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**RESEARCH ARTICLE**

***Ibrahem et al: TQ-3HQ synergy against CTX-M-15 ESBL***

# Thymoquinone and 3HQ synergy inhibits CTX-M-15 ESBL

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## ABSTRACT

Bacterial infections remain a significant cause of mortality worldwide, further aggravated by the escalating issue of antibiotic resistance. Extended-Spectrum Beta-Lactamases (ESBLs) pose a substantial challenge, capable of hydrolyzing various beta-lactam antibiotics. The slow pace of drug discovery, coupled with the rapid emergence of drug-resistant bacteria, underscores the urgent need for innovative therapeutic solutions. Thymoquinone (TQ), derived from the seeds of *Nigella sativa*, has demonstrated notable antibacterial activity against Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*. Previous research has established the efficacy of quinoxaline derivatives, such as 3-hydrazinoquinoxaline-2-thiol (3HQ), against Methicillin-Resistant *Staphylococcus aureus* (MRSA). This study investigates the potential synergy between 3HQ and TQ against various clinical strains of ESBL. The minimum inhibitory concentrations (MICs) of TQ and 3HQ were evaluated against 18 clinical ESBL strains, revealing MIC values ranging from 16 to 128 µg/mL for both compounds. Furthermore, the interaction between TQ and 3HQ was assessed using a checkerboard assay, which demonstrated a 100% synergistic interaction, with a fractional inhibitory concentration index (FICI) of less than 0.5 against the ESBL strains. Docking and molecular dynamics simulations indicated that TQ exhibits a strong binding affinity and interaction profile comparable to that of RPX-7063. In contrast, 3-hydrazinoquinoxaline-2-thiol targets a different active site, potentially enhancing thymoquinone's binding efficiency. Collectively, these compounds may effectively inhibit CTX-M-15, as evidenced by their docking scores and interaction profiles. Further investigations, including *in vivo* studies, are essential to validate these findings. This research suggests a promising strategy for developing more effective treatments for ESBL infections, emphasizing the need for in vivo validation.

**Keywords:** AMR, ESBL, FICI, 3HQ, thymoquinone.

## INTRODUCTION

The enduring threat of bacterial infections remains a major factor contributing to mortality rates worldwide, presenting an ongoing challenge compounded by the increasing issue of antibiotic resistance [1]–[3]. Extended-Spectrum Beta-Lactamases (ESBLs) pose a significant challenge within contemporary healthcare environments, primarily due to their correlation with multidrug-resistant organisms. This association poses a significant and multifaceted challenge, requiring integrated and effective strategies for control and management in healthcare environments [4], [5]. ESBLs, which are prevalent worldwide, exhibit a higher prevalence in regions such as Europe and the United States. They are synthesized by various members of the Enterobacteriaceae family, as well as *Pseudomonas aeruginosa*, and possess the ability to hydrolyze several beta-lactam antibiotics, including aztreonam and third-generation cephalosporins [6]–[8]. The rise and widespread occurrence of ESBLs carry significant consequences for treatment strategies, given that these enzymes grant resistance to a wide array of antibiotics, especially those belonging to the beta-lactam class. This poses a considerable threat, limiting treatment options and challenging traditional approaches to combating bacterial infections [9], [10]. CTX-M extended-spectrum β-lactamase (ESBL)-producing Klebsiella pneumoniae isolates are rarely reported in the United States. CTX-M-type ESBL enzymes have been identified in non-E. coli Enterobacteriaceae species, including *Klebsiella spp*., *Proteus mirabilis*, *Enterobacter* spp., *Salmonella* spp., *Shigella* spp., and *Morganella morganii*. Recently, the emergence of these enzymes has also been documented in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [11], [12].

The slow advancement in drug discovery, along with the rapid spread of drug-resistant bacteria, raises serious concerns for global health. In 2019, bacterial AMR caused 1.27 million deaths globally, with highest rates in sub-Saharan Africa. Six key pathogens drove most deaths including, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa—accounted for most of these fatalities. Methicillin-resistant *S. aureus* alone caused over 100,000 deaths, with other major contributors including drug-resistant *E. coli*, *K. pneumoniae*, and *A. baumannii* [13]. Projections indicate that infections caused by drug-resistant organisms could result in 10 million fatalities by

the year 2050. Moreover, this trend may lead to treatment failures, elevated medical expenses, prolonged hospital stays, and heightened socioeconomic burdens [14]–[16]. Therefore, there is an immediate and critical need for innovative approaches to address these challenges [17]. The combination of antibiotics are well-established, efficient, and economically viable strategy for tackling resistant bacterial infections [18]. Utilizing a synergistic approach to drug development, particularly through the repurposing of existing medications rather than the creation of entirely new compounds from scratch, has been demonstrated to offer significant advantages [19], [20]. Notably, this approach has the potential to yield savings exceeding $1 billion and slash the time required for FDA approval by half [21]. By leveraging existing drugs in novel combinations, researchers can capitalize on their established safety profiles and known pharmacological properties, streamlining the drug development process and accelerating the availability of effective treatments for resistant bacterial infections [22], [23]. This innovative strategy not only addresses the urgent need for novel antimicrobial agents but also offers a practical and efficient pathway to combat the growing threat of antibiotic resistance [24].

TH, an active constituent extracted from the seeds of Nigella sativa, commonly referred to as black cumin or black seed, is a naturally derived compound with significant biological activity. This bioactive substance, revered for its medicinal properties, is inherent to the seeds of the Nigella sativa plant, which have been utilized for centuries in traditional medicine and culinary practices across various cultures [25]. Thymoquinone's natural occurrence within these seeds demonstrates its potential as a therapeutic agent, with research exploring its diverse pharmacological effects and potential applications in healthcare and wellness [26]. It has demonstrated notable antibacterial effects against both Gram-negative bacterial strains. Additionally, its antimicrobial properties extend to combating biofilm formation, particularly in Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. This dual action against bacterial growth and biofilm formation illustrates the potential therapeutic significance of TH in addressing microbial infections, particularly those caused by Gram-negative pathogens known for their resilience and ability to form biofilms, which can exacerbate antibiotic resistance and treatment challenges [27], [28]. A study conducted earlier by Elfadil et al. showcased the significant efficacy of

quinoxaline derivatives, particularly emphasizing 3HQ, against a diverse range of clinical strains commonly linked to Methicillin-Resistant *Staphylococcus aureus* (MRSA) [2], [29]. Additionally, our research has unveiled that 3HQ possesses activity against Gram-negative strains producing Extended-Spectrum Beta-Lactamases (ESBL) (unpublished data).

Based on prior research, we suggest that pairing quinoxaline derivatives with TQ could boost their efficacy against a range of ESBL clinical strains. This inventive approach may tackle the complexities of ESBL infections and introduce fresh treatment prospects. Our study seeks to analyze the *in vitro* antimicrobial effective combination 3HQ with TQ across diverse ESBL clinical strains. Our goal is to reveal any potential synergy between these compounds, thereby improving treatment outcomes and introducing innovative strategies to combat ESBL infections.

## MATERIALS AND METHODS

### Antibacterial compounds

The compounds subjected to testing, namely 3HQ, were procured from Fluorochem Ltd., situated in the United Kingdom. Similarly, TQ powder utilized in the study was sourced from Sigma. To facilitate their experimental use, both compounds were dissolved in dimethyl sulfoxide (DMSO) (DMSO 5%), a solvent obtained from Sigma. This meticulous sourcing and preparation process ensured the integrity and reliability of the compounds utilized in the experimental procedures.

