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Antifungal Potential of *Streptomyces*-Derived Metabolites Against Fluconazole-Resistant Oral *Candida albicans*: In vitro Evaluation and Mechanistic Insights

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ABSTRACT

Background: Oropharyngeal candidiasis, primarily caused by *C. albicans*, is the most common opportunistic fungal infection in patients with head and neck cancer. The increasing emergence of FLZ resistance has led to higher morbidity and mortality rates. *Streptomyces*, a genus of *Actinomycetes*, produces bioactive molecules with antimicrobial effects. This study investigated the antifungal potential of *S. monomycini* strain 615 against FLZ-resistant *C. albicans* clinical isolates in vitro.

Methods: *S. monomycini* strain 615 was cultured, and an aqueous crud extract containing its metabolites was prepared. The effects of extract were tested on five FLZ-resistant *C. albicans* isolates. Key pathogenic factors such as protease activity, biofilm formation, and gene expressions related to virulence (*SAP1*, *SAP2*, *HWP1*, and *ERG11*) and azole resistance (ERG11) were evaluated. Cytotoxicity of the extract (1.8-0.0008 µg/ml) was assessed on KYSE-30 esophageal epithelial cells using the MTT assay.

Results: Strain 615 showed strong antifungal activity with MIC values of 0.0008-0.0035 µg/ml and MFC values of 0.0017-0.0035 µg/ml after 48 hours. The extract significantly reduced ergosterol content by 31.81%, completely inhibited phospholipase and proteinase activities at 0.0035 µg/ml and suppressed biofilm formation at 0.0035-0.0140 µg/ml. Expression of all tested virulence genes decreased except for *ERG11*, indicating a possible mechanism to overcome azole resistance. The highest extract concentration caused 76.7% cytotoxicity in KYSE-30 cells after 72 hours.

Conclusion: *S. monomycini* strain 615 could serve as an alternative or adjunct therapy for FLZ-resistant OPC in head and neck cancer patients, warranting further research to confirm safety and efficacy.
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Keywords: Actinobacteria, *Candida albicans*, Drug resistance

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List of Abbreviations:
C. albicans: *Candida albicans*; **cDNA:** complementary DNA; **CFCF:** cell-free culture filtrate; **DHE:** dehydroergosterol; **EtOAc:** ethyl acetate; **FLZ:** fluconazole; **ISP2:** international *Streptomyces* Project media 2; **MBIC:** minimum biofilm inhibitory concentration; **MFC:** minimum fungicidal concentration; **MIC:** minimum inhibitory concentration; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **OD:** optical density; **OPC:** oropharyngeal candidiasis; **PBS:** phosphate-buffered saline; **Prz:** proteinase zone; **Pz:** precipitation zone; ***S. monomycini***: *Streptomyces monomycini*; **SEM:** scanning electron microscopy; **TEM:** transmission electron microscopy; **XTT:** 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide

INTRODUCTION

Fungal infections are common in the general population and pose remarkable risks, particularly for patients who are severely ill, immune-compromised, or hospitalized. The rise in fungal resistance is mainly attributed to the unnecessary use of antibiotics. If left untreated, fungal infection is escalated into a serious condition, resulting in substantial economic costs and placing a burden on healthcare system and patients. Recent evidence indicates that both geographic spread and frequency of fungal infections are increasing, along with a rise in the resistance to antifungal agents. The main contributors to antifungal resistance arise from the misuse of these agents in human health and agriculture. This situation is further complicated by the lack of sufficient resources and attention devoted to fungal diseases, as well as the growing impact of climate change, which facilitates the spread and severity of these infections. Therefore, One Health approach that integrates human, animal, and environmental health is essential for effectively addressing to this issue[1,2].

