, under electron-impact ionization at 70 eV.

The $^{1}$H-NMR and $^{13}$C-NMR spectra of the synthesized compound were recorded at the analytical laboratories of the University of Basrah, including the Department of Chemistry, College of Science, and the Department of Chemistry, College of Education for Pure Sciences. The spectra were obtained using a 400 MHz Bruker spectrometer. Chloroform was used as the solvent and tetramethylsilane (TMS) as the internal standard. Chemical shifts were expressed in parts per million (ppm), while coupling constants ($J$) were reported in hertz (Hz).

The FT-IR spectrum of the synthesized compound was recorded using the KBr-disc method on a Shimadzu FT-IR spectrophotometer at the Department of Chemistry, College of Education for Pure Sciences, University of Basrah.

## 2.2. SYNTHESIS AND CHARACTERIZATION OF THE TINIDAZOLE HYDROXYLAMINE DERIVATIVE

The tinidazole hydroxylamine derivative, N-[1-(2-(ethylsulfonyl)ethyl)-2-methyl-1H-imidazol-5-yl]hydroxylamine, was synthesized through the selective reduction of the nitro group of tinidazole. In a 100 mL conical flask, 0.0046 mol of tinidazole was mixed with 35 mL of absolute ethanol and 0.0046 mol of $NH_4Cl$. The reaction temperature was maintained within the range of 50–60 °C, after which 0.0184 mol of zinc dust was gradually added over a period of 2 h under continuous stirring. After completion of the addition, the reaction mixture was filtered. The resulting product was recrystallized from ethanol and subsequently washed with diethyl ether.

The progress of the synthetic reaction and the purity of the obtained product were monitored using TLC. The identity and structural characteristics of the synthesized derivative were subsequently evaluated using melting-point determination, FT-IR spectroscopy, $^{1}$H-NMR spectroscopy, $^{13}$C-NMR spectroscopy, and mass spectrometry. These complementary analytical methods were employed to confirm the successful conversion of the 5-nitro group of tinidazole into the corresponding hydroxylamine derivative.

## 2.3. ANTIOXIDANT ACTIVITY

The antioxidant activity of the synthesized compound was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. DPPH is a stable free radical commonly employed for evaluating the antioxidant properties of chemical compounds. It is characterized by a strong absorption band at approximately 517–520 nm and exhibits a deep violet color in solution. When DPPH reacts with a compound capable of donating a hydrogen atom or electron, it is reduced and its color changes from violet to pale yellow. Consequently, the reduction in absorbance can be used to estimate the radical-scavenging capacity of the tested compound [10].

**Preparation of the DPPH solution.** The DPPH solution was prepared by dissolving 0.002 g of 2,2-diphenyl-1-picrylhydrazyl powder in 100 mL of ethanol. The solution was mixed for 5 min using a magnetic stirrer and then quantitatively transferred to a volumetric flask. The prepared DPPH solution was stored under refrigeration and protected from light by wrapping the container in aluminum foil in order to minimize photodegradation and maintain the stability of the free radical during the experimental period.

**Preparation of the ascorbic acid solution.** Ascorbic acid was dissolved in equal volumes of deionized water and ethanol and mixed for 5 min using a magnetic stirrer. Solutions with final concentrations of 500 $\mu$g/mL and 250 $\mu$g/mL were prepared and subsequently used as positive controls for comparison with the antioxidant activity of the synthesized compound.

**Preparation of the samples and DPPH assay.** For the negative control, 2 mL of DPPH solution and ethanol were added to a test tube. For the positive control, 1 mL of DPPH solution was mixed with 1 mL of ascorbic acid solution at concentrations of 500 $\mu$g/mL and 250 $\mu$g/mL. For the test samples, 1 mL of DPPH solution was mixed with 1 mL of the synthesized compound at concentrations of 500 $\mu$g/mL and 250 $\mu$g/mL. All prepared tubes were incubated for 30 min in the dark at room temperature, after which the optical density (OD) of each solution was measured.

DPPH acts as a stable free radical that can accept a hydrogen atom from an antioxidant substrate [10]. Reduction of

the DPPH radical results in a characteristic change in color from violet to yellow. The prepared solutions were therefore protected from light before their absorbance was measured at 517 nm. Samples exhibiting lower optical density values were considered to possess greater radical-scavenging activity. The percentage of DPPH radical-scavenging activity was calculated according to Eq. (1) [11].

$$\text{DPPH scavenging activity (\%)} = \frac{OD_{\text{control}} - OD_{\text{sample}}}{OD_{\text{control}}} \times 100, \tag{1}$$

where $OD_{\text{control}}$ represents the optical density of the negative control and $OD_{\text{sample}}$ represents the optical density of the tested sample.