### Bacterial strains, growth media and condition

For this study, bacterial strains were meticulously selected from a pool of 18 ESBL-producing isolates originating from King Abdulaziz University Hospital, located in Jeddah, Saudi Arabia. These isolates were carefully preserved in glycerol and stored at a temperature of -80°C to maintain their viability and integrity. Upon retrieval, a thawing process was meticulously conducted to ensure the optimal recovery of the bacterial cultures. Subsequently, the isolates were cultured on blood agar plates obtained from HiMedia or MacConkey agar, a renowned supplier based in India. Each isolate underwent identification and susceptibility testing using the Vitek 2 system (bioMerieux, France) with the Gram-negative strain card type AST-N417, following the manufacturer's guidelines meticulously. The cultivation process was carried out

meticulously overnight at a temperature of 37°C under aerobic conditions to promote optimal bacterial growth. It is important to note that ethical approval was not required for this study, as it solely involved the analysis of bacterial isolates devoid of any patient-specific data or history. The study exclusively focused on bacterial specimens, obviating the need for ethical clearance.

## Sensitivity test

In order to evaluate the sensitivity of antimicrobials, we employed a broth microdilution assay. This meticulous procedure commenced with the preparation of a two-fold serial dilution of the antibiotics under investigation in Mueller Hinton Broth (MHB), meticulously sourced from Sigma-Aldrich in the United States. Subsequently, precise aliquots of 100 µl of the prepared antibiotic solutions were meticulously dispensed into each well of 96-well plates, sourced from Italy. The original stock solution for each drug was prepared at a concentration of 10 mg/ml. A solution of 128 µg/ml was prepared with MHB from this original stock solution. This solution was then subjected to a series of twofold serial dilutions and pipetted into each corresponding well.

To ensure the accuracy of our inoculum suspension, its density was meticulously adjusted to 0.5 McFarland units using a Biosan Densitometers DEN-1B suspension turbidity detector. Following this calibration process, precise volumes of 5 µl of the prepared inoculum were meticulously added to each well containing varying concentrations of antibiotics. The meticulously prepared plates were then subjected to overnight incubation at a temperature of 37°C. It is important to note that antibiotic susceptibility testing was conducted in triplicate to ensure robustness and reliability of the results. Subsequently, mean values were meticulously recorded for subsequent analysis and interpretation. This meticulous approach ensured the accuracy and reliability of our antibiotic susceptibility testing methodology [2].

## Checkerboard assay

To evaluate the interactions between the antimicrobial agents, the checkerboard broth assay was used. In this method, a twofold serial dilution of each compound was prepared in MHB. Subsequently, 50 µl of each dilution was partitioned into 96-well

plates. The inoculum suspension density was accurately adjusted to 0.5 McFarland using a Biosan Densitometer DEN-1B suspension turbidity detector. Then 5 µl of the diluted bacteria were added to each well of the 96-well plates [1]. The checkerboard test was performed three times and the average values were recorded for subsequent analysis.

### Assessment of the interactions between the tested antimicrobial agents

A checkerboard assay was applied to assess the interaction among antimicrobial drugs. This assay was used to evaluate all possible drug combinations of the two drugs within the designated concentration range. The interaction of the two drugs was assessed quantitatively by the fractional inhibitory concentration index (FICI), which was computed according to the following formula: FICI = [(MIC 3HQ in combination)/MIC 3HQ alone] + [(MIC TQ in combination)/MIC TQ alone].

The FICI results are to be evaluated as described below: Readings below or equivalent to 0.5 indicate synergy, readings greater than 0.5 but not above 1 indicate an additive effect, readings higher than 1 but not above 2 indicate indifference and readings higher than 2 indicate antagonism. In practice, synergy as determined by this calculation corresponds to a reduction in the MIC of each drug by at least two dilution levels when the drug is combined [30], [31].

### Statistical analysis

GraphPad Prism was used for the statistical analysis. At least three replicates were performed for each experiment and the mean and standard deviation (SD) values were estimated. An unpaired t-test was performed to assess significant differences and statistical analysis between the experimental groups. The p-value was evaluated as 0.05, and a significant p-value was defined as $\leq 0.05$. The p-values were presented as follows: *$p<0.05$, **$p<0.01$ and ***$p<0.001$. The details of the statistical analysis are given in the legend to each figure. Due to the limited sample size, no formal normality tests (e.g., Shapiro–Wilk) were performed; instead, we assumed approximate normality based on the consistent distribution of MIC values. Independent samples (unpaired) t-tests were applied for predefined, hypothesis-driven comparisons. No correction for multiple comparisons was applied, as the analysis was limited in scope and not exploratory in nature

## Docking study

In this study, the structure of the CTX-M-15 enzyme bound to the ligand RPX-7063 was retrieved from the Protein Data Bank (PDB) with the PDB ID 7TI0 (https://www.rcsb.org/structure/7TI0). This structure, resolved at a 1.5 Å resolution, the ligands Thymoquinone and 3-Hydrazinoquinoxaline-2-thiol were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Thymoquinone was retrieved with PubChem ID 10281, while 3-Hydrazinoquinoxaline-2-thiol was retrieved with PubChem ID 781224.

Prior to docking, the protein and ligands underwent a series of preparations involving the removal of all water molecules and hydrogen atoms were added to both the protein and ligands to accurately represent the protonation states under physiological conditions, which is crucial for forming hydrogen bonds during docking [32]. The protein-ligand complexes were then subjected to energy minimization to relieve any steric clashes and to ensure that the structures were in their lowest energy conformations. These prepared structures were then used in molecular docking studies using the Maestro interface, XP docking protocol was used to explore the potential binding modes of Thymoquinone and 3-Hydrazinoquinoxaline-2-thiol with the CTX-M-15 enzyme, setting the stage for further analysis through molecular dynamics simulations and interaction profiling.

## MD simulations

The MD simulation was used for the understanding of how Thymoquinone and 3-Hydrazinoquinoxaline-2-thiol interact with the CTX-M-15 enzyme and their potential as inhibitors. These simulations were performed using the Desmond on Maestro tool [33], a comprehensive molecular modeling platform. The MD simulations were run for a total of 50 nanoseconds (ns), a time frame sufficient to observe the dynamic behavior of the complexes and to assess their stability over time.

During the simulations, the atomic motions of the protein and ligands were tracked, allowing for the analysis of key interactions such as hydrogen bonds, hydrophobic interactions, and water bridges. The RMSD (Root Mean Square Deviation) values were calculated for both the protein and the ligands, providing insights into the structural stability of the complexes throughout the simulation period. The resulting

data were then used to generate interaction histograms, illustrating the frequency and nature of these interactions over the course of the simulation.

## RESULTS

### Assessing the minimum inhibitory concentrations (MICs) of both 3HQ and TQ

Before moving forward with the checkerboard method, it is imperative to determine MICs of both 3HQ and TQ. This preliminary step holds significant importance as it establishes the baseline effectiveness of each compound against the target pathogen. By accurately determining the MICs, we can ensure optimal concentrations are utilized in subsequent combination studies, thereby maximizing the potential synergistic effects between the two compounds. This meticulous approach lays the groundwork for a thorough and comprehensive evaluation of their combined antimicrobial activity, ultimately contributing to a more robust and reliable assessment of their efficacy against the target pathogen.

As detailed in Table 1, both TQ and 3HQ exhibited MIC values ranging from 16 to 128 µg/mL. Following the meticulous acquisition of these MIC values, a meticulously crafted checkerboard test protocol was subsequently devised. This comprehensive approach entailed the meticulous blending of varying quantities of TQ and 3HQ, meticulously calibrated to explore potential synergistic interactions between the two compounds.