Oropharyngeal candidiasis significantly enhances the risk of systemic candidiasis in cancer patients. It often arises as a complication of radiation therapy for head and neck cancers, typically beginning with the colonization of *Candida* in the oral mucosa. *C. albicans*, a normal component of human flora, is responsible for the majority of candidiasis cases, accounting for over 80% of OPC cases. Moreover, radiation treatment can elevate the likelihood of oral *Candida* colonization as much as 74.5%[3,4]. *Candida* infections are a global health concern, and the rising issue of antimicrobial resistance complicates their treatment. Development of antifungal drug resistance among *Candida* species leads to the increase in morbidity and mortality rates, particularly in immunocompromised patients. Since fungi are eukaryotic organisms, there are limited molecular targets for antifungal drug development[5-7]. Currently, four major classes of FDA-approved antifungal agents (polyenes, flucytosine, azoles, and echinocandins) are used to treat invasive fungal infections[8]. Unfortunately, in addition to the emergence of drug resistance among *Candida* populations, cases of multidrug resistance have also been reported for this pathogen. A recent study involving 300 fungi isolates revealed a resistance rate of 80% against FLZ, followed by 23% against amphotericin and 7% against micafungin. Furthermore, 1% of all isolates exhibited resistance to all classes of antifungal agents, while about 24% demonstrated resistance to at least two classes of these agents[9].

Actinomycetes are Gram-positive bacteria known for their ability to produce a wide range of natural products, making them a valuable resource for the discovery of new drugs derived from their abundant secondary metabolites. Many *Actinomycetes*, especially those belonging to the genus *Streptomyces*, synthesize a broad spectrum of antibiotic compounds, including macrolides, β-lactams, polyenes, aminoglycosides, tetracycline, peptides, and polyethers[10]. Each year, new species of *Streptomyces* and other *Actinomycetes* are identified, offering promising opportunities for the development of new antibiotics and antifungals capable of combating drug-resistant pathogens through different mechanisms of action[11-14]. However, a significant gap exists between the discovery of a biological metabolite with a novel mode of action and the development of an antibiotic that can safely be used in clinical settings. In this regard, the selection of fast-growing *Actinomycetes*, the choice of an appropriate solvent for metabolite extraction, and the identification of metabolites that do not adversely affect human cells are critical steps toward obtaining safe antifungal agents.

The present study examines the potential of metabolites isolated from soil *Actinomycetes* as biocontrol agents against drug-resistant *C. albicans* isolates, which are associated with OPC in patients with head and neck cancer. The study aimed to investigate the effects of soil *Actinomycetes* on the growth rate and pathogenic factors of drug-resistant *C. albicans* isolates, with the ultimate goal of identifying natural compounds that can help combat the increasing threat of antimicrobial resistance.

MATERIALS AND METHODS

Microorganisms and culture condition

Among the various *Actinomycete* isolates obtained from rhizosphere soil in a previous study[15], we selected strain 615 (GenBank accession number MN888940) due to its significant effectiveness in controlling the soil-borne fungal pathogens. This bacterium, with more than 99.65% similarity to *S. monomycini* NRRL B-24309^T (JNYL01000005), produces cellulase and siderophores[15]. Strain 615 was cultured on a 8 cm plastic Petri plate containing ISP2 medium[16] consisting of 10 g/l of malt extract, 4 g/l of yeast extract, 4 g/l of glucose, and 18 g/l of agar, with pH adjusted to 7.2. The plates were incubated at 28 °C for 7 days. Bacteria were then scraped off the surface of the medium, suspended in a sterile saline solution (0.9% NaCl), and adjusted to a 10^6 CFU/ml concentration. Next, 100 µl of the spore suspension was added to Erlenmeyer flasks (250 ml) containing 50 ml of ISP2 broth. The flasks were incubated in a rotary shaker incubator at 150 rpm and 28 °C. After four days, the content of the flasks was centrifuged at 12,000 ×g for 5 minutes. The resulting

supernatant CFCF was used for either direct cell treatment or preparation of the aqueous phase [17]. Five FLZ-resistant clinical isolates of *C. albicans*, which were obtained from OPC in patients with head and neck cancer, were involved in this study. *Candida* strains were identified by culturing on CHROMagar Candida and PCR using ITS_1-ITS_4 primers. *C. albicans* ATCC10231 was used as a control in all experiments[18].