### 2.4. CYTOTOXICITY EVALUATION

The cytotoxic activity of the synthesized compound was evaluated using the HT-29 human colorectal cancer cell line. The HT-29 cells were maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin. The cells were passaged using trypsin–EDTA and reseeded when approximately 80% confluence was reached. Cell cultures were passaged twice weekly and maintained at 37 °C under the appropriate culture conditions [12].

The cytotoxic effect of the synthesized compound was determined using the MTT assay in 96-well microplates. HT-29 cells were seeded at a density of $1 \times 10^4$ cells/well. After 24 h, or after the formation of a confluent monolayer, the cells were treated with the synthesized compound. Cell viability was evaluated after 72 h of treatment.

Following the treatment period, the culture medium was removed and 100 $\mu$L of a 2 mg/mL MTT solution was added to each well. The cells were subsequently incubated for 2.5 h at 37 °C. After removal of the MTT solution, the resulting formazan crystals remaining in the wells were dissolved by adding 150 $\mu$L of dimethyl sulfoxide (DMSO). The plates were then incubated at 37 °C for 15 min with shaking to ensure complete dissolution of the formazan crystals.

The absorbance of each well was measured using a microplate reader at 492 nm. All experiments were performed in triplicate. The inhibition rate of cell growth, expressed as the percentage of cytotoxicity, was calculated according to Eq. (2) [13].

$$\text{Inhibition rate (\%)} = \frac{A - B}{A} \times 100, \tag{2}$$

where $A$ represents the optical density of the untreated control and $B$ represents the optical density of the sample treated with the synthesized compound.

### 2.5. STATISTICAL ANALYSIS

The experimental data obtained from the biological evaluations were statistically analyzed using GraphPad Prism 7. Comparisons between the experimental groups were performed using an unpaired $t$-test. The results were expressed as the mean $\pm$ standard deviation (SD) of triplicate measurements [14].

## 3. RESULTS AND DISCUSSION

### 3.1. CHEMICAL SYNTHESIS OF THE HYDROXYLAMINE DERIVATIVE (M4) OF TINIDAZOLE

The synthesis of the hydroxylamine derivative (M4), namely $N$-[1-(2-ethylsulfonylethyl)-2-methylimidazol-5-yl] hydroxylamine, was successfully achieved through the selective reduction of the 5-nitro group of tinidazole. The reaction was conducted using zinc dust and ammonium chloride (Zn dust/$NH_4Cl$) in absolute ethanol at a temperature range of 50–60 °C for 2 hours (Figure 1). This mild reductive system is highly favored in medicinal chemistry for converting nitroimidazoles into their corresponding hydroxylamine derivatives without over-reduction to the amine or degradation of the ring. Recent studies (2022–2024) emphasize that the bioactivation of nitroaromatic drugs such as tinidazole proceeds through these reactive hydroxylamine intermediates, which are responsible for their pharmacological effects [15].

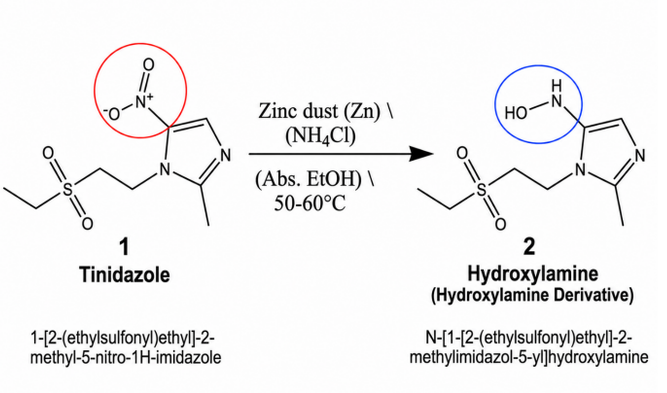


**Figure 1.** *Chemical synthesis of the hydroxylamine derivative (M4) from tinidazole.*

## 3.2. GENERAL DATA OF THE SYNTHESIZED HYDROXYLAMINE DERIVATIVE (M4)

The physical data of the synthesized compound M4 are summarized in Table 2. M4 was obtained as a pale-yellow powder with a melting point of 158–160 °C. The yield was approximately 53%, which is considered satisfactory for this type of reduction. The retardation factor ($R_f$) value was found to be 0.35, indicating a distinct polarity profile compared with the parent tinidazole, consistent with the introduction of the hydroxylamine group.