### 3HQ and TQ exhibit synergistic effects against diverse ESBL clinical strains

To assess the potential synergistic effective combination 3HQ and TQ against a variety of ESBL clinical strains, a comprehensive checkerboard test was conducted. In this assay, the MICs of TQ and 3HQ individually ranged from 16 to 128 µg/ml for inhibiting ESBL growth. However, the most intriguing findings emerged when the two compounds were combined. The MICs of 3HQ were reduced by a factor of 4 to 16 when paired with TQ against ESBL clinical isolates. Equally noteworthy were the observations regarding TQ when paired with 3HQ. Across all tested ESBL strains, the MICs of TQ were notably reduced, exhibiting decreases of 4 to 8 times compared to its standalone MIC values (as illustrated in Figure 1). For example, in *Escherichia coli* strain 4, the MIC of 3HQ decreased significantly from

32 µg/mL to 4 µg/mL when combined with TQ. Similarly, the MIC of TQ was reduced from 64 µg/mL to 8 µg/mL in the presence of 3HQ, indicating a pronounced synergistic effect

These results strongly suggest that the presence of 3HQ enhances the efficacy of TQ against ESBL strains, as highlighted in Table 2. This phenomenon reveals the potential for synergistic interactions between these compounds in combating ESBL infections, particularly against ESBL strains (Table 2).

## Molecular docking

The docking study examines the potential synergistic effects of Thymoquinone and 3-Hydrazinoquinoxaline-2-thiol on inhibiting the CTX-M-15 protein. The RPX-7063 was used as control and for validation of our docking protocol. The 2D interaction of the control as shown in Figure 2, shows a robust network of interactions that stabilize its binding to the CTX-M-15 protein. RPX-7063 forms multiple hydrogen bonds with key amino acids such as Ser212, Gly211, Thr210, and Ser105. Additionally, it engages in hydrophobic interactions with residues like Tyr80 and Asn79, further enhancing its binding affinity. This extensive interaction profile highlights the effectiveness of RPX-7063 in binding to the CTX-M-15 protein, serving as a benchmark for evaluating other potential inhibitors.

The binding interaction profile of compound 3HQ showed similarity to that of RPX-7063. The 2D interaction graphic demonstrates that, like the control ligand, 3HQ forms hydrogen bonds with important residues including Ser212 and Ser45. Furthermore, it forms hydrophobic bonds with residues such as Tyr80 and Asn79, which are crucial for maintaining the binding's stability (Figure 3). 3HQ and RPX-7063 exhibit comparable patterns of interaction, indicating that 3HQ may have the ability to function as a powerful inhibitor of the CTX-M-15 protein when used alone or in conjunction with other medications. The ability of 3HQ to mimic the control's interactions indicates its potential to bind effectively and inhibit the protein's activity. This is further supported by the docking score of -3.56 (Table 3), which reflects a favorable binding affinity and supports the visualized interaction profile.

On the other hand, thymoquinone shows a different interaction profile on the other site, with fewer interactions overall compared to both RPX-7063 and 3-

Hydrazinoquinoxaline-2-thiol. However, the overall extent of hydrogen bonding and hydrophobic interactions is less pronounced in thymoquinone. Interestingly, thymoquinone binds to a distinct active site on the CTX-M-15 protein, which may contribute to a potential synergistic effect when used in combination with 3-Hydrazinoquinoxaline-2-thiol. thymoquinone may change the stability or shape of the protein by binding at a different site, which would increase 3-Hydrazinoquinoxaline-2-thiol binding affinity at the primary active site. When combined with 3-Hydrazinoquinoxaline-2-thiol, the docking score of -3.8 (while marginally higher than that of 3-Hydrazinoquinoxaline-2-thiol) remains indicative of a respectable binding affinity that may help to suppress CTX-M-15 overall.

## MD simulation study

The protein-ligand complex stability over time is clearly seen by the RMSD graphs (Figures 4A-6A). The RMSD for the protein (blue line) for the control compound RPX-7063 (red) stays comparatively steady, suggesting that the protein structure is well-maintained during the simulation. While there are normal variations in the ligand (red line), with time, the line stabilizes, indicating a constant interaction with the protein (Figure 4A). Thymoquinone exhibits a similar pattern in its RMSD profile. The protein shows stability over time, with the RMSD remaining below 2 Å, indicating that the protein-ligand complex is well-stabilized [34]. The ligand's RMSD fluctuates initially but stabilizes, suggesting that Thymoquinone establishes a steady binding conformation during the simulation (Figure 5A).

In contrast, 3-Hydrazinoquinoxaline-2-thiol shows a higher degree of fluctuation in both the protein and ligand RMSD, particularly the ligand, which indicates that the binding may be less stable or that the ligand undergoes significant conformational changes throughout the simulation. This could be due to its interaction with a different active site compared to Thymoquinone and the control, potentially affecting the overall complex stability (Figure 6B).

The histograms (Figures 4B-5B), reveal the types and frequencies of interactions that occur during the 50 ns simulation. The histogram for the control compound RPX-7063 demonstrates a high frequency of hydrogen bonding (green bars), which are essential to the protein-ligand complex's stability. Thymoquinone's histogram displays a comparable pattern to the control, with a substantial fraction of hydrogen bonds and

water bridges. This suggests that Thymoquinone, like the control, forms a stable and consistent interaction network within the CTX-M-15 binding site. The presence of hydrophobic interactions also supports the stability of the complex.

On the other hand, the histogram for 3-Hydrazinoquinoxaline-2-thiol shows a lower frequency of hydrogen bonds and water bridges compared to both the control and thymoquinone. This lower interaction frequency, particularly in hydrogen bonds, correlates with the observed higher RMSD fluctuations, indicating a less stable interaction. However, the presence of hydrophobic interactions, while less frequent, suggests that there might still be regions of stable binding, particularly at the different active sites it targets.

The residues and ligands interaction analysis revealed that both 3-Hydrazinoquinoxaline-2-thiol (3HQ) and the control compound (RPX-7063) occupy the canonical active site of the CTX-M-15 enzyme. The control ligand interacts with key active site residues such as Asn145, Thr210, Ser212, and Arg249, while 3HQ similarly engages residues including Tyr80, Pro82, Lys86, Tyr104, Asn107, and Arg249, indicating that it binds within the same functional pocket involved in enzymatic catalysis. In contrast, Thymoquinone (TH) binds to a distinct site involving residues Arg14, Tyr35, Arg40, Arg136, Gly150, and Gln163, spatially separated from the catalytic center. This suggests that TH may target an allosteric pocket, potentially modulating enzyme activity indirectly. The binding of TH at this secondary site may induce conformational changes that enhance the accessibility or binding efficiency of 3HQ. As shown in molecular dynamics (MD) simulations, there is an increase in RMSD fluctuations in the protein backbone when TH is bound. This could allosterically modulate the geometry of the active site, thereby enhancing 3HQ binding affinity and stability, as supported by the lower RMSD and more consistent hydrogen bond formation of 3HQ in combined binding scenarios. This hypothesis aligns with the observed synergistic reduction in MICs and FICI values.

