Unveiling the Efficacy of 3-Hydrazinoquinoxaline-2-Thiol Against *Pseudomonas aeruginosa*

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ABSTRACT

***Pseudomonas aeruginosa* (*P. aeruginosa*) is a serious opportunistic pathogen, especially in immunocompromised individuals and those with cystic fibrosis. Its ability to acquire resistance poses challenges in clinical settings. *P. aeruginosa* has increasingly developed resistance to the last-resort antibiotics. Repurposing, also known as drug repositioning, is a strategic approach in pharmaceutical research aimed at discovering new therapeutic applications for existing medications. This study assesses the *in vitro* efficacy of 3-hydrazinoquinoxaline-2-thiol (3HTQ) against *P. aeruginosa*, following promising results with quinoxaline derivatives on other bacterial strains. Broth microdilution-assay was utilised to assess the activity of 3HTQ against 63 different clinical isolates of *P. aeruginosa.* The Minimum Inhibitory Concentrations (MICs) of the tested antibiotic were determined for 63 isolates of *P. aeruginosa*. The MIC levels ranged from 8 µg/mL to 128 µg/mL, whereas the majority isolates (n=32, 50.8%) were at 64 µg/mL, highlighting diverse antibiotic susceptibility among P. aeruginosa isolates. Furthermore, we noticed that sixteen isolates (25.4%) and twelve isolates (19%) demonstrated an MIC of 32 µg/mL and 128 µg/mL respectively. Notably, three isolates (4.8%) displayed an MIC of 8 µg/mL, reflecting the highest level of susceptibility among the tested isolates. These findings enforce the requirement of further tests of the genetic variability of these strains with high MIC values to comprehend the resistance mechanisms and their virulence determinants. Further mechanistic studies are required to elucidate the reasons behind these variations in susceptibility. Moreover, exploring the potential of 3HTQ in combination therapies could enhance its overall antimicrobial activity.**

Key words: *Pseudomonas aeruginosa,* Repurposing, 3-hydrazinoquinoxaline-2-thiol, Antimicrobial resistance, Gram-negative bacteria.

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INTRODUCTION

P. aeruginosa is a gram-negative-rod-shaped, anaerobic bacterium that tests positive for oxidase and does not ferment lactose. Known for its versatile metabolism and significant antibiotic resistance [1], [2]. It is widespread and commonly found in soil, water, and on plant surfaces. However, it is a notorious opportunistic organism creaing various infections, particularly in immunocompromised individuals and patients diagnosed with cystic fibrosis [3], [4]. This pathogen can form biofilms and develop antimicrobial resistance mechanisms leading to significant health challenges in clinical settings, due to the development of multi-drug resistance species. *P. aeruginosa*poses a major threat in both hospital and community settings, causing wide range of healthcare-associated and community-acquired bloodstream infections. These infections are often associated with delays in effective antibiotic treatment and higher mortality rates [4], [5]. Hospital acquired infections primarily impact on patients in intensive care units, as well as those undergoing catheterization, suffering from burns, or dealing with chronic illnesses [6].

It has been recognized as the second most frequent cause of ventilator-associated pneumonia, the fourth leading cause of catheter-associated urinary tract infections, the fifth most major responsible for surgical site infections, and the seventhcause of central line-associated bloodstream infections [7]. *P. aeruginosa* isolates are one of the most frequently isolated pathogens in Saudi hospital. Around 11% of all nosocomial infections and up to 31% of those caused by gram-negative bacteria. This pathogen is found in 13-25% of wound infections, up to 16%of bacterial respiratory infections,6-16% of urinary tract infections (UTIs),and7-11% of bacteraemia. There are significant challenges in the management of *P. aeruginosa* infection due to its inherent resistance to many drugfamilies and its ability to obtain additional resistance genes through mobile elements. The prevalence and resistance patterns of *P. aeruginosa* vary widely depending on the period, location, and type of species [8]–[10]. According to the 2015 antimicrobial resistance surveillance data, the development of carbapenem resistance across *P. aeruginosa* was notably high, with a prevalence of 66% in Romania. Across Europe, the average rate of carbapenem resistance in *P. aeruginosa* was 17.8% [11]. In the United States, a previous study utilizing data from nationally representative sample of microbiological laboratories reported that 22% of *P. aeruginosa* isolates causing pneumonia were classified as multidrug-resistant [12].