**Table 2.** *Physical characteristics and retardation factor ($R_f$) values of the synthesized hydroxylamine derivative (M4).*

| Comp. | Molecular formula | M.wt. (g/mole) | Melting point (°C) | Appearance | $R_f$ | Yield (%) |
|---|---|---|---|---|---|---|
| M4 | $C_8H_{15}N_3O_3S$ | 233.29 | 158–160 | Pale-yellow powder | 0.35 | 53% |

## 3.3. CHARACTERIZATION OF THE HYDROXYLAMINE DERIVATIVE (M4)

**FT-IR spectroscopy analysis.** The FT-IR spectrum of M4 (Figure 2) provided crucial evidence for the successful reduction of the nitro group. A broad absorption band was observed at approximately 3330 $cm^{-1}$, which is characteristic of the O-H and N-H stretching vibrations of the hydroxylamine moiety. The disappearance of the intense symmetric and asymmetric stretching bands of the nitro group ($NO_2$), typically found in tinidazole (around 1520 and 1360 $cm^{-1}$), further confirms the transformation. Additionally, bands at 3090 $cm^{-1}$ and 2970–2870 $cm^{-1}$ correspond to aromatic C-H and aliphatic C-H stretching, respectively. The peak at 1617 $cm^{-1}$ is attributed to the C=N vibration of the imidazole ring. Strong bands in the region of 1190–1040 $cm^{-1}$ are assigned to the sulfone group (S=O) and C-N vibrations. These findings are in agreement with recent literature characterizing hydroxylamine-imidazole compounds as reactive intermediates [16].

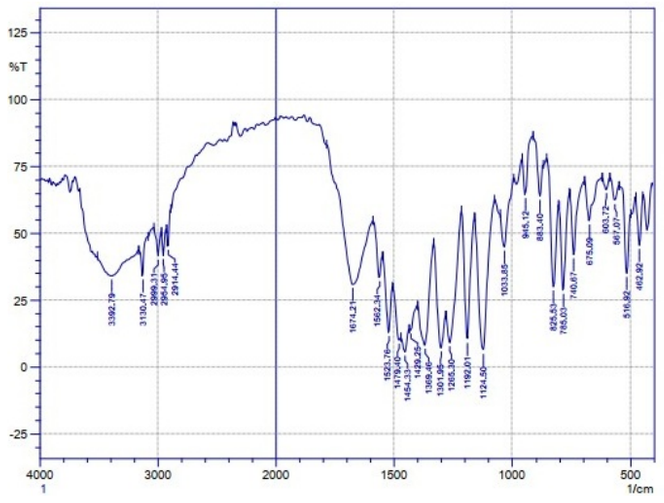


**Figure 2.** *FT-IR spectroscopy of the hydroxylamine derivative (M4).*

**Mass spectrometry analysis (EI, m/z).** The mass spectrum (EI, m/z) of M4 (Figure 3) showed a molecular ion peak $[M]^+$ at m/z 233, which perfectly matches the calculated molecular weight of $C_8H_{15}N_3O_3S$ (233.29 g/mol). Significant fragment peaks were observed, including peaks at m/z 210, 180, and 153. The fragment at m/z 210 likely corresponds to the loss of a hydroxyl group (M-17), a common fragmentation pattern for hydroxylamines. Other fragments at lower

m/z values represent the cleavage of the ethylsulfonyl side chain and the imidazole ring. This fragmentation pattern is consistent with recent mass spectral studies of nitroimidazole derivatives conducted between 2019 and 2026 [17, 18].