Preparation of aqueous phase

All solvents used in this section were HPLC grade. The residues of *Streptomyces* cells and mycelia were removed from the CFCF via suction filtration using Whatman® No. 5 filter paper, followed by a 0.22 µm Millipore Durapore® membrane filter. The obtained solution was dissolved in 250 ml of H_2O and extracted three times with 250 ml of EtOAc. The EtOAc and aqueous phases were separated with the separatory funnel, and the aqueous phase was dried using the lyophilization method and then weighed. The dried extract dissolved in minimal H_2O (10 mg/ml) was used for *Candida* treatments[17].

Growth inhibitory effect of CFCF

The freshly prepared CFCF of strain 615 was added to a 96-well plate at two-fold concentrations (V/V). Subsequently, 100 µl of *C. albicans* suspension (0.5 to 2.5 × 10^3 CFU/ml) in RPMI 1640 medium (Sigma, USA) was added to each well, bringing the total volume to 200 µl. The plates were then incubated at 35 °C for 48 h. After the incubation time, the MIC value of CFCF was evaluated. Wells without *Candida* cells were considered as negative controls, while FLZ at a concentration of 64 µg/ml was used as a positive control[19].

Determination of MIC of aqueous phase

The antifungal activity of the aqueous phase from strain 615 was evaluated using the broth microdilution method according to CLSI standard M27-A3[20,21]. Briefly, aliquots of the fungal suspension containing 0.5-2.5 × 10^3 cell/ ml were distributed into the wells of a microtiter plate containing RPMI 1640 medium supplemented with 0.165 MOPS buffer and adjusted to pH 7.0-7.2. Two-fold serial dilutions of aqueous phase (1.8 to 0.0008 µg/ml) were prepared in RPMI 1640 and added to 96-well plates. The plates were then incubated at 35 °C for 24-48 h. For quality control, *C. albicans* strain ATCC 10231 was tested alongside FLZ-resistant clinical isolates. After incubation, the OD was measured at 530 nm using a plate reader (EPOCH2, BioTek, USA). The MIC was determined by comparing the growth of *C. albicans* in the treated samples to that of the controls. All experiments related to MIC and MFC determination were performed in triplicate.

Effect of aqueous phase of strain 615 on *C. albicans* phospholipase activity

The effect of the aqueous phase on the phospholipase activity of *C. albicans* was evaluated using the egg yolk agar plate consisting of Sabouraud dextrose agar, 57.3 g of NaCl, 0.55 g of $CaCl_2$, and 8% sterile egg yolk, as described previously[22,23]. To assess phospholipase activation, *C. albicans* cultures (OD_{600} = 1.0) were treated with compounds at both the MIC and twice the MIC (2× MIC). Treated cells were then spotted in triplicate onto egg yolk agar plates and incubated at 37 °C for 7 days. Phospholipase activity was determined by measuring the diameters of both the colonies and the Pz surrounding the colonies. *C. albicans* ATCC 10231 was served as the positive control, while *C. glabrata* ATCC 90030 was utilized as the negative control. The mean Pz was calculated for all concentrations tested, and the results were categorized into three groups: Pz = 1, no phospholipase activity; 0.64 > Pz < 1, moderate phospholipase activity (+); Pz < 0.64 high phospholipase activity (++). A higher Pz value indicated low enzymatic activity.

Effect of aqueous phase of strain 615 on *C. albicans* proteinase activity

The proteolytic activity of the treated *Candida* isolates was investigated by bovine serum albumin agar medium, as described before[24]. This experiment was carried out according to the protocol used in the previous section. Each sample was cultured three times and compared with *C. albicans* ATCC 10231 as a positive control. Enzyme activity was estimated based on the diameter of the white halo surrounding each colony, which was caused by the protein degradation zone. The total diameter indicated the presence of Prz. The Prz values were categorized as follows: 1 (no activity), 0.64 to 1 (moderate activity), and <0.64 (high activity).