P. aeruginosa presents significant treatment challenges due to its intrinsic and acquired antibiotic resistance mechanisms, such as impermeable outer membranes and efflux pumps [4]. These mechanisms can complicate infections and contribute to their severity. This bacterium utilizes several resistance strategies to combat antibiotics [13]. Efflux pumps actively expel antibiotics from the bacterial cell, reducing their intracellular concentration and rendering them ineffective against a broad range of antibiotics, including β-lactams and aminoglycosides [14], [15]. Additionally, the outer membrane of *P. aeruginosa* has reduced permeability compared to other gram-negative bacteria, resulting in limiting antibiotics entry and diminishing their efficacy [16]. The bacterium also produces antibiotic-degrading enzymes, such as β-lactamases, which hydrolyze and inactivate antibiotics, conferring resistance to penicillin, cephalosporins, and carbapenems [17], [18]. Furthermore, *P. aeruginosa* can form biofilms to create a protective environment, shielding bacterial cells from antibiotics and the host immune system, thereby fostering chronic infections [19]. Recently, *P. aeruginosa* has increasingly developed resistance to colistin, a last-resort antibiotic [20]. Thus, alternative treatment methods are immediately required to address this issue [21]. Repurposing, also known as drug repositioning, is a strategic approach in pharmaceutical research aimed to discover new therapeutic applications for existing medications [21].

In a previous study, Elfadil et al. displayed the remarkable effectiveness of quinoxaline derivatives versus different clinical methicillin-resistant *Staphylococcus aureus* (MRSA) strains [22]. More recently, another investigation assessed the efficacy of these derivatives against ESBL-producing bacteria [23]. Building on this research, we assume that quinoxaline derivatives may also show activity against clinical *P. aeruginosa*. To our knowledge, the *in vitro* efficacy of 3HTQ against *P. aeruginosa* has not been studied before. Therefore, this study aims to examine the *in vitro* activity of 3HTQ against *P. aeruginosa.*

MATERIAL AND METHODS

Antibacterial Compounds

The compounds used in this paper, specifically 3HTQ, were procured from Fluorochem Ltd., a reputable supplier based in the United Kingdom. The choice of 3HTQ was chosen by its promising antibacterial properties, which have been noted in previous studies. Upon receipt, the compound was stored under appropriate conditions as specified by the supplier to ensure its stability and integrity prior to experimentation. For all experiments, the compound was accurately weighed using a high-precision analytical balance to obtain the desired concentration for subsequent testing. To prepare the working solution, 3HTQ was dissolved in DMSO, a solvent known for its ability to effectively dissolve a wide range of organic compounds. The dissolution process involved adding the measured quantity of 3HTQ to a pre-determined volume of DMSO, followed by vigorous stirring to ensure complete dissolution. The resulting solution was then aliquoted and kept at -20°C for further assays. Before each experiment, the solution was brought to room temperature and thoroughly mixed to ensure homogeneity. The concentration of DMSO in the final test solutions was carefully controlled and kept at a minimum to avoid any potential solvent-related effects on bacterial cells. This method of preparation was consistent across all experiments to ensure reproducibility and reliability of the results. The use of DMSO as a solvent was based on its compatibility with biological assays and its minimal interference with antibacterial activity measurements.

Bacterial isolates

This study focused on the examination of sixty-two *P. aeruginosa*, encompassing various species. These isolates were sourced from Microbiology Culture in Clinical and Molecular Microbiology Laboratories in King Abdulaziz University Hospital in Jeddah, Saudi Arabia.. Upon acquisition, the bacterial isolates were maintained in glycerol and stored at a temperature of -80°C to maintain their viability. Prior to any experimental procedures, the bacteria were carefully thawed and cultivated on sheep blood agar by (Saudi prepared Media Laboratory Company). These cultures were then incubated for 20 hours at 35-37°C in an aerobic condition to ensure optimal bacterial environement. Identification and antibiotic susceptibility testing of the isolates were conducted using the Vitek 2 system (BioMerieux, France), utilizing GN Card for identification of GNB and AST-N419 & AST-N417 for susceptibility testing of clinically significant aerobic Gram negative bacilli. This system follows the manufacturer's guidelines to ensure accurate and reliable results.