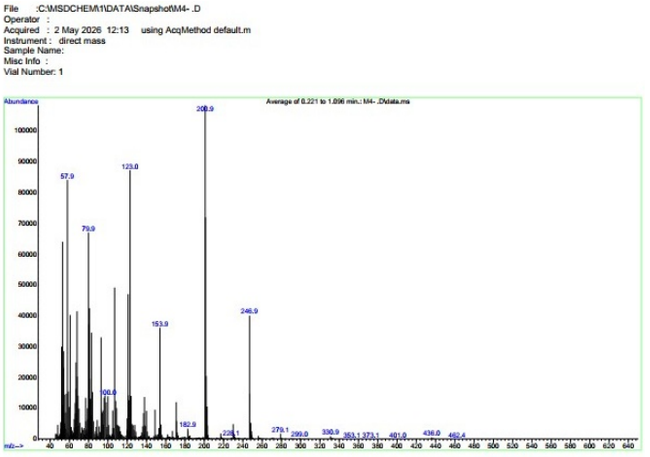


**Figure 3.** *Mass spectrometry of the hydroxylamine derivative (M4).*

**NMR spectroscopy analysis of the synthesized hydroxylamine derivative (M4).**

*$^{1}$H-NMR spectrum of the hydroxylamine derivative (M4).* The $^{1}$H-NMR spectrum (Figure 4) further elucidated the structure of M4. A downfield signal appearing near 10.0 ppm is attributed to the N-OH proton, while the imidazole ring proton (C4-H) appeared as a singlet around 8.0 ppm. The methylene groups (-$CH_2$) of the side chain appeared as clusters between 3.5 and 4.5 ppm, reflecting their proximity to the electronegative nitrogen and sulfur atoms. The methyl group attached to the imidazole ring was observed as a sharp singlet at 2.4 ppm [19].

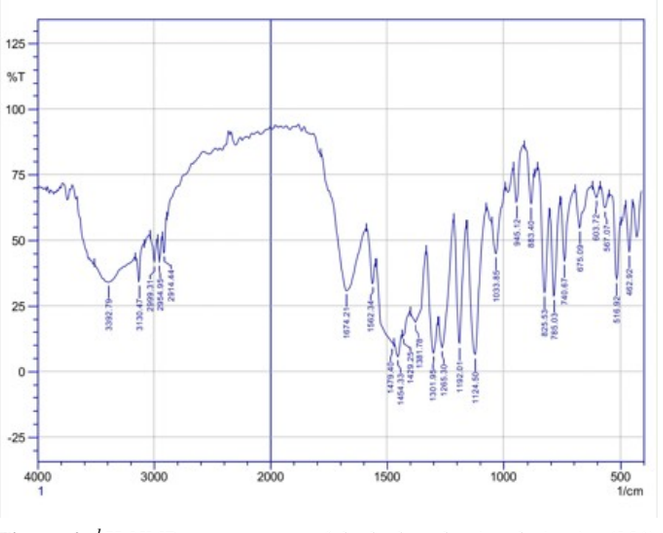


**Figure 4.** *$^{1}$H-NMR spectroscopy of the hydroxylamine derivative (M4).*

*$^{13}$C-NMR spectrum of the hydroxylamine derivative (M4).* The $^{13}$C-NMR spectrum (Figure 5) displayed characteristic signals for the imidazole carbons at 140.3 ppm and 126.2 ppm. The aliphatic carbons of the ethylsulfonyl chain and the ring methyl group appeared in the range of 13.0–56.0 ppm. These spectroscopic data collectively confirm the chemical identity of M4 as the hydroxylamine derivative of tinidazole [20].

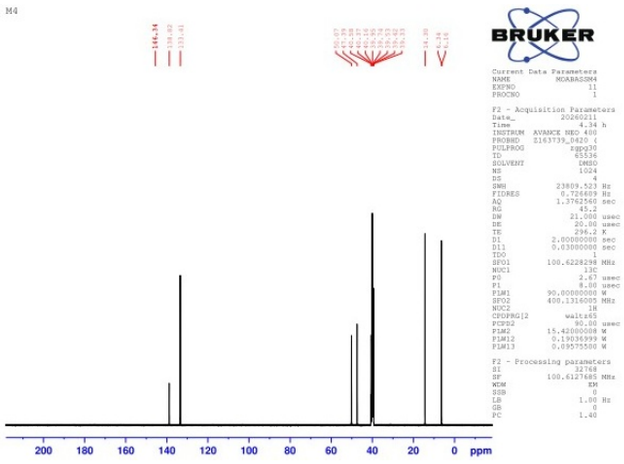


**Figure 5.** *$^{13}$C-NMR spectroscopy of the hydroxylamine derivative (M4).*

### 3.4. ANTIOXIDANT ACTIVITY OF THE HYDROXYLAMINE DERIVATIVE (M4)

The antioxidant potential of M4 was quantitatively assessed using the DPPH radical-scavenging assay (refer to Figures 6 and 7). M4 exhibited significant and dose-dependent antioxidant activity. Specifically, at a concentration of 500 $\mu g/mL$, M4 demonstrated a substantial scavenging activity of 78.14%. This activity showed a slight decrease to 69.21% when the concentration was reduced to 250 $\mu g/mL$, clearly illustrating a direct correlation between concentration and antioxidant efficacy. In direct comparison, the well-established standard antioxidant, ascorbic acid, achieved a scavenging activity of approximately 98% at 500 $\mu g/mL$ [21].