## DISCUSSION

To the best of our knowledge, this study is the first to document a synergistic interaction between TQ and 3HQ derivatives against various ESBL clinical strains. This discovery may enhance our understanding of ESBL therapeutics and indicates a promising approach for combating antibiotic resistance. The concurrent administration of TQ and 3HQ resulted in a significant decrease in the MIC of TQ, with reductions of up to 8-fold observed. Similarly, when thymoquinone was administered alongside 3HQ, the MIC of the 3HQ derivatives was reduced by up to 16-fold. This robust synergistic interaction between 3HQ and TQ was consistently demonstrated across experiments involving various clinical ESBL strains. These findings strongly suggest that the combined therapeutic approach with TQ and 3HQ elicits a more potent antimicrobial response against ESBL strains compared to the individual efficacy of each compound alone. Previous studies have suggested that incorporating a second antibiotic into the treatment regimen can address the limitations of the initial antibiotic [24], [35]. This is consistent with our findings, as we demonstrated that the drug combination reduced the MICs by up to 16-fold.

The MIC of TQ alone was 64 µg/mL, while that of 3HQ was 32 µg/mL. However, in combination, only 8 µg/mL of TH and 2 µg/mL of 3HQ were required to achieve the same inhibitory effect against *E. coli*. This suggests that the combination allows for lower MICs, indicating that effective therapeutic outcomes can be achieved with lower drug doses, potentially reducing the risk of adverse effects. Further investigations are essential to confirm and expand upon this promising observation. Given the potential toxicity associated with high concentrations of 3HQ, TQ, and other drugs, an emerging strategy involves using lower doses of each drug synergistically to mitigate this concern [31]. The effectiveness of TQ has also been demonstrated against *Proteus vulgaris* [36], [37]. We propose that combining antimicrobial therapies could broaden the spectrum of coverage, although additional tests are necessary to substantiate this claim.

Through our research, we have uncovered a compelling synergy between the combination of 3HQ and TQ, particularly in their impact on a wide array of clinical ESBL strains. This synergistic effect appears to be multifaceted, stemming from

several mechanisms that operate concurrently to inhibit bacterial growth. TQ, in particular, emerges as a pivotal player in this synergistic relationship. It exerts its antimicrobial prowess through a cascade of actions, primarily targeting the fundamental structure and function of bacterial cells. One prominent mechanism involves the induction of irreversible damage to bacterial morphology. This entails a series of disruptions, starting with the compromise of cell membrane integrity. As a consequence, there is a leakage of essential cellular components, notably proteins, which are vital for bacterial survival. Furthermore, TH penetrates the intracellular domain, where it disrupts crucial proteins essential for various cellular processes [38]–[40].

In contrast, our investigation has unveiled the pivotal role played by 3HQ in impeding the process of DNA synthesis within bacterial cells. This discovery sheds light on a critical mechanism by which 3HQ exerts its antimicrobial effects. By targeting DNA synthesis, 3HQ disrupts the fundamental process necessary for bacterial replication and proliferation. The inhibition of DNA synthesis represents a strategic approach in combating bacterial infections, as it directly hinders the ability of bacteria to propagate and spread. Through its actions on DNA synthesis, 3HQ effectively interferes with the replication of genetic material within bacterial cells, leading to their eventual demise [41]–[43].

This may underscore the multifaceted nature of the 3HQ and TQ combination in combating bacterial pathogens. While TQ disrupts bacterial morphology and cellular functions, 3HQ intervenes at the genetic level, disrupting DNA synthesis. Together, these complementary mechanisms synergistically enhance the antimicrobial efficacy of the combination, offering promising prospects for the development of novel therapeutic interventions against bacterial infections.

Another potential explanation for the increased efficacy observed with the combination of TQ and 3HQ against various ESBL clinical strains lies in the enhanced generation of reactive oxygen species (ROS) [44]. ROS are highly reactive molecules that can wreak havoc within bacterial cells by disrupting crucial cellular processes. One notable effect of ROS is their interference with cellular electron

transport, leading to a cascade of events culminating in sustained oxidative stress and, ultimately, cell death [45], [46].

The ability of TQ to induce the formation of ROS is well-documented in numerous studies. This property is pivotal in its antimicrobial activity, as ROS serve as potent weapons in the battle against bacterial pathogens. By triggering the production of ROS, TH effectively unleashes a barrage of oxidative assaults on bacterial cells, overwhelming their defense mechanisms and rendering them vulnerable to destruction [27], [47]. In combination with 3HQ, TQ capacity to induce ROS formation may be further potentiated, leading to an intensified antimicrobial effect against ESBL strains. This synergistic interplay between TQ and 3HQ underscores the complexity of their interactions and highlights the diverse mechanisms by which they target bacterial pathogens.

Overall, the enhanced generation of ROS represents yet another facet of the multifaceted approach employed by TQ and 3HQ in combating ESBL infections. By exploiting the oxidative vulnerabilities of bacterial cells, this combination holds significant promise for the development of novel therapeutic strategies against drug-resistant pathogens. Moreover, this study explores the potential synergistic effects of thymoquinone and 3-Hydrazinoquinoxaline-2-thiol on inhibiting the CTX-M-15 protein, using RPX-7063 as a control. Docking and MD simulations revealed thymoquinone's strong binding affinity and interaction profile similar to RPX-7063, forming hydrogen bonds and hydrophobic interactions with key residues. In contrast, 3-Hydrazinoquinoxaline-2-thiol binds to a different active site, potentially enhancing Thymoquinone's binding. This combination may effectively inhibit CTX-M-15, as supported by docking scores and interaction profiles. The study’s findings suggest that thymoquinone and 3-Hydrazinoquinoxaline-2-thiol, when used together, could provide a promising strategy to combat CTX-M-15-mediated antibiotic resistance.

Further tests are necessary to comprehensively characterize the combination of TQ and 3HQ against ESBL strains. One such critical test is the Time Kill assay, which is essential for thoroughly examining the bactericidal effects of the TH and 3HQ combination over a specified duration. This assay will provide valuable insights into the potential of this combination as a long-term treatment strategy [1]. Additionally, a

resistance assay is crucial to assess the likelihood of bacteria developing resistance to the combination. This evaluation ensures the sustained effectiveness of the treatment by identifying any potential for resistance development [31]. A proteomic analysis is also essential to achieve a comprehensive understanding of the genetic responses when bacteria are challenged with the TQ and 3HQ combination. This test will identify which genes are upregulated or downregulated, thereby elucidating the underlying mechanisms responsible for the observed synergy.

Notably, TH has shown significant antibiofilm activity against *P. aeruginosa*. This observation warrants further investigation into the combined efficacy of thymoquinone and 3HQ against ESBL biofilm formation [27]. Given that this study primarily includes *E. coli* strains, with only single strains of *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, it is important to include a greater variety of strains in future studies. This broader inclusion will better assess the efficacy of this combination against these resistant and challenging organisms. Expanding the range of tested organisms will provide a more comprehensive evaluation of the therapeutic potential of the TQ and 3HQ combination, ultimately contributing to more robust and effective treatment strategies against resistant bacterial infections. Future studies will also explore the in vivo efficacy and pharmacodynamic properties of this combination to better understand its therapeutic applicability.

## Limitation

One limitation of our study is the disproportionate representation of bacterial strains, as we included a large number of *E. coli* isolates but only a single isolate each of *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. This imbalance occurred because we relied on the hospital to provide isolates, and these were the strains that infected patients at the time of our request. This study was limited to *in vitro* assessments of the antibacterial activity of thymoquinone and 3-hydrazinoquinoxaline-2-thiol. Therefore, further preclinical studies are necessary to determine the safety and therapeutic applicability of this combination.