Effect of aqueous phase of strain 615 on *C. albicans* biofilm formation

The in vitro biofilm formation assay was carried out on five FLZ-resistant *C. albicans* isolates, as described previously[18]. In brief, *C. albicans* cells (1.0 ×10^6 cells/ml) were suspended in RPMI 1640 medium and added to a 96-well tissue culture plate, allowing for 90 min for adhesion at 37 °C. After the initial adhesion, the medium was aspirated to remove non-adherent cells, and then fresh medium containing two-fold concentrations of the aqueous phase of strain 615 was added to the adherent cells. The plates were subsequently incubated at 37 °C for 24 h until the formation of mature biofilm. *C. albicans* ATCC10231 was used as a control (FLZ-susceptible). After incubation, the biofilms were washed three times with

sterile PBS, and biofilm formation was estimated using the XTT assay[25]. In brief, after PBS washing, a 100 µl aliquot of XTT-menadione (0.5 g/l in Ringer's lactate-1 µM) was added to each test, as well as to the control wells. The plates were then incubated in the dark at 37 °C for 1 h. The OD was determined at 490 nm using a microtiter plate reader. The MBIC was determined as the lowest concentration of the drug that inhibited mature biofilm formation. All samples were tested in triplicate.

Effect of aqueous phase of strain 615 on *C. albicans* ergosterol content

Sterols were extracted using a previously modified method[26]. Briefly, spore suspensions of five FLZ-resistant *C. albicans* isolates were cultured at different concentrations (0.0035, 0.0018, and 0.0009 µg/ml) of the aqueous phase. The cultures were incubated at 180 ×g at 35 °C for 72 h. After incubation, the fungal mass was collected by centrifugation and washed three times with distilled water, and the weight of the fungi was determined. Saponification was performed by adding 3 ml of 25% alcoholic KOH solution (25 g of KOH mixed with 35 ml of sterile distilled water, and ethanol) to make a total of 100 ml for each sample. Each tube was vortex-mixed for 1 min and then refluxed in 85 °C water bath for 1 h. After incubation, the tubes were cooled at room temperature. Non-saponifiable sterols were extracted by adding a mixture of 1 ml of distilled water and 3 ml of *n*-hexane, which was then shaken vigorously for 3 min. The hexane layer was collected, transferred to clean tubes, and stored at -20 °C for 18-24 h. Before analysis, the sterol extracts were diluted five folds in 100% ethanol. Following dilution, the presence of ergosterol was assessed using spectrophotometry in the range of 200 to 300 nm with the help of a Perkin–Elmer EZ 301 spectrophotometer (PerkinElmer, USA). A distinctive four-peaked curve confirmed the presence of ergosterol and the late sterol intermediate 24(28)DHE in the extract, while a flat line indicated the absence of detectable ergosterol. The amount of ergosterol was calculated as a percentage of the wet weight of the fungus, and the quantification was performed by the following formula:

$$\%\ \text{ergosterol} + \%24(28)\ \text{DHE} = [(A\ 281.5/290) \times F]/\text{pellet weight},$$

$$\%24(28)\ \text{DHE} = [(A230/518) \times F]/\text{pellet weight},$$

$$\%\text{ergosterol} = [\%\ \text{ergosterol} + \%24(28)\ \text{DHE}] - \%24(28)\ \text{DHE}$$

where F is the factor for dilution in ethanol, and 290 and 518 are the E values (percentages per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

Esophageal epithelial cell viability after treatment with the aqueous phase of strain 615