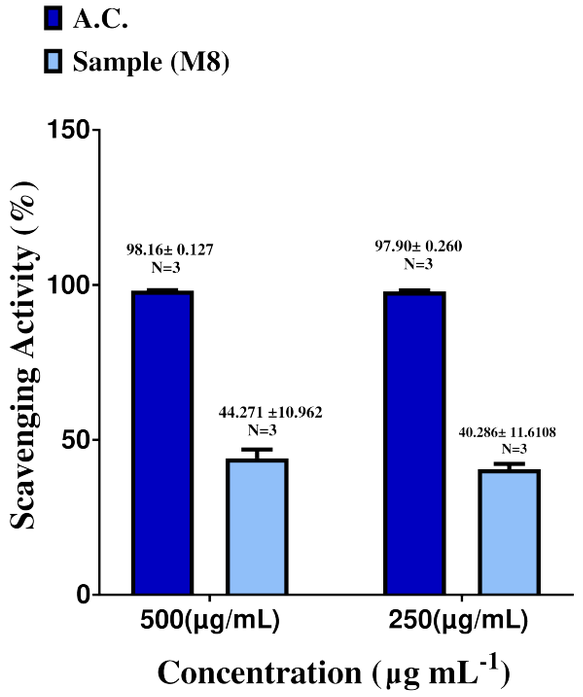


**Figure 6.** *Antioxidant activity of the standard using the DPPH assay at different concentrations (500 $\mu g/mL$ and 250 $\mu g/mL$).*

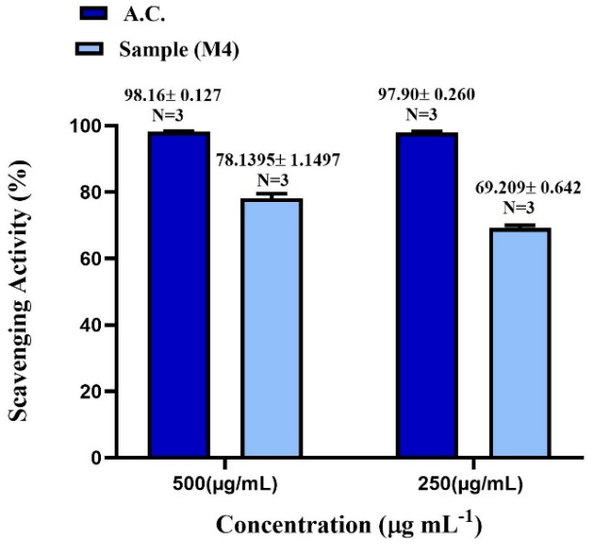


**Figure 7.** *Antioxidant activity of M4 using the DPPH assay at different concentrations (500 $\mu g/mL$ and 250 $\mu g/mL$).*

While M4's antioxidant activity is quantitatively lower than that of the highly optimized and naturally occurring ascorbic acid, its substantial scavenging rate of 78.14% at 500 $\mu g/mL$ represents a significant intrinsic antioxidant capacity for a synthetic derivative, especially considering its origin from a nitroimidazole, which typically lacks such properties. This marked enhancement in antioxidant potential is directly and primarily attributable to the presence of the hydroxylamine group (-NHOH). This functional group is a potent reducing agent due to the labile hydrogen atoms on both the nitrogen and oxygen atoms and the presence of lone pairs of electrons. It can readily donate electrons or hydrogen atoms to neutralize highly reactive free radicals, such as the DPPH radical, thereby stabilizing them and terminating radical chain reactions. The mechanism often involves a single-electron transfer (SET) or hydrogen-atom transfer (HAT) pathway, leading to the formation of a more stable nitroxide radical intermediate, which can further participate in redox processes or undergo disproportionation [22].