## CONCLUSION

Our study offers the first evidence of synergy between 3HQ and TQ against a variety of ESBL strains. Although these findings indicate promising clinical applications, additional tests and a comprehensive proteomic analysis are necessary to further characterize and understand the full potential of this combination. Moreover, *in vivo* studies are essential to assess their toxicity, pharmacokinetics, and overall suitability as potential therapeutic agents.


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## TABLES AND FIGURES WITH LEGENDS

**Table 1. Comparative MIC values of 3HQ and TQ (measured in µg/ml) for clinical ESBL species**

| Number of strain | ESBL producing organism | MIC of 3HQ | MIC of TQ |
|---|---|---|---|
| 1 | *Pseudomonas aeruginosa* | 128 | 128 |
| 2 | *Klebsiella pneumoniae* | 64 | 128 |
| 3 | *Klebsiella pneumoniae* | 64 | 128 |
| 4 | *Escherichia coli* | 32 | 64 |
| 5 | *Acinetobacter baumannii* | 32 | 128 |
| 6 | *Escherichia coli* | 64 | 32 |
| 7 | *Escherichia coli* | 32 | 32 |
| 8 | *Escherichia coli* | 32 | 32 |
| 9 | *Escherichia coli* | 32 | 64 |
| 10 | *Escherichia coli* | 32 | 64 |
| 11 | *Escherichia coli* | 32 | 16 |
| 12 | *Escherichia coli* | 16 | 32 |
| 13 | *Escherichia coli* | 32 | 64 |
| 14 | *Escherichia coli* | 32 | 32 |
| 15 | *Escherichia coli* | 32 | 16 |
| 16 | *Escherichia coli* | 64 | 32 |
| 17 | *Escherichia coli* | 32 | 32 |
| 18 | *Escherichia coli* | 16 | 16 |

Abbreviations: MICs, minimum inhibitory concentrations; ESBLs, extended-spectrum β-lactamases; 3HQ, 3-hydrazinoquinoxaline-2-thiol; TQ, thymoquinone.

**Table 2. Synergy assessment of 3HQ and TQ versus ESBL strains determined by FICI analysis**

| Number of strain | ESBL producing organism | FIC of 3HQ | FIC of TQ | FICI |
|---|---|---|---|---|
| 1 | *Pseudomonas aeruginosa* | 0.146 | 0.169 | 0.315 |
| 2 | *Klebsiella pneumoniae* | 0.167 | 0.198 | 0.365 |
| 3 | *Klebsiella pneumoniae* | 0.167 | 0.169 | 0.365 |
| 4 | *Escherichia coli* | 0.125 | 0.125 | 0.25 |
| 5 | *Acinetobacter baumannii* | 0.208 | 0.125 | 0.333 |
| 6 | *Escherichia coli* | 0.125 | 0.188 | 0.313 |
| 7 | *Escherichia coli* | 0.188 | 0.156 | 0.344 |
| 8 | *Escherichia coli* | 0.2 | 0.291 | 0.491 |
| 9 | *Escherichia coli* | 0.084 | 0.094 | 0.178 |
| 10 | *Escherichia coli* | 0.149 | 0.125 | 0.274 |
| 11 | *Escherichia coli* | 0.146 | 0.167 | 0.313 |
| 12 | *Escherichia coli* | 0.167 | 0.177 | 0.344 |
| 13 | *Escherichia coli* | 0.146 | 0.188 | 0.334 |
| 14 | *Escherichia* | 0.104 | 0.208 | 0.312 |

| | | | | |
|---|---|---|---|---|
| | *coli* | | | |
| 15 | *Escherichia coli* | 0.167 | 0.094 | 0.261 |
| 16 | *Escherichia coli* | 0.208 | 0.146 | 0.354 |
| 17 | *Escherichia coli* | 0.208 | 0.208 | 0.416 |
| 18 | *Escherichia coli* | 0.167 | 0.208 | 0.375 |

**Note:** Synergy is considered present when the FICI is less than 0.5. Abbreviations: FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; ESBLs, extended-spectrum β-lactamases; 3HQ, 3-hydrazinoquinoxaline-2-thiol; TQ, thymoquinone.

**Table 3. Docking scores for compound–protein interactions**

| Compound | PubChem ID | Docking score |
|---|---|---|
| Thymoquinone | 10281 | -3.80 |
| 3-Hydrazinoquinoxaline-2-thiol | 781248 | -3.56 |
| RPX-7063 | - | -6.9 |

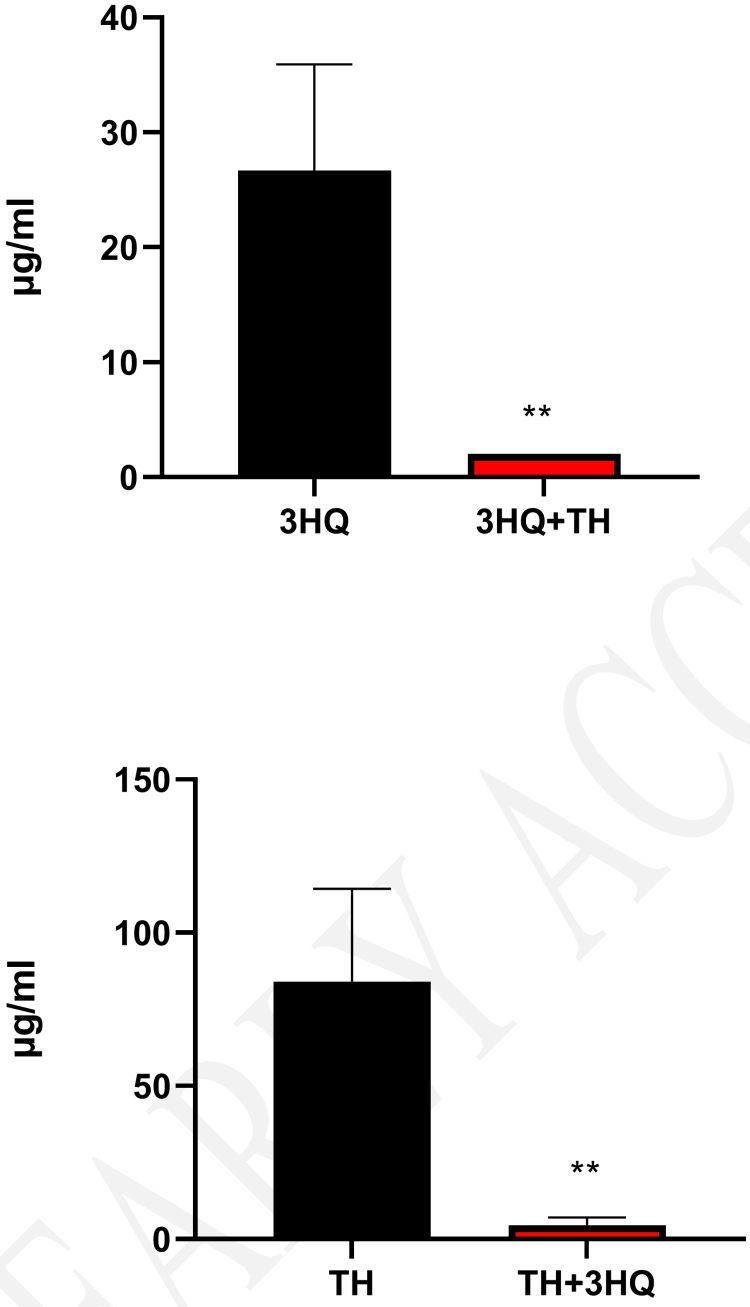





**Figure 1. Minimum inhibitory concentrations (MICs) of 3-hydrazinoquinoxaline-2-thiol (3HQ), thymoquinone (TQ) and their combination against the tested isolate, *Escherichia coli* strain 4.** Statistical comparisons were performed using unpaired t-tests. Significant differences are indicated by asterisks on the bars:

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The MICs for 3HQ combined with TQ show a $p$ value of 0.0098 when compared to

the MICs of 3HQ alone. Similarly, the MICs for TQ combined with 3HQ have a p-value of 0.002 when compared to the MICs of TQ alone. Data are presented as mean ± standard deviation (SD) from triplicate experiments. A *p* value of less than 0.05 is considered statistically significant.

