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ORIGINAL ARTICLE

Evaluation of the antagonistic effect of *Pseudomonas aeruginosa* toxins on azole antifungal resistance in *Candida albicans* species isolated from clinical samples in Iran

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ABSTRACT

Background and Objectives: The azole antifungals are the most frequent class used to treat *Candida* infections. It is essential to elucidate the potential of natural compounds as an alternative in eliminating *Candida albicans (C. albicans).* Therefore, in the present study, the antagonistic effect of *Pseudomonas aeruginosa* toxins on azole antifungal resistance in *C. albicans* species was investigated.

Materials and Methods: In this study, 28 *C. albicans* species with azole antifungal resistance were obtained from patients at Shohadaye Tajrish Hospital. The effect of toxins, such as phenazine, pyocyanin, pyoverdine, and fluorescein, was examined on *C. albicans* species. The antifungal activity of these toxins against *C. albicans* spp. was determined using methods such as minimal inhibitory concentration (MIC_{90}), radial diffusion assay (RDA), and detection of reactive oxygen species (ROS).

Results: The prevalence of *C. albicans* strains in urinary catheters, surgical wounds, respiratory tracts, blood, and standard strains was 46.3%, 21.4%, 25%, 7.14%, and 3.57%, respectively. The MIC values were reported as 32 µg/ml for phenazine, and 128 µg/ml for pyoverdine, pyocyanin, and fluorescein. The results showed that phenazine exhibited higher inhibitory effects against *C. albicans* isolated from clinical samples compared to the other toxins. After exposure to phenazines (20 µg/ml), 65-70% of yeast cells of *C. albicans* spp. showed rhodamine 123 fluorescence, indicating high intracellular reactive oxygen species (ROS) production.

Conclusion: The antifungal effect of different toxins in *C. albicans* spp. may be due to ROS-mediated apoptotic death. The results suggest that phenazine has high potential in controlling *C. albicans*. This natural compounds are a potential alternative for eliminating this yeast.

Keywords: Antagonistic effect; Toxins of *Pseudomonas aeruginosa*; *Candida albicans*

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INTRODUCTION

In recent years, there has been a significant increase in the morbidity and mortality associated with invasive fungal infections, posing a serious challenge to overcome. Approximately 75% of all fungal infections affecting humans are caused by *Candida* spp. (1) The reported mortality rate for candidiasis worldwide ranges from 5% to 71%, with crude mortality rates as high as 81%. (2).

The interaction between different species of pathogens in these interkingdom communities can have a significant impact on microbe-host interactions, the effectiveness of antimicrobial treatments, and even the overall outcome of the infection. It is important to understand and consider these interactions when developing treatment strategies. The presence of multiple species within an infection can complicate treatment approaches, as different pathogens may respond differently to specific antimicrobials. Additionally, the interactions between these species can contribute to the development of antimicrobial resistance, making treatment even more challenging. Therefore, it may be necessary to develop adapted treatment strategies that take the specific interactions between pathogen species in interkingdom microbial communities into account. This approach can help optimize treatment outcomes and minimize the development of antimicrobial resistance in these complex infections (3).

C. albicans and *P. aeroginosa* are opportunistic pathogens that can be found in similar sites of infection such as in burn wounds and most importantly in the lungs of CF and mechanically ventilated patients. *C. albicans* is particularly difficult to treat because of the paucity of antifungal agents, some of which lack fungicidal activity (4).

Emphasizing the medical importance of fungal diseases is crucial, as they contribute to the increasing rate of all Gram-negative bacterial septicemias. Unfortunately, the number of invasive fungal infections is on the rise, particularly among immunocompromised hosts such as those with autoimmune diseases, AIDS, burns, undergoing chemotherapy or radiotherapy, and transplantation. Another concerning factor is the development of resistance against currently used antifungal drugs. Additionally, existing drugs have drawbacks such as acute and chronic side effects, and limited clinical efficacy due to their impact on non-targeted cells. These factors have worsened the situation highlighting the urgent need to search for new antifungal agents (5).