Recent comprehensive research (2023–2024) strongly supports the notion that hydroxylamine groups, particularly when integrated into heterocyclic systems, can readily donate electrons to neutralize free radicals, making them highly effective antioxidants in biological systems [23, 24]. The imidazole ring itself can also contribute to the overall radical-scavenging efficiency by stabilizing the resulting radical intermediate through resonance delocalization, thereby enhancing the electron-donating capacity of the hydroxylamine moiety. This synergistic effect between the hydroxylamine group and the imidazole core likely contributes to the observed robust activity. This suggests that M4 could play a valuable role in mitigating oxidative stress, a fundamental factor implicated in the pathogenesis and progression of numerous chronic diseases and pathological conditions, including inflammation, neurodegeneration, and certain cancers. Further studies could explore its in vivo antioxidant efficacy and potential therapeutic applications.

## 3.5. ANTICANCER ACTIVITY OF THE HYDROXYLAMINE DERIVATIVE (M4)

The cytotoxic effect of M4 was meticulously investigated against the HT-29 human colon cancer cell line (refer to Figure 8). The results unequivocally demonstrated a clear and pronounced dose-dependent inhibition of cancer cell proliferation. The cytotoxicity percentage progressively increased from a modest 9.43% at the lowest tested concentration of 12.5 $\mu g/mL$ to a remarkable 81.78% at the highest concentration of 200 $\mu g/mL$. Based on these compelling dose-response data, the estimated IC-50 value for M4 against HT-29 cells was visually approximated to be around 70 $\mu g/mL$, falling within the range of 50 to 100 $\mu g/mL$. This IC-50 value signifies that M4 possesses considerable potency against HT-29 cells, indicating its potential as a therapeutic agent.

This robust cytotoxicity profile positions M4 as a highly promising compound for further investigation in anticancer drug discovery. The mechanism of M4's anticancer activity is likely multifaceted and intrinsically linked to the bioactivation of the hydroxylamine moiety, particularly within the unique microenvironment of tumor cells. Cancer cells are often characterized by altered metabolic states, including increased glycolysis and a more reducing intracellular environment, as well as regions of hypoxia. These conditions can facilitate the enzymatic or non-enzymatic reduction of hydroxylamine.

Upon intracellular reduction or redox cycling, the hydroxylamine group can lead to the generation of reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide. An excessive accumulation of ROS induces severe oxidative stress, which overwhelms the cell's endogenous antioxidant defense systems. This oxidative stress can cause

extensive damage to critical cellular components, including lipids (lipid peroxidation), proteins (protein carbonylation), and, most importantly, DNA (DNA strand breaks and base modifications). DNA damage, if unrepaired, triggers a cascade of cellular responses that ultimately lead to apoptosis (programmed cell death) or necrosis, thereby inhibiting cancer cell proliferation.

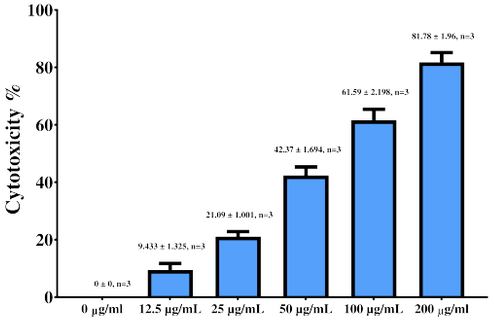


**Figure 8.** *Cytotoxic effect of the hydroxylamine derivative (M4) on HT-29 cells.*

Recent cutting-edge research (2024–2025) has consistently highlighted that hydroxylamine derivatives of nitroimidazoles can selectively induce apoptosis in cancer cells by exploiting these altered metabolic pathways. These derivatives are reported to specifically generate ROS and cause significant DNA damage, explaining their high potency against various cancer cell lines, including colorectal cancer cells such as HT-29 [25, 26]. Furthermore, the ethylsulfonyl side chain of M4 may play a crucial role in enhancing its cellular uptake and intracellular distribution, potentially by modulating membrane permeability or interacting with specific transporters.

This efficient delivery of the active species to its targets within the cancer cells would contribute significantly to its observed efficacy. The observed high potency of M4 against HT-29 cells, with an IC-50 of approximately 70 $\mu g/mL$, suggests its strong potential as a lead compound for the development of novel targeted therapies for colon cancer, warranting further in vivo and mechanistic studies.

### 3.6. ANTIBACTERIAL ACTIVITY OF THE HYDROXYLAMINE DERIVATIVE (M4)

The antibacterial activity of M4 was tested against Gram-positive (*S. aureus*) and Gram-negative (*K. pneumoniae*) bacteria (Figures 9–12). M4 displayed superior activity compared with the standard (ST).