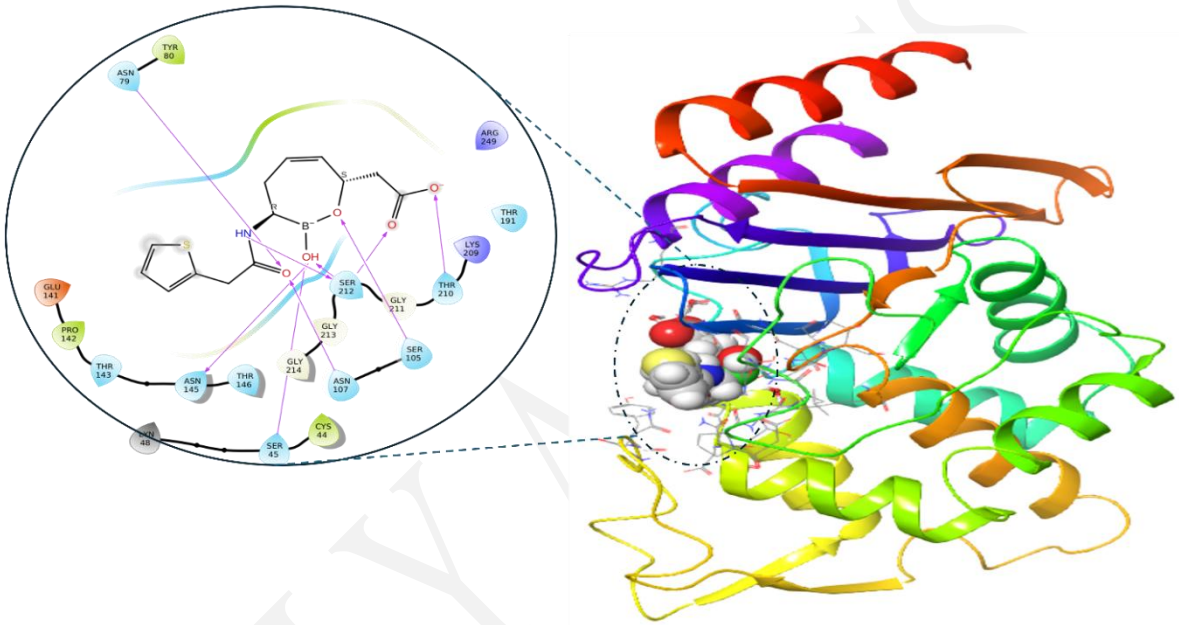



**Figure 2. 3D and enlarged 2D representation of the interaction of CTX-M-15 protein and co-crystalized inhibitor (RPX-7063).** In the 3D representation (right), the protein is shown as a rainbow-colored cartoon ribbon, where each color represents a different region of the protein's secondary structure. The ligand RPX-7063 is displayed in space-filling (ball) representation with carbon atoms in gray, oxygen in red, nitrogen in blue, and sulfur in yellow. In the 2D interaction diagram (left), hydrogen bonds are indicated by purple arrows, pointing from donor to acceptor. Hydrophobic interactions are shown as curved light lines, while amino acid residues are labeled and colored by property.

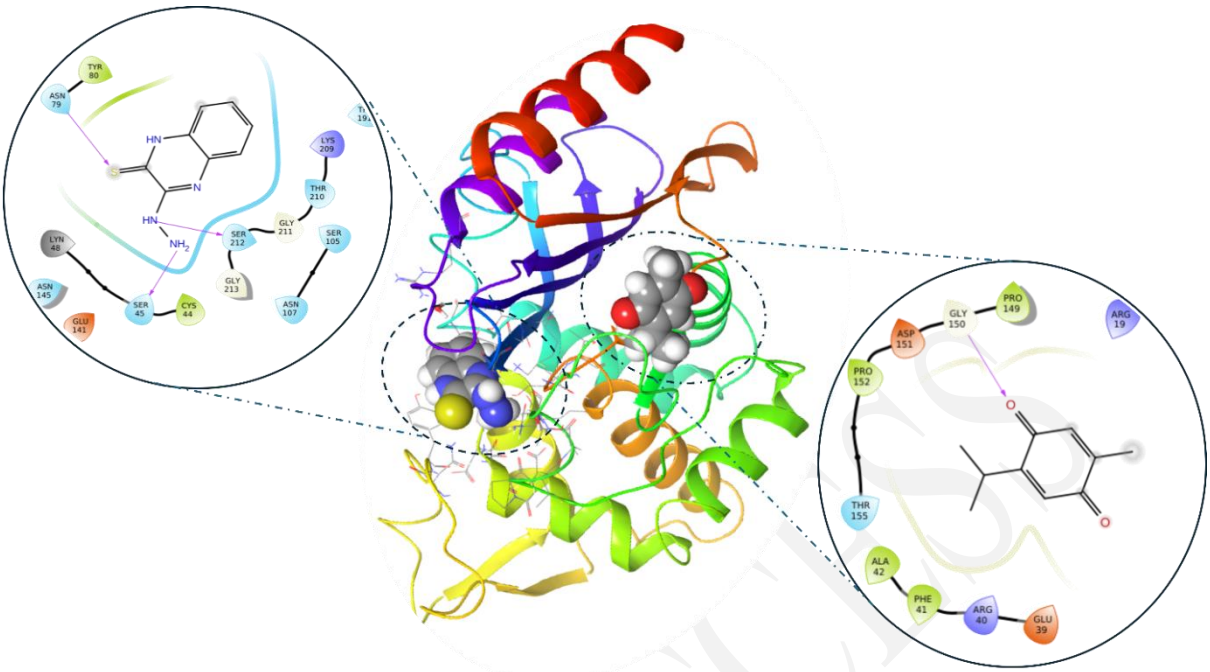



**Figure 3. 3D and enlarged 2D representation of the interaction of CTX-M-15.** Purple lines indicate hydrogen bonds, the ligand in 3D representation is shown in balls, while protein is shown in cartoon colored representation. On the upper left enlarged shot showed 3-Hydrazinoquinoxaline-2-thiol interacting with protein main active site, while the lower enlarged shot showed thymoquinone interacting with other binding grove on the protein.