The cytotoxicity assay was performed using the KYSE-30 cell line, which was derived from the mucosal surface of the well-differentiated squamous cell carcinoma. To assess the inhibitory effect of the aqueous phase of strain 615 on the growth and proliferation of KYSE-30 cells, the ability of reduction to formazan was determined. KYSE-30 cells (2-9 × 10^4) were seeded in 96-well plates (Orange Scientific, Brussels, Belgium) containing 100 µl of RPMI 1640 medium, along with aqueous phase at concentrations ranging from 0.10 to 3.5 ng/ml. After incubation at 37 °C, the medium was removed from the control, and the cells were treated for 24, 48 and 72 h. Next, 50 µl of MTT solution (0.5 mg/ml) was added to each well and the cells were further incubated at 37 °C for 2 h. For solubilization of the precipitated formazan, we added 100 µl of DMSO and measured the OD at 550 nm wavelength. The inhibition rate of the compounds was calculated using the following equation: inhibition rate (%) = 1 - OD_{exp}/OD_{con} × 100, where OD_{exp} and OD_{con} represent the OD of the treated and untreated cells, respectively[17].

RNA extraction and gene expression

Total RNA was extracted from FLZ-resistant clinical isolates of *C. albicans* treated with the aqueous phase of strain 615 using the RNAX plus kit (Sina Clone, Iran). To prepare the fungal cell mass, *C. albicans* isolates were treated with different concentrations of the aqueous phase in 24-well plates. After 24 h of incubation at 35 °C, total RNA was extracted from the cultures. The RNA concentration was measured using a spectrophotometer (EPOCH2, BioTek). First-strand cDNAs were synthesized using a cDNA synthesis kit (Parstous, Mashhad, Iran). The expression levels of *ERG11, SAP1, SAP2,* and *HWP1* genes were evaluated by real-time PCR with specific primers (Table 1). Real-time PCR was performed on the Rotor-Gene 3000 real-time PCR system (Applied Biosystem, USA). The results were analyzed by relative quantification, with *ACT1* expression serving as the reference gene. ΔCT values were calculated using the following formula: ΔCT = CT (target) - CT (reference). Gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method, and fold increases were assessed using the relative threshold method ($2^{-\Delta\Delta CT}$)[18].

Scanning and transmission electron microscopy

To study the effects of the aqueous phase of strain 615 on cellular damage, a culture of *C. albicans* was

Table 1. Primer sequences and their PCR products size

Gene	Sequences	Product size (bp)	References
ACT1	GACCGAAGCTCCAATGAATC AATTGGGACAACGTGGGTAA	270	[37]
HWP1	CTCCAGCCACTGAAACACCA GGTGGAATGGAAGCTTCTGGA	136	[19]
SAP1	TCAATCAATTTACTCTTCCATTTCTAACA CCAGTAGCATTAACAGGAGTTTTAATGACA	161	[37]
SAP2	AACAACAACCCACTAGACATCACC TGACCATTAGTAACTGGGAATGCTTTAGGA	177	[3]
ERG11	AATGATTTTGTTTATAATTTAAGATGGACTATTGA AATGATTTCTGCTGGTTCAGTAGGT	91	[36]

prepared using the Sabouraud dextrose agar (40 g of glucose, 10 g of peptone, and 0.5 g of chloramphenicol). The plates were incubated at 37 °C for 24 h, after which one colony was selected for the treatment with bacterial aqueous phase at MIC concentration. Next, the samples were incubated at 37 °C for 24 hand then placed in 2.5% glutaraldehyde solution overnight, washed using PBS buffer, and fixed by osmium tetroxide. Each slide was then dehydrated using gradient series of ethanol for 20 min and treated with hexamethyldisilazane for another 20 min. To ensure complete drying, we incubated the slides at 28 °C overnight. Also, a thin layer of gold was applied on the slides to prevent surface charge during SEM analysis. The images were captured using a field-emission SEM (Quanta 200, 3 kV, 10.0 mm). For TEM, after drying, samples were pre-fixed in 2% osmium tetroxide buffer and embedded in epoxy resin overnight. An ultramicrotome was employed for sample preparation, and sections were stained with 2% uranyl acetate and 4% lead citrate. These sections were evaluated using a transmission electron microscope (EM208s, 100KV, Philips, Netherland)[27].