Pyocyanin, for instance, has demonstrated antibacterial effects against both Gram-positive and Gram-negative bacteria. Its mechanism of action involves interacting with the cell membrane respiratory chain, leading to the loss of metabolic transport process ability in bacterial cells (6).

Studies on bacteria and fungi have revealed that microorganisms possess a vast array of unique structures with potential therapeutic applications. However, the increasing prevalence of antibiotic-resistant pathogens poses a significant challenge in the treatment of infectious diseases. Consequently, there is a pressing need for research focused on the development of more effective antibiotics. Antifungal activity has been observed in clinical strains, particularly in cases where cystic fibrosis patients are infected with fungal complications. These findings suggest that certain microorganisms, such as *P. aeroginosa*, may exhibit antifungal properties. Numerous literature reviews have explored the interactions between fungi and bacteria, specifically their production of secondary metabolites in the environment, and their implications for medicine and technology (7-9).

Resistance to antifungal drugs is increasing among *Candida* isolates. Lack of correct diagnosis of *Candida* and the experimental use of antifungal drugs are the main causes of this resistance. *Candida* isolates showed the highest sensitivity to voriconazole and ketoconazole and the lowest sensitivity to fluconazole (10).

Given the emergence of multi-drug resistant *C. albicans*, it is crucial to implement a rational drug prescription approach that is based on the principles of antifungal stewardship and therapeutic drug monitoring. Antifungal stewardship involves the responsible and judicious use of antifungal drugs to optimize patient outcomes while minimizing the development of resistance. This approach aims to prevent the overuse or misuse of antifungal medications, which can contribute to the emergence of drug-resistant strains. It is important for healthcare professionals to stay updated on the latest guidelines and recommendations for antifungal stewardship and therapeutic drug monitoring in order to provide the most effective and individualized treatment for patients with multi-drug resistant *C. albicans* infections (11). Among the great panoply of interactions found within the context of human infections, the communication between fungi

and bacteria has been the focus of great interest in the last years. *C. albicans* and *P. aeroginosa* comprise an example of a clinically relevant fungal-bacterial consortium commonly found in the respiratory tract and skin (12-14).

In this study, we aim to explore the potential of microbial interactions in nature to discover novel therapeutic drugs. Specifically, we evaluated the antagonistic effect of *P. aeroginosa* toxins on azole antifungal resistance in *C. albicans* species isolated from clinical samples in Iran.

MATERIALS AND METHODS

Patients and setting. This study was a descriptive study conducted in the laboratory of Tajrish Shohadayeh Hospital from October 2021 to the end of September 2022. A total of 28 non-repetitive *C. albicans* strains resistant to azole antifungal drugs were collected from various clinical samples, including bronchial aspiration, urine, wound, sputum, and blood samples. Only patients with a positive culture for *C. albicans* were included, while those who had been recently or currently treated with antifungal medication were excluded. Demographic and medical data of the patients were obtained from their medical records. *C. albicans* (ATCC 10231) served as reference strains.

Phenotypic methods. *C. albicans* identification was initially carried out through phenotypic traits. However, the reproducibility and differentiation levels of these approaches are very low, which limits their reliable diagnosis and epidemiological analysis (15, 16).

The germ-tube test (GTT) method, which is a widely used, is an effective technique for identifying *C. albicans* based on the formation of germ tubes in response to serum incubation. Its simplicity, rapidity, and high sensitivity makes it a valuable tool in clinical settings for *C. albicans* identification (17).

The chlamydospore formation test is a phenotypic method used to identify *C. albicans* based on its ability to produce chlamydospores under specific culture conditions. While this test can be a valuable tool, it is recommended to use it in conjunction with other identification techniques to ensure accurate and reliable identification of *Candida* species (18).

The carbon assimilation test was a phenotypic method used to identify *Candida* species based on their ability to assimilate and utilize different carbon sources. Despite being economical and relatively simple, it can be time-consuming and should be used in conjunction with other identification techniques for accurate and reliable identification of *Candida* species (19).