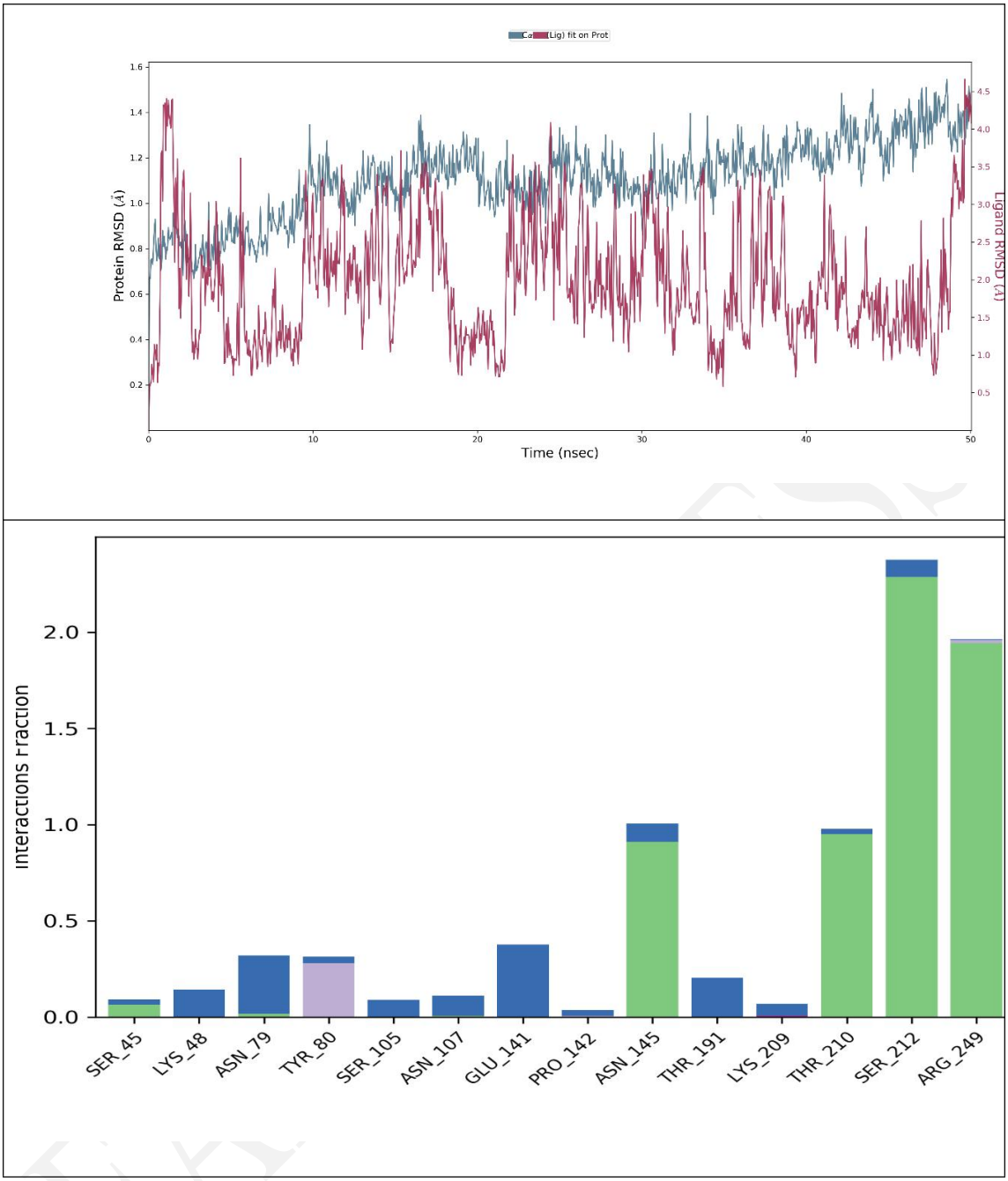



**Figure 4.** A: The RMSD for the protein (blue line) for the control compound RPX-7063 (red), B: a varied interaction profile is also shown by the notable contributions from hydrogen bonds (green), hydrophobic contacts (purple bars) and water bridges (blue bars).

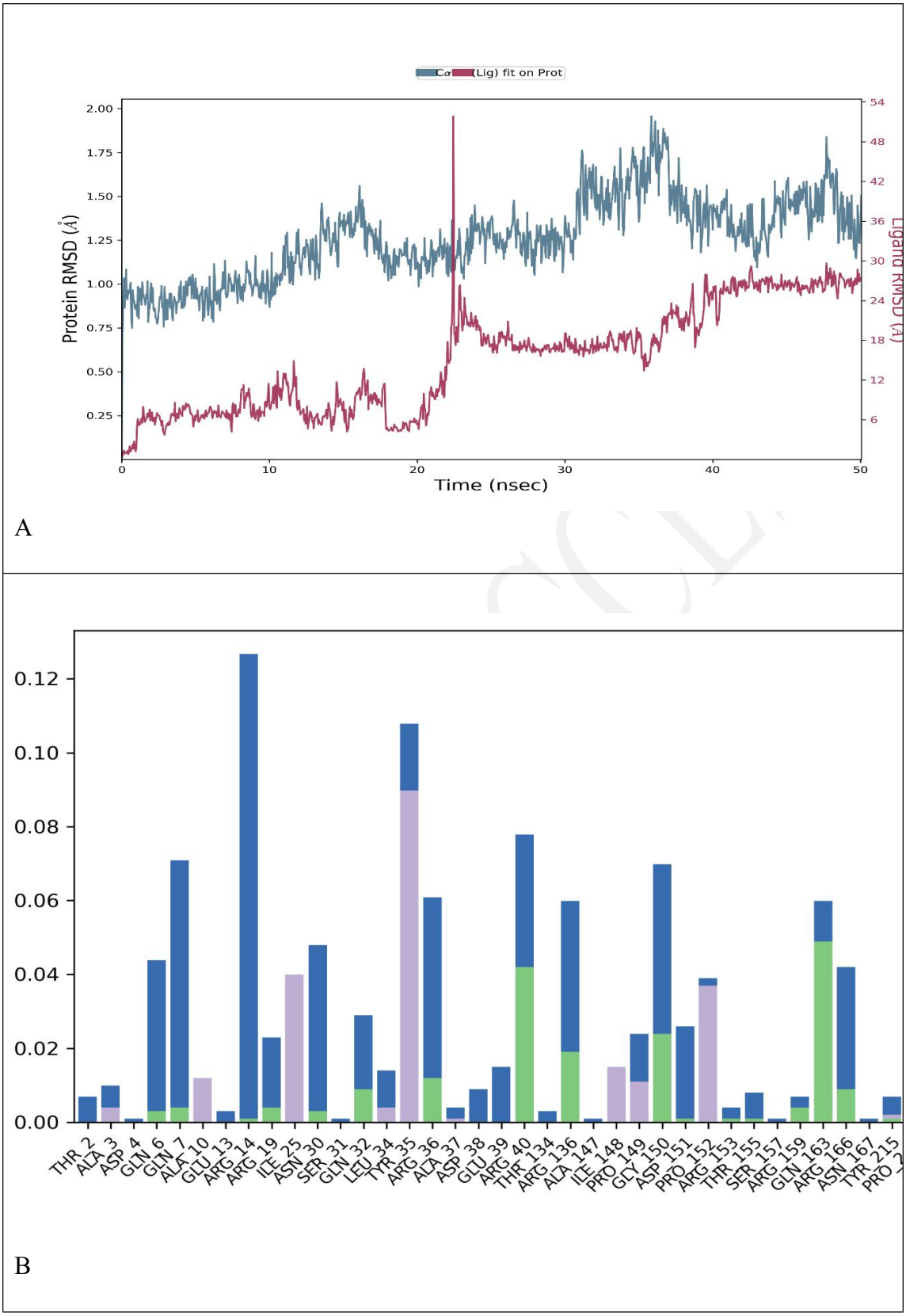
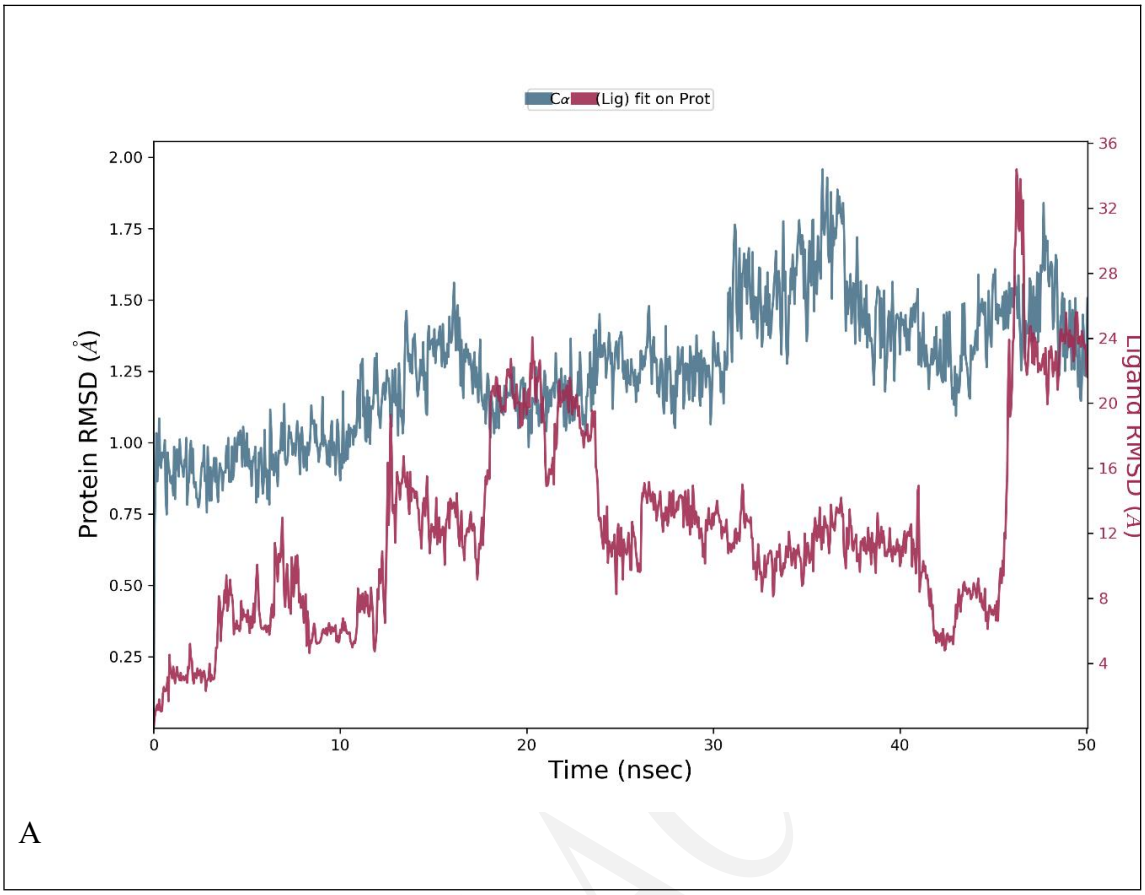