The sample collection process adhered to the guidelines established by the Ethics and Research Committee of the Faculty of Applied Medical Sciences at King Abdulaziz University (Reference No 301-24), in compliance with the Declaration of Helsinki. Since the clinical isolates used in this study were obtained as part of the hospital's standard

laboratory procedures, the ethics committee waived the requirement for informed consent.

Broth Microdilution Assay

To assess the antimicrobial sensitivity of the tested compounds, we employed a detailed and rigorous broth microdilution assay. This assay is a cornerstone in antimicrobial research, providing precise measurements of the inhibitory effects of antimicrobial agents. Firstly, we prepared two-fold serial dilutions of the tested compound in MHB (Sigma-Aldrich, United States). This medium is widely recognized for its suitability in antimicrobial susceptibility testing due to its nutrient composition, which supports the growth of a broad range of bacterial species. The serial dilutions were meticulously prepared to ensure accurate concentration gradients for the assay. Next, we dispensed 100 µl aliquots of each prepared antimicrobial solution into the individual wells of sterile 96-well microtiter plates (Corning, Italy). These plates are designed to provide a uniform environment for bacterial growth and to facilitate precise measurement of antimicrobial activity. The use of high-quality microtiter plates is essential to minimize potential contamination and ensure reliable results. The bacterial inoculum was prepared and its density was standardized to 0.5 McFarland, using a suspension-turbidity-detector (Biosan Densitometers DEN-1B). The McFarland standard is a turbidity reference used to approximate the number of bacteria in a liquid suspension, ensuring that the inoculum size is consistent across all wells. This standardization is essential as it directly affects the reproducibility and accuracy of the antimicrobial susceptibility testing. Following the calibration of the bacterial suspension, we transferred 5 µl aliquots of the standardized inoculum into the wells containing the antimicrobial solutions. Accurate inoculation is critical to the validity of the MIC determination. The inoculated plates were then incubated for 20 hours at thirty seven °C in an aerobic condition. This incubation period allows for sufficient bacterial growth to occur, enabling the observation of any inhibitory effects exerted by the antimicrobial agents. The temperature and incubation conditions were carefully controlled to mimic the optimal growth conditions for the bacterial isolates under study [23], [24]. To ensure the reliability and reproducibility of our findings, we conducted the antimicrobial susceptibility testing in triplicate. Each set of tests was independently performed, and the mean-values were noted for subsequent interpretation. This triplicate approach helps to account for any variability and ensures the robustness of the results. The key outcome of this assay, the MIC, was identified by identifying the lowest concentration of the antimicrobial agent that completely inhibited visible bacterial growth. The MIC levels were evaluated using the exact broth microdilution technique, adhering strictly to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI). The CLSI guidelines provide a standardized framework for conducting and interpreting antimicrobial susceptibility tests, ensuring that our results are accurate and comparable to other studies in the field [25]. This thorough and methodologically rigorous approach ensures that the study outcomes are precise and aligned with established standards in microbiological assessment. By adhering to these standards, we enhance the reliability and validity of our findings, contributing valuable data to the field of antimicrobial research.

Assessment of Cytotoxicity of 3-Hydrazinoquinoxaline-2-Thiol in HepG2 Cells Using the MTT Assay

The cytotoxicity of 3HTQ was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Solarbio-China). HepG2 human hepatocellular carcinoma cells were cultured in a 96-well plate at a density of 5,000 cells per well. The cells were treated with increasing concentrations of 3-Hydrazinoquinoxaline-2-Thio (100, 200, and 400 µg/ml) and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. Following this, the incubation period, the treatment media was carefully removed, and the cells were washed once with phosphate-buffered saline (PBS). MTT solution, made atfinal concentration of 0.5 mg/ml. The cells were then incubated for an additional 3-4 hours at 37°C to allow for MTT reduction. After the incubation, the media was removed, and the resulting formazan crystals were dissolved in DMSO. The absorbance was weighed at 590 nm via a microplate-reader (BioTek 800 TS Absorbance Reader-United States) to quantify cell viability.