Statistical analysis

The data of the biofilm formation and gene expression, as well as cytotoxicity test, were analyzed by two-way ANOVA in GraphPad PRISM 6 (GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Inhibitory effect of bacterial aqueous phase on fungal growth

Antifungal activity of the CFCF and the aqueous phase of the *S. monomycini* strain 615 was evaluated against five FLZ-resistant clinical isolates of *C. albicans*. The MIC and MFC values for the CFCF and the aqueous phase are summarized in Table 2. The aqueous phase exhibited potent antifungal activity with MIC values ranging from 0.0008 to 0.0035 µg/ml and MFC values between 0.0017 to 0.0035 µg/ml for the resistant clinical isolates. In contrast, the FLZ-susceptible strain *C. albicans* ATCC 10231 showed significantly higher MIC and MFC values, indicating the presence of metabolites other than FLZ in the aqueous phase (Table 2).

Table 2. MIC, MFC, and MBIC of CFCF and aqueous phase of strain 615 against five FLZ-resistant clinical isolates of *C. albicans*

Organism	Fluconazole	Bacterial supernatant		Aqueous phase extract		
	MIC (µg/ml)	MIC (v/v %)	MFC (v/v %)	MIC (µg/ml)	MFC (µg/ml)	MBIC (µg/ml)
C. albicans 1878	>64	1.562	1.562	0.0017	0.0017	0.0070
C. albicans 2314	64	3.125	3.125	0.0035	0.0035	0.0070
C. albicans 1028	64	0.781	1.562	0.0008	0.0017	0.0035
C. albicans 4573	32	3.125	3.125	0.0035	0.0035	0.0140
C. albicans 9854	32	3.125	3.125	0.0035	0.0035	0.0140
Standard	0.5	12.50	12.50	0.0140	0.0140	0.0562

FLZ susceptible control strain: *C. albicans* ATCC 10231

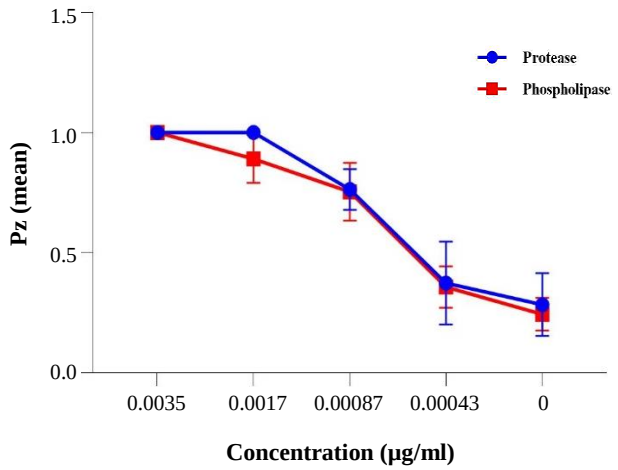

Fig. 1. Effect of aqueous phase of strain 615 on proteinase and phospholipase activity of five FLZ-resistant *C. albicans* isolates. All concentrations, except for 0.00043 µg/ml, represented significant difference with control ($p < 0.0001$).

Enzyme activity inhibition

The inhibition of phospholipase and proteinase activities was evaluated by the aqueous phase. As illustrated in Figure 1, the activity of both enzymes at a concentration of 0.0035 µg/ml was significantly (100%) inhibited. The phospholipase activity showed a significant reduction; at highest concentration of the aqueous phase, it was almost completely eliminated. Additionally, a significant reduction was observed in proteinase activity, indicating the potential of the aqueous phase in reducing the virulence of *C. albicans* (Fig. 1).