**Figure 5.** A: The RMSD for the protein (blue line) for the compound thymoquinone (red), B: a varied residual interaction profile is also shown by the notable contributions from hydrogen bonds (green), hydrophobic contacts (purple bars) and water bridges (blue bars).


Cα (Lig) fit on Prot
Protein RMSD (Å)
Ligand RMSD (Å)
Time (nsec)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
36
32
28
24
20
16
12
8
4
0
10
20
30
40
50

A

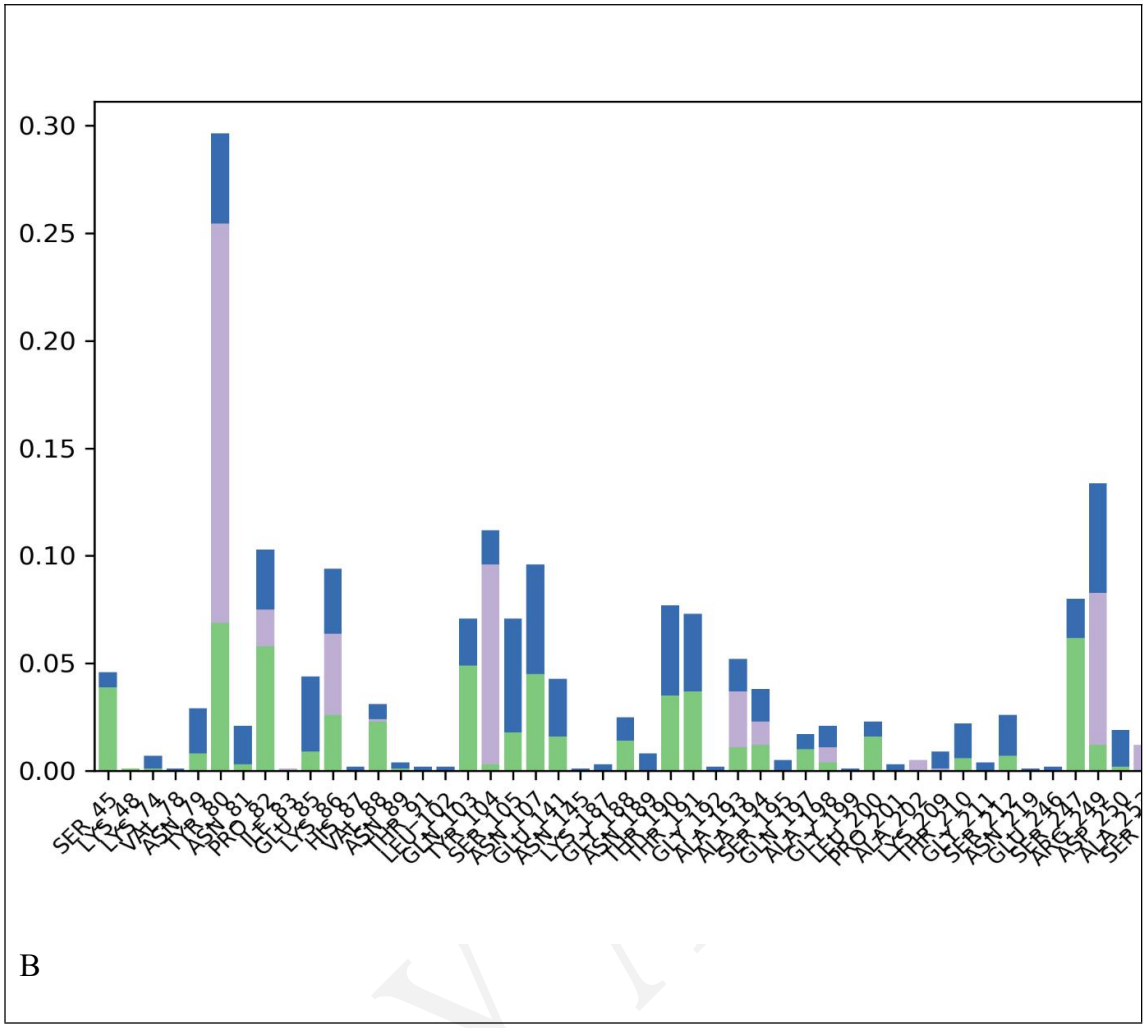



**Figure 6.** A: The RMSD for the protein (blue line) for compound 3-Hydrazinoquinoxaline-2-thiol (red), B: a varied interaction residual profile is also shown by the notable contributions from hydrogen bonds (green), hydrophobic contacts (purple bars) and water bridges (blue bars).