Statistical Analysis

The statistical analysis for this study was meticulously conducted utilising GraphPad Prism version 8, a robust and widely recognized statistical software. Each experiment was carried out at least three times to ensure the reproducibility and reliability of our results. From these repetitions, we calculated the average values and standard deviations (SD), which provided measures of central tendency and dispersion, respectively. These statistical measures are essential for understanding the variability and consistency of our data. To compare differences between groups, we utilized an unpaired t-test. This test is specifically important for assessing whether there is a significant difference between the means of two independent groups. Additionally, to evaluate differences in survival rates between experimental groups, we employed both the log-rank test and the Gehan-Breslow-Wilcoxon test. These tests are specifically designed to analyse time-to-event data, allowing us to assess the statistical significance of survival distributions. The threshold for statistical-significance was set at a P-value of 0.05. finding were considered significant if the P-value was equal to 0.05 or less. To convey the levels of significance, we used a system of asterisks: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. This notation helps to quickly and clearly communicate the strength of the statistical evidence. Detailed information on the statistical analyses, including the specific tests used and the interpretation of the results, is provided in the figure legends. This comprehensive approach ensures that the reader can fully understand the statistical context and the significance of the findings presented in the figures. Statistical analysis was done utilising one-way ANOVA to assess significant differences in cell viability among the different concentrations of 3HTQ. A p-value < 0.05 was considered statistically significant.

RESULTS

The MICs of the tested antibiotic were determined against 63 isolates of *P. aeruginosa*.

The MIC values, measured in micrograms per millilitre (µg/ml), are presented in (Table 1).

Table 1. The antibiotic susceptibility testing using broth microdilution of 63 isolates of *P. aeruginosa*. MIC: minimum inhibitory concertation (µg/ml)

Number of P. aeruginosa strains	MIC (µg/ml)
1	64
2	64
3	64
4	64
5	64
6	128
7	64
8	64
9	64
10	32

11	64
12	32
13	8
14	64
15	64
16	128
17	64
18	32
19	32
20	64
21	64
22	64
23	64
24	64
25	32
26	32
27	64
28	64
29	64
30	32
31	64
32	32
33	8
34	64
35	32
36	32
37	128
38	128
39	32
40	64
41	64
42	128
43	32
44	32
45	64
46	64
47	64
48	32
49	128
50	128
51	128
52	128
53	32
54	64
55	128
56	128
57	32
58	8
59	64
60	64
61	64
62	32
63	64

The distribution of MIC values shows a vary from 8 µg/ml to 128 µg/ml. Notably, most of the isolates had MIC values of 64 µg/ml, indicating a moderate level of susceptibility to the antibiotic. The MIC levels of 32 µg/ml and 128 µg/ml were also frequently observed, while a small number of isolates displayed an MIC of 8 µg/ml, suggesting higher susceptibility. The variability in MIC values highlights the diverse response of *P. aeruginosa* isolates to the antibiotic, underscoring the importance of individualized susceptibility testing for effective treatment planning.

The distribution of MIC values shows a vary from 8 µg/ml to 128 µg/ml. To better understand the prevalence of each MIC value, the percentages for the most common values are calculated (Table 2).

Table 2. MIC values of 3HTQ against clinical isolates of *P. aeruginosa*. The table shows the distribution of isolates based on their MIC values, with the number and percentage of isolates for each concentration. MIC values are represented in micrograms per milliliter (µg/mL).