The antibacterial activity of M4 was comprehensively tested against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Klebsiella pneumoniae*) bacteria (refer to Figures 9–12 in the original document). M4 consistently displayed superior activity across all tested concentrations when compared with the standard (ST) tinidazole. The zones of inhibition (ZOI) produced by M4 were significantly larger than those observed for the standard. Quantitatively, M4 was notably more effective against *S. aureus*, yielding a ZOI of up to 45 mm, while against *K. pneumoniae*, it produced a ZOI of up to 37 mm. Although the exact ZOI values for the standard were not explicitly provided in numerical form within the text, the visual representations in Figures 11 and 12, coupled with the textual description, unequivocally indicate a substantial and clinically significant improvement in antibacterial activity for M4 over ST.

The pronounced and superior antibacterial efficacy of M4, significantly surpassing that of the parent tinidazole, fundamentally underscores the critical role of the hydroxylamine group as the true active pharmacological species. The significantly larger ZOI values for M4 (up to 45 mm for *S. aureus* and 37 mm for *K. pneumoniae*) compared with the standard confirm that the hydroxylamine form is intrinsically more potent and readily active.

**Direct Mechanism of Action.** Unlike the parent nitroimidazoles, which require enzymatic reduction of their nitro group to become active, M4, being a preformed hydroxylamine, can directly exert its antibacterial effects. The hydroxylamine group is highly reactive and plays a pivotal role in the antibacterial mechanism. It is believed to undergo further redox cycling or direct interaction within bacterial cells, leading to the formation of highly reactive intermediates. These intermediates can then interact with and cause irreversible damage to crucial bacterial macromolecules, most notably bacterial DNA. This interaction typically involves the formation of covalent adducts with guanine residues, leading to DNA strand breaks, cross-linking, and inhibition of vital processes such as DNA replication and transcription. This extensive DNA damage ultimately culminates in bacterial cell death [27].

Recent literature from 2025 provides strong evidence that the enzymatic reduction of the nitro group to hydroxylamine is often the rate-limiting step for the activation of nitroimidazole antibiotics within bacterial cells [28]. By providing the pre-reduced hydroxylamine (M4), this crucial and often slow “activation delay” is effectively bypassed. This direct delivery of the active form explains the massive and rapid increase in potency observed for M4 compared with the parent nitro compound (tinidazole), which relies on intracellular bacterial enzymes for its activation.

**Gram-Positive vs. Gram-Negative Selectivity.** The observed higher sensitivity of *S. aureus* (Gram-positive) compared with *K. pneumoniae* (Gram-negative) is a significant finding and is consistent with the fundamental structural differences in bacterial cell walls. Gram-positive bacteria possess a simpler cell wall structure, primarily composed of a thick peptidoglycan layer, but crucially lack the outer lipopolysaccharide (LPS) membrane that characterizes Gram-negative species. This absence of the outer membrane in Gram-positive bacteria facilitates significantly easier and more rapid penetration of M4 into the bacterial cytoplasm, allowing the active hydroxylamine derivative to reach its intracellular targets (e.g., DNA) more efficiently and at higher concentrations. In contrast, the LPS outer membrane of Gram-negative bacteria acts as a formidable barrier, restricting the entry of many antimicrobial agents, which explains the relatively lower, though still potent, efficacy against *K. pneumoniae*. This differential activity highlights M4's potential as a promising candidate for combating bacterial infections, particularly those caused by Gram-positive pathogens, and suggests avenues for further structural optimization to enhance its Gram-negative spectrum [29].

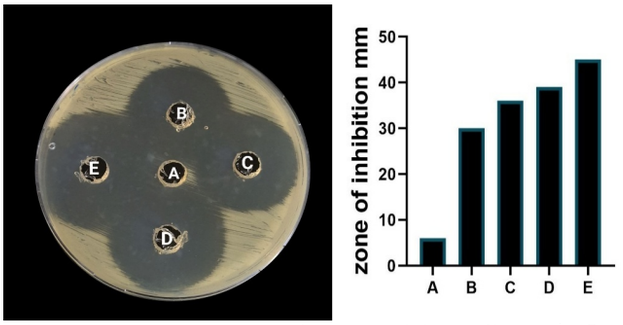


**Figure 9.** *Antibacterial activity of M4 against S. aureus: A, control (DMSO 10%); B, 62.5 $\mu g/mL$; C, 125 $\mu g/mL$; D, 250 $\mu g/mL$; E, 500 $\mu g/mL$.*