Carbohydrates fermentation. Fermentation tests, particularly carbohydrate fermentation tests, have been traditionally used for *Candida* species differentiation based on acid and carbon dioxide formation. After differentiation, storing selected colonies at -75 degree centigrade allows for further analysis and characterization of the *Candida* isolates (20-22). *P. aeroginosa* toxins [Phenazine (Fenazyna) 820973; Pyoverdine: P8124-1MG; Fluorescein (O-methacrylate): 568864; Pyocyanin: P0046] were purchased from Sigma Aldrich, Germany.

Preparation of *C. albicans* suspension. Harvesting *C. albicans* colonies involved suspending the collected cells in a saline solution, and the fungal cell concentration was determined by measuring the optical density (OD) of the suspension. An OD between 0.08 and 0.13 was considered favorable for achieving a 0.5 McFarland concentration, which was a common reference point for microbial cell concentrations (23).

Radial diffusion assay (RDA). The radial diffusion assay (RDA) was used to determine the antifungal activity of substances against *Candida* species. The assay involved dispersing fungal cells in agar plates, creating wells, adding different concentrations of substances, and incubating the plates. The inhibition zones around the wells were then observed and photographed to assess the antifungal activity (24, 25).

Determination of minimal inhibitory concentration (MIC). After obtaining the probable MIC using the well diffusion assay, a broth microdilution assay was performed following the methods described by the Clinical and Laboratory Standards Institute (CLSI). The minimal inhibitory concentration (MIC) was determined using the broth microdilution method for *C. albicans* and the toxins. Briefly, microtubes in a sterile microtube plate were filled with 10 µL inoculum of *C. albicans* (0.5 McFarland standard; 1-5 $\times 10^6$ CFU/mL), 90 µL of Sabouraud dextrose broth (SDB) (Scharlau, Turkey), and 100 µL of serially-diluted suspensions of each toxin, from 32-512 µg/mL.

One microtube was prepared by adding normal saline instead of each toxin (positive control), and one was filled with sterile SDB without the *C. albicans* inoculum (negative control). The optical density (OD) of each microtube was read and documented at 640 nm immediately after preparation, and after a 24-hour incubation at 37°C. The minimum drug concentrations for which the post-incubation change in OD was <1% and <50% were considered as the MIC_{90} and the MIC_{50}, respectively (26, 27).

Determination of minimum fungicidal concentration (MFC). The Minimum Fungicidal Concentration (MFC) determination involves adding solutions of the natural compounds or extracts at 1 × MIC (Minimum Inhibitory Concentration) and 2 × MIC concentrations to separate agar plates. These agar plates are then incubated under suitable conditions for the growth of *C. albicans*. After incubation, the plates are examined, and the number of colonies that have grown on each plate is counted. The MFC is defined as the lowest concentration of the natural compound or extract that either shows no growth or exhibits fewer than three colonies. This indicates a high level of killing activity against *C. albicans*, typically representing approximately 99 to 99.5% efficacy. By determining the MFC, researchers can assess the ability of the natural compounds or extracts to not only inhibit the growth of *C. albicans* (MIC) but also effectively kill the fungal cells. This information is crucial in evaluating the potential of these natural products as antifungal agents. It is worth noting that the MFC determination is a standard method used to evaluate the fungicidal activity of various antimicrobial agents, including natural compounds and extracts. This approach helps in understanding the potency and efficacy of these agents against specific fungal pathogens, such as *C. albicans* (26-28).