MIC value	Number of isolates	Percentage
64 µg/mL	32 isolates	50.8%)
32 µg/mL	16 isolates	25.4%
128 µg/mL	12 isolates	19.0%
8 µg/mL	3 isolates	4.8%

There was a significant difference in the susceptibility profiles between sensitive and resistant strains of *P. aeruginosa* (*P value*< 0.05) (Figure 2). The higher resistance values observed in certain strains against 3-hydrazinoquinoxaline-2-thiol could be attributed to the acquisition of different antimicrobial resistance mechanisms. This significant variation in MIC values between resistant and susceptible strains indicates that the resistant *P. aeruginosa* may have developed or acquired specific genetic or biochemical pathways that confer resistance to this compound. Such mechanisms might include the upregulation of efflux pumps, which actively expel the antibiotic from bacterial cells, the modification of target sites, rendering the antibiotic to be less effective, or the production of enzymes that can degrade or modify the compound. These findings underscore the complexity of bacterial resistance and highlight the necessity for comprehensive studies to elucidate the precise mechanisms underlying this resistance. Understanding these pathways is crucial for developing more effective therapeutic strategies and optimizing the use of 3-hydrazinoquinoxaline-2-thiol in clinical settings. Further research should focus on genetic and biochemical analyses to pinpoint the exact resistance mechanisms and evaluate potential combination therapies that could enhance the efficacy of this promising compound against resistant *P. aeruginosa* strains.

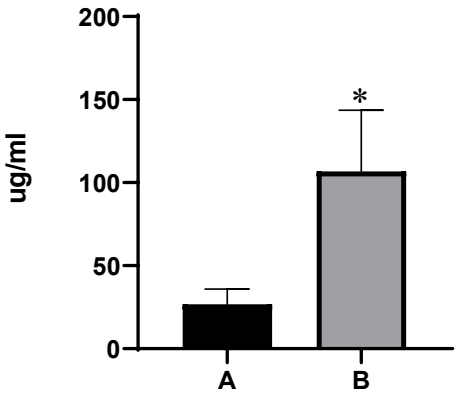

Figure 1. Comparison of MIC values for 3-hydrazinoquinoxaline-2-thiol between sensitive and resistant *P. aeruginosa* strains. The MIC values exhibit significant variation, with resistant strains showing higher resistance levels. The statistical analysis indicates a significant difference between the sensitive and resistant strains, with a P value of 0.022 (*P < 0.05). This suggests that resistant strains may have developed or acquired specific genetic or biochemical pathways conferring resistance to 3-hydrazinoquinoxaline-2-thiol. *P. aeruginosa* sensitive strain (A) and resistant strain (B).

Effect of 3HTQ on HepG2 Cell Viability at Different Concentrations

The figure presents the cytotoxicity of 3-Hydrazinoquinoxaline-2-Thio at concentrations of 100, 200, and 400 µg/ml on HepG2 cells, compared to a control group. Viability of the cells was examined using the MTT assay, and the findings are determined as a percentage relative to the control group, which is considered 100% viable. The graph shows that at all tested concentrations (100, 200, and 400 µg/ml), there was no statistically significant decrease in cell viability in comparison with the control group, as indicated by the "ns" labels (not significant). This suggests that 3HTQ, at the concentrations tested, did not significantly affect the viability of HepG2 cells following 24-hours of therapy.

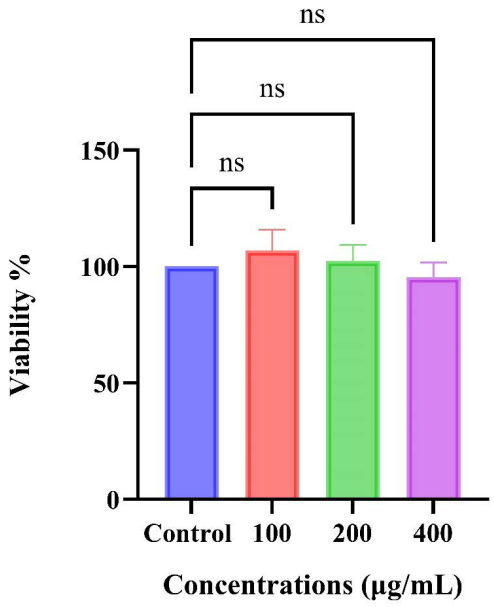

Figure 2. Cytotoxicity of 3-Hydrazinoquinoxaline-2-Thiol on HepG2 cells following 24 hours of treatment at a concentrations of 100, 200, and 400 µg/ml. Findings are expressed as mean ± standard error of the mean (SEM). Statistical analysis was done utilising one-way ANOVA, with no significant differences observed between the control and treated groups (ns = not significant, $p > 0.05$).