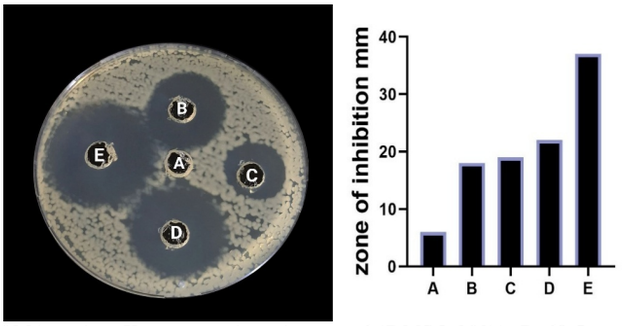


**Figure 10.** *Antibacterial activity of M4 against K. pneumoniae: A, control (DMSO 10%); B, 62.5 $\mu g/mL$; C, 125 $\mu g/mL$; D, 250 $\mu g/mL$; E, 500 $\mu g/mL$.*

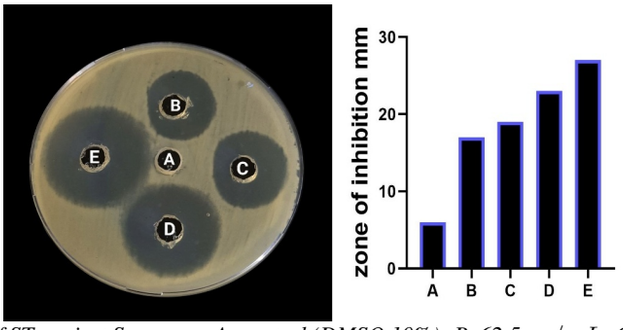


**Figure 11.** *Antibacterial activity of ST against S. aureus: A, control (DMSO 10%); B, 62.5 $\mu g/mL$; C, 125 $\mu g/mL$; D, 250 $\mu g/mL$; E, 500 $\mu g/mL$.*

**Table 3.** *Antibacterial analysis based on the zone of inhibition (mm).*

| Sample | | A | B | C | D | E |
|---|---|---|---|---|---|---|
| *S. aureus* | M4 | 6 | 30 | 36 | 39 | 45 |
| *K. pneumoniae* | | 6 | 18 | 19 | 22 | 37 |

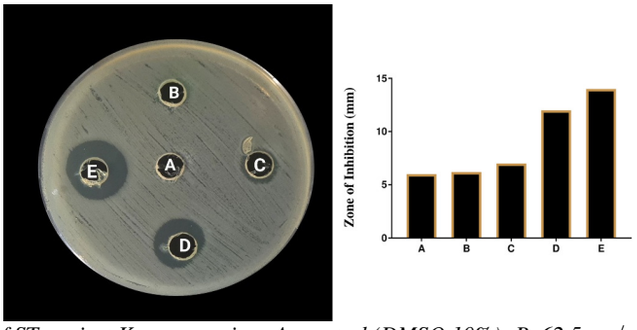


**Figure 12.** *Antibacterial activity of ST against K. pneumoniae: A, control (DMSO 10%); B, 62.5 $\mu g/mL$; C, 125 $\mu g/mL$; D, 250 $\mu g/mL$; E, 500 $\mu g/mL$.*

## 4. CONCLUSION

This research successfully synthesized and characterized a novel hydroxylamine derivative of tinidazole (M4), demonstrating a strategic advancement in combating antimicrobial resistance. The meticulous spectroscopic analysis confirmed the successful transformation of tinidazole into its hydroxylamine counterpart. Crucially, the in vitro biological evaluation revealed that M4 exhibits significantly superior antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* compared with the parent drug. This enhanced potency is primarily due to the hydroxylamine moiety's ability to directly exert antimicrobial effects, circumventing the enzymatic activation pathway that often contributes to tinidazole resistance. The findings underscore the therapeutic potential of hydroxylamine-modified nitroimidazoles and pave the way for the development of more effective and resistance-evading antimicrobial agents. Future work should focus on in vivo studies, toxicity profiling, and further optimization of the hydroxylamine scaffold to broaden its antimicrobial spectrum and clinical applicability.

## DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

In the course of preparing this manuscript, the authors employed ChatGPT to improve the linguistic quality and readability of the content. After utilizing this AI tool, the authors conducted comprehensive review and necessary revisions to ensure the accuracy, integrity, and scientific rigor of the manuscript. All authors bear full responsibility for the final content of the published work, including any errors or inconsistencies that may arise.

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