Intracellular reactive oxygen species (ROS). In this study, the fluorometric method was utilized to measure the levels of reactive oxygen species (ROS). The cells were treated with DCFH-DA (2', 7'-dichlorofluorescin diacetate), which is a non-fluorescent compound that can be oxidized by ROS to produce the fluorescent compound DCF (2',7'-dichlorofluorescein). After adding DCFH-DA to the cells, an incubation period allowed the compound to enter the cells and be deacetylated by intracellular esterases. Once inside the cells, DCFH-DA is converted to DCFH, which is non-fluorescent. In the presence of ROS, DCFH is oxidized to DCF, a fluorescent compound that can be detected using spectrometry. To evaluate ROS production more specifically, dihydrorhodamine 123 staining was employed. This staining method allows for the detection of hydrogen peroxide and other specific ROS. The stained cells were then analyzed using flow cytometry, which directly measures the levels of ROS in the harvested cells. By using these fluorescence-based techniques, the study was able to quantify and assess the levels of ROS in the cells. This information is crucial for understanding the oxidative stress response and the potential impact of the natural compounds or extracts being studied on ROS production and cellular oxidative stress. Overall, the fluorometric method, along with DCFH-DA staining, and flow cytometry, provided valuable tools to measure and analyze ROS levels in the context of this study. These techniques offer insights into the cellular response to oxidative stress and the potential antioxidant activity of the natural compounds or extracts under investigation (27, 28).

Ethics statement. All experiments were conducted in accordance with the relevant guidelines and regulations. (Ethics code: IR.SBMU.RETECH.REC.1399.1211).

Statistics. The data obtained from the study were analyzed using one-way ANOVA followed by Dunnett's post hoc test in GraphPad Prism Version 5.10 for Windows. (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

Study population. The *C. albicans* samples collected in this study included 21 female (75%) and 7 male (25%). The mean age of the studied women was 38 and the mean age of the men was 56. This suggests that *C. albicans* infections are more prevalent in females within the studied population. Additionally, Fig. 1 indicates that the most common source from which *C. albicans* samples were obtained was urinary catheters. This finding suggests that urinary catheters may play a significant role in *C. albicans* infections in the studied population. It implies that the use of urinary catheters may increase the risk of *C. albicans* colonization and subsequent infections in patients.

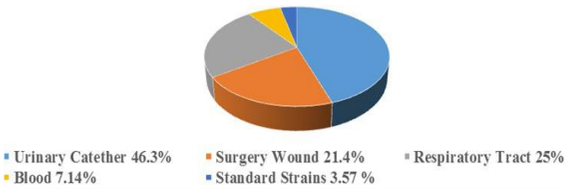

Fig. 1. The prevalence of azole antifungal resistance *C. albicans* species isolated from clinical samples referred to laboratory of Tajrish Shohadayeh Hospital

These findings highlight the importance of considering gender differences and the use of urinary catheters as potential risk factors for *C. albicans* infections in clinical settings. Antifungal susceptibility testing was performed on the *C. albicans* isolates for four different antifungal drugs: itraconazole, fluconazole, voriconazole, and ketoconazole. The results of these tests, indicating the percentage of resistance observed for each antifungal, were obtained from the laboratory. Specifically, the resistance rates in 28 clinical samples were found to be 3 (10.7%) for itraconazole, 6 (21.4%) for fluconazole, 1 (3.57%) for voriconazole, and 1 (3.6%) for ketoconazole.

Radial diffusion assay (RDA). According to Fig. 2, the results of the radial diffusion assay indicated that phenazines exhibited the highest antifungal activity. Larger zones of inhibition were observed for phenazines compared to the other tested compounds, which suggested that phenazines have a stronger inhibitory effect on fungal growth compared to the other compounds tested in this study.

Minimal inhibitory concentration (MIC). According to Fig. 3, the results indicated that phenazines exhibited the maximum antifungal activity with a minimum inhibitory concentration (MIC) of 32 µg/mL. This means that at a concentration of 32 µg/mL, phenazines were able to inhibit the growth of the fungal strain being tested. On the other hand, other toxins such as pyoverdine, pyocyanin, and fluorescein showed antifungal activity but at a higher concentration of 128 µg/mL. This suggested that these toxins have a weaker antifungal effect compared to phenazines, as they required a higher concentration to inhibit fungal growth. Overall, the results from Fig. 3 supported the finding that phenazines had the maximum antifungal activity among the tested compounds, with a lower MIC compared to the other toxins.