DISCUSSION

The emergence of antibiotic-resistant bacteria represents a significant and growing threat to public health globally, driving the urgent need to identify and develop new compounds capable of effectively combating bacterial infections [26]. This issue is particularly acute given the widespread prevalence of multidrug-resistant (MDR) strains, which render many conventional antibiotics ineffective [27]. Within this context, it becomes imperative to explore and evaluate novel antimicrobial agents with the potential to address this pressing concern [21].

In our study, we have focused on assessing the activity of 3HTQ, a compound that has not previously been investigated for its activity versus a diverse range of *P. aeruginosa* clinical strains. This marks a significant gap in the existing research literature, as the evaluation of this compound against such resistant pathogens is unprecedented.

Our findings indicate that 3HTQ exhibits notable antimicrobial activity with against the tested panel of *P. aeruginosa* clinical strains. This promising activity suggests that the compound holds potential as a candidate for further development and use in the fight against antibiotic-resistant infections. However, one of the most remarkable notices from our study was the considerable variation in the MIC values of 3HTQ across different bacterial strains. This variability highlights the complex interactions between the compound and the diverse bacterial targets, suggesting that its efficacy may be influenced by specific genetic and phenotypic characteristics of the pathogens.

Such findings underscore the necessity for continued research to better understand the mechanismsunderlying the observed variations in antimicrobial activity. Detailed investigations into the genetic and molecular determinants of resistance in these bacterial strains will be crucial in elucidating the factors that contribute to the differing MIC values. This knowledge will, in turn, inform the optimization and potential clinical application of 3HTQ as a therapeutic agent versus resistant infections.

The distribution of MIC values shows a range from 8 µg/mL to 128 µg/mL. Notably, the majority of the isolates had MIC values of 64 µg/mL, indicating a moderate level of susceptibility to the antibiotic. The MIC levels of 32 µg/mL and 128 µg/mL were also frequently observed, while a small number of isolates showed an MIC of 8 µg/mL, suggesting higher susceptibility. The variability in MIC values highlights the diverse response of *P. aeruginosa* isolates to the antibiotic, underscoring the importance of individualized susceptibility testing for effective treatment planning.

The distribution of MIC values reveals significant variability in the susceptibility of *P. aeruginosa* isolates to the tested antimicrobial agent. This variability indicates that while a substantial portion of isolates (50.8%) demonstrated moderate susceptibility with an MIC of 64 µg/ml, there is a notable presence of both highly resistant and more susceptible strains within the sample set. A total of 12 isolates (19.0%) exhibited an MIC of 128 µg/ml. These strains are categorized as highly resistant to the tested antibiotic, indicating a strong capability to survive even at high concentrations of the drug. The presence of these highly resistant species led to a significant threat in clinical-settings, requiring alternative treatment strategies or combination therapies. Most of the isolates, 32 out of 63 (50.8%), displayed an MIC of 64 µg/ml. These strains demonstrate moderate resistance, suggesting that while the antibiotic can inhibit their growth, it requires a relatively higher concentration to do so. This level of resistance may still be manageable with higher doses or prolonged treatment duration. 16 isolates (25.4%) displayed an MIC of 32 µg/ml, indicating a lower level of resistance. These strains are more susceptible to the antibiotic, as it can effectively inhibit their growth at a lower concentration. This finding is encouraging, as it suggests that a portion of the *P. aeruginosa* population remains treatable with standard antibiotic dosages. A small number of isolates, 3 out of 63 (4.8%), exhibited an MIC of 8 µg/ml. These strains are highly susceptible to the antibiotic, with even minimal concentrations being sufficient to inhibit their growth. The presence of these strains indicates that despite the overall trend towards resistance, some *P. aeruginosa* isolates remain highly treatable.