Inhibitory effect of bacterial aqueous phase on biofilm formation

The aqueous phase demonstrated a significant inhibitory effect on biofilm formation on both FLZ-resistant and standard isolates of *C. albicans*. The determined MBIC revealed a dose-dependent inhibition of biofilm formation. As shown in Table 2, five FLZ-resistant isolates exhibited strong anti-biofilm activity with MBIC values ranging from 0.0035 to 0.0140 µg/ml. In contrast, the FLZ-susceptible strain *C. albicans* ATCC 10231 represented higher MBIC values (0.0562 µg/ml) than those of resistant isolates (Table 2). Statistical analysis showed a significant relationship ($p < 0.0001$) in the biofilm formation ability between five FLZ-resistant and one-susceptible (control) strains after treatment with the bacterial extract (Fig. 2).

Inhibitory effect of bacterial aqueous phase on ergosterol biosynthesis

The study analyzed the impact of varying concentrations of the aqueous phase of strain 615 on the ergosterol content of FLZ-resistant *C. albicans* isolates. We observed a significant reduction in ergosterol content with increasing concentrations of the aqueous phase. At a concentration of 0.0035 µg/ml, the mean ergosterol content reduced to 0.030 ± 0.001 µg/mg, indicating a 31.81% inhibition compared to the control (0.044 ± 0.001 µg/mg; Table 3). This significant reduction ($p < 0.0001$) highlights the effectiveness of the aqueous phase in disrupting ergosterol synthesis in *C. albicans* isolates.

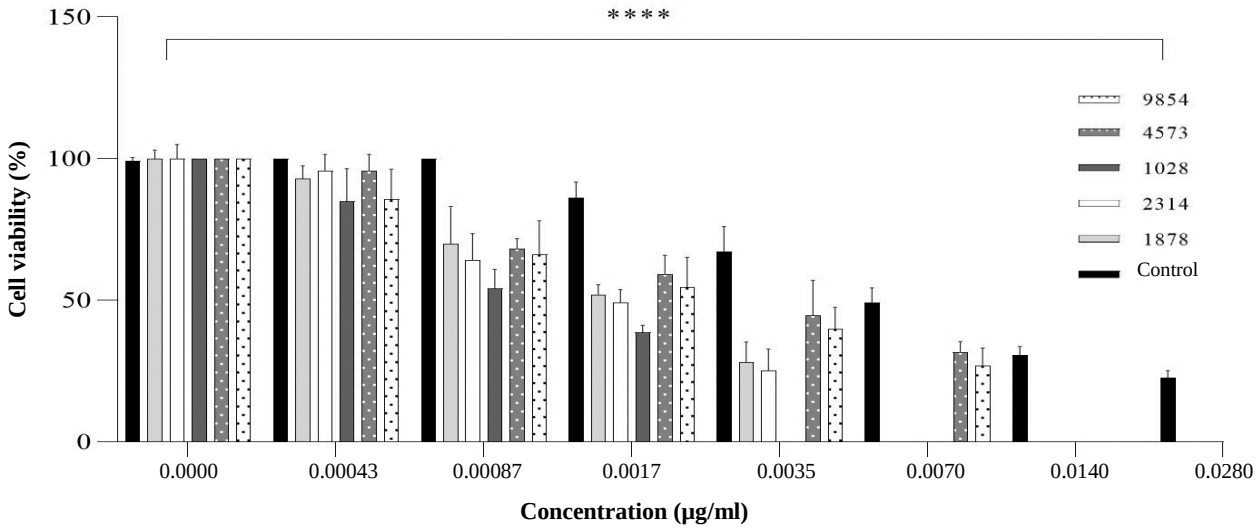

Fig. 2. The mean biofilm formation of five FLZ-resistant *C. albicans* clinical strains and *C. albicans* ATCC 10231 as control ($^{****}p < 0.0001$).