Intracellular reactive oxygen species (ROS). Based on Fig. 4, the left hand graph showed the level of reactive oxygen species (ROS) production in a control group (untreated sample). This graph serves as a baseline or reference for comparison with the other experimental conditions. The MIC of phenazine graph (middle) likely showed the level of ROS production at the minimum inhibitory concentration (MIC) of phenazine. This graph indicated the effect of phenazine on ROS production at the concentration that inhibited the growth of the fungal strain being tested. The right hand graph depicted the

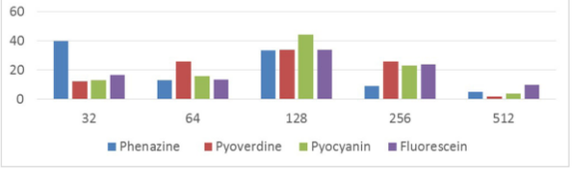

Fig. 3. Antagonistic effect of *P. aeroginosa* toxins on azole antifungal resistance in *C. albicans* species isolated from clinical samples (with dilution of 32, 64, 128, 256, and 512 µg/ml) by broth microdilution method

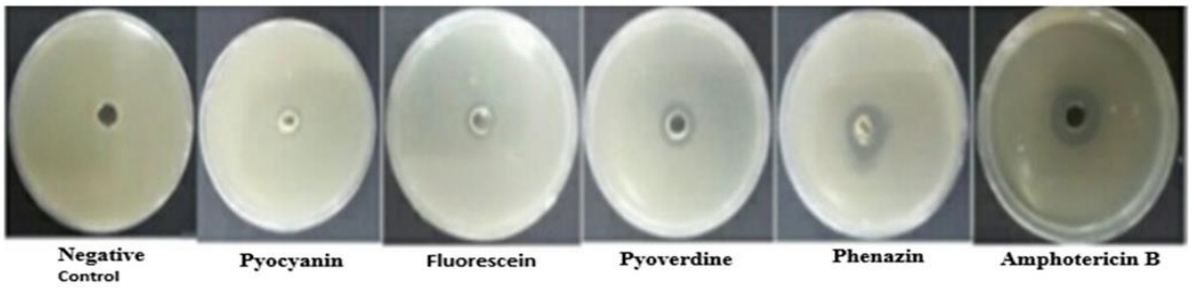

Fig. 2. Antagonistic effect of *P. aeroginosa* toxins on azole antifungal resistance in *C. albicans* species isolated from clinical samples by radial diffusion assay. A clear zone of inhibition due to toxins and amphotericin B around the wells were compared to each other. First well (Left hand) was the negative control without any toxins. The last well (Right hand) was amphotericin B.

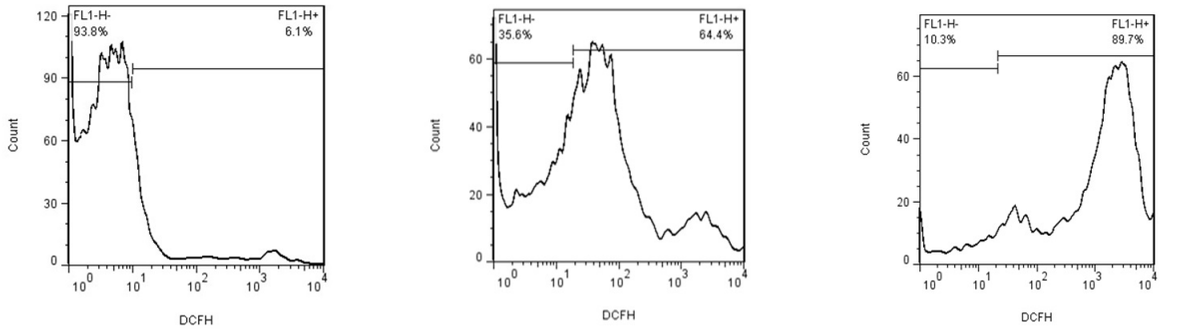

Fig. 4. Reactive oxygen species (ROS): The graphs show ROS signal for control sample (in Left), ROS signal for *C. albicans* exposed to phenazine at one-fold the minimum inhibitory concentration (in Middle), ROS signal for *C. albicans* exposed to phenazine at 2-fold the minimum inhibitory concentration (MIC) (in Right).

level of ROS production when the concentration of phenazine was twice the MIC. This graph can provide insights into the effect of higher concentrations of phenazine on ROS production.