To gain a deeper understanding of the observed MIC variation, we further categorized the isolates into two groups: those known to be resistant, including carbapenem-resistant Enterobacteriaceae (CRE) strains, and those identified as susceptible. This distinction allowed us to analyze the antibiotic's efficacy across different resistance profiles. Among the isolates identified as resistant, including CRE strains, we observed a higher frequency of elevated MIC values. Specifically, these strains exhibited MICs predominantly in the range of 64 to 128 µg/mL, underscoring their robust defense mechanisms against the

antibiotic. The prevalence of high MIC values in this group highlights the need for more aggressive or alternative therapeutic approaches to manage infections caused by these resistant pathogens. Conversely, the susceptible strains showed a trend towards lower MIC values, primarily within the 8 to 32 µg/mL range. This group responded well to the antibiotic, with effective inhibition at lower concentrations. The presence of these susceptible strains within the clinical isolates offers a glimmer of hope, suggesting that the antibiotic remains a viable option for treating a subset of *P. aeruginosa* infections.

In this study, 3HTQ was tested for cytotoxicity on HepG2 cells at concentrations of 100, 200, and 400 µg/ml, with no significant reduction in cell viability observed at any of these doses in comparison with the control group. This indicates that 3HTQ exhibits minimal cytotoxic effects on HepG2 cells within this concentration range. Importantly, the MIC against *P. aeruginosa* was determined to be 256 µg/ml, a concentration higher than those tested for cytotoxicity. Given that the compound was well tolerated at lower concentrations, its antimicrobial efficacy at 256 µg/ml against *P. aeruginosa* may be achievable without significant toxicity, supporting its potential as a therapeutic candidate.

The higher resistance values observed in certain strains against 3HTQ could be attributed to the acquisition of different resistance mechanisms. This significant variation in MIC values between resistant and susceptible strains indicates that the resistant *P. aeruginosa* may have developed or acquired specific genetic or biochemical pathways that confer resistance to this compound.

Further studies are essential to delve into the resistance mechanisms employed by these resistant strains. Understanding the molecular and genetic bases of their resistance can provide critical insights into how these bacteria withstand the effects of 3HTQ. Such research will not only enhance our comprehension of the compound's efficacy but also aid in devising strategies to overcome resistance, ensuring the continued utility of this promising antimicrobial agent in clinical settings.

The activity of 3HTQ against Gram-positive bacteria, specifically MRSA, has been previously demonstrated, showcasing its potential as a potent antimicrobial agent [28]. Moreover, it has been demonstrated the good activity of 3HTQ versus certain strains of Gram-negative, extended-spectrum beta-lactamase (ESBL)-producing bacteria [23]. The results of our study are promising and indicate a potentially new therapeutic option with several distinct advantages. One of the most significant findings of our research is the demonstrated activity of 3-hydrazinoquinoxaline-2-thiol against various *P. aeruginosa*. These pathogens are known for their high resistance to many commonly used antibiotics, making the search for effective treatments particularly urgent. The ability of 3-hydrazinoquinoxaline-2-thiol to effectively inhibit certain strains of *P. aeruginosa* suggests that it could play a crucial role in managing infections caused by multidrug-resistant organisms. Furthermore, the broad-spectrum efficacy of 3HTQ, encompassing both Gram-positive and Gram-negative bacteria, indicates its potential as a versatile antimicrobial agent. This broad coverage within a range of bacterial species could make it a valuable addition to the current arsenal of antibiotics, particularly in the treatment of mixed bacterial infections or in scenarios where the causative pathogen has not yet been identified.

The mechanisms by which 3HTQ exerts its antibacterial effects appear to be multifaceted. Preliminary evidence suggests that it may inhibit bacterial growth through several pathways, including the prevention of DNA synthesis and the induction of reactive oxygen species (ROS) [29], [30]. These mechanisms disrupt critical cellular processes within the bacteria, leading to their eventual death [31]. The ability to target multiple bacterial processes simultaneously is a desirable trait in an antibiotic, as it reduces the likelihood of resistance development. Given these findings, further study is warranted to fully reveal the detailed mechanisms of action of 3-hydrazinoquinoxaline-2-thiol. Understanding how it interferes with bacterial cellular machinery will not only enhance our knowledge of its antimicrobial properties but also aid in optimizing its use in clinical settings. Additionally, studies focusing on the pharmacokinetics, safety, and efficacy of this compound *in vivo* are essential to determine its suitability for use in humans [32].