The scale bars in the images represented a length of 5 µm and were applicable to all the images. This information helps to understand the size and dimensions of the structures or cells being observed in the images. Fig. 4 suggested that at this higher concentration of phenazine, there was an increased production of ROS in the *C. albicans* cells. To measure the fluorescence intensity of the ROS indicator, a plate reader was used with an excitation wavelength (λex) of 485 nm and an emission wavelength (λem) of 528 nm, with a gain of 35. This method allows for the quantification of ROS levels in the samples. The data presented in the bar graphs represent the means ± the standard error of the mean (SEM) of three biological replicates. Statistical significance was evaluated using a one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, which compared each condition to the control group. This statistical analysis helped to determine if there were significant differences in ROS production between the control and the phenazine-exposed conditions.

DISCUSSION

In the 1970s, the inhibitory effect of *P. aeroginosa* on the growth of *C. albicans* was first reported. Hogan and Kolter later reported that *P. aeroginosa* can kill hyphal cells of *C. albicans*, but it does not have the same effect on fungal yeasts (29, 30). Additionally, it was reported that the deadly effect of *P. aeroginosa* toxins on *C. albicans* was largely dependent on the different morphotypes of the fungus (31, 32). In our study, the prevalence of *C. albicans* strains in various sources showed the most prevalence in urinary catheters 13 (46.3%), followed by surgical wounds 6 (21.4%), respiratory tracts 7 (25%), blood 2 (7.14%), and standard strains 1 (3.57%). The prevalence of *C. albicans* in different sources indicated its potential involvement in various infections, with urinary catheters being the most common source. These results provided insights into the interactions between *P. aeroginosa* and *C. albicans* and their implications in infection control and treatment strategies.

The MIC_{90} (Minimum Inhibitory Concentration at which 90% of growth is inhibited) values were reported as 32 µg/ml for phenazine, and 128 µg/ml for pyoverdine, pyocyonine, and fluorescein. This indicated that phenazine exhibited the lowest MIC value, suggesting that it has the strongest inhibitory activity against *C. albicans* compared to the other toxins and amphotericin B. However, it is mentioned that for some isolates and substances, reading the MIC values can be complicated due to the occurrence of MIC-phenomena. MIC-phenomena refers to growth effects that hinder the clear determination of a MIC due to factors such as trailing (reduced turbidity compared to the positive growth control) or incomplete inhibition of growth. To overcome these challenges, guidelines were suggested to be followed for accurate MIC reading, which may involve considering factors like growth inhibition and optical clarity of the wells. Marr et al. reported the occurrence of trailing by *C. albicans* when exposed to fluconazole. They were able to eliminate trailing by lowering the pH of the medium (33). This suggests that the pH of the medium can influence the trailing phenomenon.

In the study conducted by Marcos-Zambrano et

al., they found a trailing frequency of 6.8% for fluconazole and *C. albicans* (34). This indicates that a small percentage of *C. albicans* isolates showed trailing behavior when exposed to fluconazole.

It is important to note that MIC-phenomena, including trailing, are known to be drug adaptations. The frequencies of these phenomena can vary not only between different species and compounds tested but also between patient cohorts and hospitals. This suggestd that the ability of *C. albicans* to exhibit trailing and other MIC-phenomena can be influenced by various factors, such as patient characteristics and local microbial environments (35).

The similar resistance adaptation observed in isolates exhibiting trailing may suggest that these isolates could lead to similar therapeutic failures for azole drugs, similar to resistant isolates. However, studies using in vivo murine models have shown contradictory results. These studies suggested that isolates exhibiting trailing actually respond to azole therapy (36-40).