Despite demonstrating a weaker effect against certain strains of *P. aeruginosa*, 3-hydrazinoquinoxaline-2-thiol represents a promising candidate for further exploration, particularly in the context of combination therapies and the advancement of treatment modalities. The potential for this compound to be used in conjunction with established antibiotics is an avenue worth investigating, as it may lead to synergistic effects that enhance overall antimicrobial efficacy. By combining 3HTQ with other antibiotics, it is possible to achieve a more robust and comprehensive antibacterial strategy. This approach could not only improve the effectiveness of treatment regimens but also help in mitigating the risk of bacterial resistance developing against both the new compound and existing antibiotics. The exploration of such synergistic combinations is critical, as it addresses one of the significant challenges in contemporary antimicrobial therapy—the rapid emergence of bacterial resistance. By leveraging the unique properties of 3-hydrazinoquinoxaline-2-thiol in combination with other therapeutic agents, we may be able to prolong the clinical utility of current antibiotics and introduce novel treatment paradigms. This strategy could lead to more effective interventions against resistant bacterial strains, thereby enhancing patient outcomes and public health. Moreover, these combination therapies could provide a dual mechanism of action, disrupting bacterial growth through multiple pathways and reducing the likelihood of resistance [33]. The investigation into these synergistic effects and the development of combination treatments should be prioritized in future research to fully realize the potential of 3-hydrazinoquinoxaline-2-thiol and to address the pressing issue of antibiotic resistance in *P. aeruginosa* and other pathogenic bacteria.

In summary, this study highlights the promising potential effect of 3-hydrazinoquinoxaline-2-thiol as an effective antimicrobial agent against certain strains of *P. aeruginosa*. Its broad-spectrum activity and multifaceted mechanisms of action make it a compelling candidate for further development and clinical application. As antibiotic resistance continues to pose a critical challenge to global public health, the introduction of new therapeutic options like 3-hydrazinoquinoxaline-2-thiol could be promising in our ongoing battle against bacterial infections.

CONCLUSION

The MIC distribution among the 63 of *P. aeruginosa* isolates underscores the complex landscape of antibiotic resistance within this bacterial species. While a significant portion of the isolates exhibited moderate to high resistance, there remains a subset that is highly susceptible to the tested antibiotic. This variability necessitates a tailored approach to treatment, taking into account the specific resistance profiles of the infecting strains. The differentiation between resistant and susceptible strains provides valuable insights for developing targeted therapeutic strategies, ultimately aiming to improve clinical outcomes in the face of rising antibiotic resistance.

Abbreviations

MUH, Muller Hinton broth, 3-hydrazinoquinoxaline-2-thiol ,3HTQ, ESBL, Extended-Spectrum Beta-Lactamases, MIC, minimum inhibitory concentration, MDR, multidrug resistant, ROS, Reactive oxygen species, *P. aeruginosa, Pseudomonas aeruginosa*

Funding: This research paper was conducted independently and did not receive any financial support or funding from external sources. All aspects of the research, including design, data collection, analysis, and interpretation of results, were carried out solely by the authors without reliance on any financial grants or contributions. The independence of this work underscores the objectivity and impartiality of the research findings presented in this paper.

Author contributions: The study was conceptualized by, Jawahir A. Mokhtar, and Abdelbagi Alfadil. Methodology was developed by Ahmad M. Sait, Karem Ibrahem and Dalya Attallah. Data collection and analysis were carried out by Ahmad M. Sait, Karem Ibrahem and Abdelbagi Alfadil. Manuscript writing was undertaken by Jawahir A. Mokhtar, Ahmad M. Sait and Karem Ibrahem. Manuscript criticising, revision, editing and formatting was done by Hanouf A. Niyazi, Hatoon A. Niyazi, Noof R. Helmi, Hussam Daghistani, Yousef Almoghrabi, Khalil Alkuwaity, Mohammed Mufrrih, Mazen A. Ismail, Ohood S. Alharbi, Wafaa Alhazmi, Mona Abdulrahman Alqarni,Tariq Ekhmimi and Bandar Hasan Saleh[1]. All authors have read and approved the final manuscript.

Potential Conflict of Interest: None

Competing Interest: None

Acceptance Date: 02-11-2024

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