The discrepancy between in vitro resistance and in vivo response to azole therapy for isolates exhibiting trailing is an intriguing finding. It may indicate that the trailing phenomenon observed in vitro does not necessarily translate to treatment failure in vivo. Other factors, such as host immune response or drug pharmacokinetics, may play a role in the observed response to azole therapy. Further research is needed to understand the underlying mechanisms and clinical implications of the contradictory findings between in vitro trailing and in vivo response to azole therapy. These studies highlight the complexity of antifungal resistance and the need for comprehensive evaluation of drug efficacy in different settings, including in vivo models and clinical trials. It is important to consider these contradictory findings when interpreting the clinical relevance of trailing and its impact on treatment outcomes in patients. This similar resistance adaptation would suggest that isolates lead to similar therapeutic failures for azole drugs as resistant isolates, however the in vivo murine models suggest that isolates that exhibit trailing respond to azole therapy, which is contractionary (36-40).

The mechanism of action of the studied toxins in *C. albicans* involved the induction of intracellular reactive oxygen species (ROS) production. In this study, a ROS probe called DCFH-DA was used. DCFH-DA is taken up by the cells and undergoes deacetylation. Upon exposure to ROS, DCFH-DA is oxidized to a fluorescent compound called 20,70-dichlorofluorescein. The addition of phenazine at a concentration of 32 µg/ml resulted in increased fluorescence, indicating higher ROS production in *C. albicans* compared to the other toxins tested. The relative intensity of fluorescence, measured by spectrofluorometry, increased from 139 arbitrary units (A.U.) for cells without the addition of phenazine to 251 A.U. with the addition of 32 µg/ml of phenazine. This increase in fluorescence suggests a higher intracellular ROS production in response to phenazine.

Furthermore, high intracellular ROS levels were observed in *Candida* cells stained with DHR123 after exposure to 32 µg/ml of phenazine. DHR123 is converted to rhodamine 123 (Rh123) in the presence of ROS, and the observation of high intracellular ROS levels in DHR123 staining further supports the findings of increased ROS production in response to phenazine. Overall, the studied toxins, particularly phenazine at a concentration of 32 µg/ml, induced higher intracellular ROS production in *C. albicans*, as evidenced by increased fluorescence intensity and DHR123 staining. These findings suggested that the toxins may exert their effects on *Candida* cells through the generation of ROS, which could have implications for their antimicrobial activity or potential therapeutic applications (38, 41).

Transcriptional analysis confirmed that, in the presence of *P. aeruginosa*, the expression of SOD2 (superoxide dismutase 2) and several other detoxifying enzymes was down-regulated. This suggests that *P. aeruginosa* toxins may simultaneously induce ROS stress while reducing the expression of SOD2, which could overwhelm the capacity of the detoxification system and lead to cell death. It is worth noting that other classes of antifungals also induce ROS production in *C. albicans* as part of their mode of action. This suggests that *P. aeruginosa* toxins may also increase the susceptibility of the fungus to other antifungal drugs (39, 40).

Overall, the findings suggest that *P. aeruginosa* toxins can enhance the antifungal susceptibility of *C. albicans* by inducing ROS stress and suppressing ROS-detoxifying enzymes. This highlights the potential for targeting ROS-related pathways as a strategy for antifungal therapy and suggests that the combination of *P. aeruginosa* toxins with other antifungal drugs could have synergistic effects in treating fungal infections (42).

CONCLUSION

The modification or accumulation of phenazines after secretion by microbes can have various biological activities that can influence antibiotic toxicity. These processes can be taken into consideration when designing phenazine-producing biostrains. These modified phenazines may exhibit enhanced antibiotic toxicity or even acquire new antimicrobial activities. Understanding and manipulating these modification pathways can be a strategy to enhance the effectiveness of phenazine-based antibiotics. This can occur if the phenazines act as antagonists or inhibitors of the antibiotic, interfering with its mode of action or reducing its effectiveness. Understanding these interactions between phenazines and antibiotics can aid in the design of biostrains that produce phenazines with optimal properties for antimicrobial activity.

The limitation of the study. Contamination of the clinical samples with various types of bacteria, finding of azoles resistant *Candida albicans* strains, and entering patients who had not taken antibiotics until the time of examination were some of the limitations of this study. In this study, we used stored *C. albicans* strains. The main reason for not using the PCR method was that we did not have the clinical samples related isolated yeasts.

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