Table 3. Effect of aqueous phase concentrations of strain 615 on the ergosterol content of FLZ-resistant *C. albicans* isolates

Aqueous phase extract (µg/ml)	Ergosterol content per fungal dry mass (µg/mg)	Ergosterol inhibition (%)
0.0035	0.030 ± 0.001	31.81
0.0018	0.035 ± 0.001*	20.45
0.0009	0.039 ± 0.002*	11.36
0.0000	0.044 ± 0.001	0.00

Results are the mean ± SD of two separate experiments in triplicate sets. *Statistically significant difference with control ($p < 0.05$).

Gene expression analysis

Real-time PCR analysis revealed a significant downregulation in the expression of genes associated with pathogenicity and azole resistance, specially *SAP1*, *SAP2*, *HWP1*, and *ERG11*, following treatment with the aqueous phase. Among the tested genes, the expression of *ERG11* showed the most substantial reduction, suggesting a direct effect on the ergosterol biosynthesis pathway (Fig. 3). The relative gene expression levels normalized to *β-actin* are depicted in Figure 3.

Cytotoxic effect of bacterial aqueous phase on esophageal epithelial cell

The cytotoxic effects of the aqueous phase on KYSE-30 cells were evaluated using the MTT assay. The results indicated a concentration-dependent reduction in cell viability. At the highest concentration, 76.7% cytotoxicity was observed after 72 hours, suggesting that the aqueous phase is effective against *C. albicans*; however, the cytotoxic effects of bacterial extract on human cells require further investigation (Fig. 4). A two-way ANOVA analysis demonstrated significant differences between untreated and all treated (0.10-3.5 ng/ml) samples.

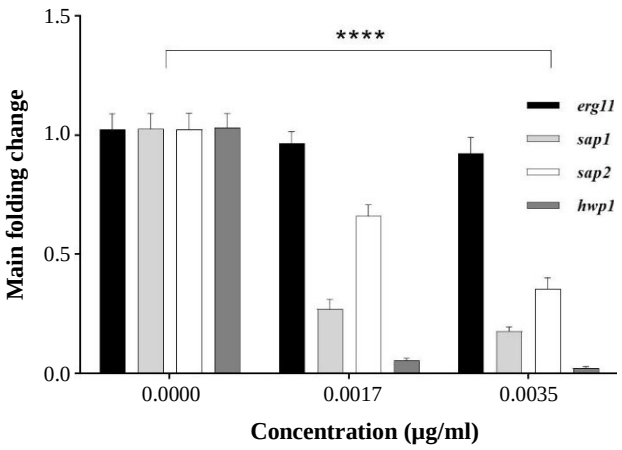

Fig. 3. Expression ratio of azole resistance, proteinase secretion, and biofilm formation responsible genes *SAP1*, *SAP2*, *ERG11*, and *HWP1* versus internal control (*β-actin*) in five FLZ-resistant *C. albicans* isolates after treatment with aqueous phase concentrations of strain 615 (****$p < 0.0001$).

Effect of the aqueous phase of strain 615 on the morphology of *C. albicans*

After treatment with the aqueous phase of strain 615, the morphology of *C. albicans* was examined using SEM and TEM. The images of *C. albicans* cells at MIC concentration are shown in Figure 5. The cells affected by the bacterial metabolite exhibited shriveled appearance, showing a loss of integrity and regularity in their cell walls. In contrast, the untreated cells indicated preserved integrity (Fig. 5A). The TEM images revealed that in the cells treated with MIC concentration of bacterial aqueous phase, the cell membrane lost its integrity, and showed irregularities in the cell membrane (Fig. 5C). In comparison, the control (non-treated) cells displayed a regular and uniform surface on both the cell wall and cell membrane, and the cytoplasm appears normal (Fig. 5B). In contrast, the treated cells showed ruptures in the cell membrane with penetration into the cytoplasm. As a result, the cytoplasm failed to maintain its normal appearance, and revealed significant changes and abnormalities within the cytoplasmic space. Furthermore, the cytoplasm of the treated cells appeared highly dense, granular, and vacuolated. Certain areas of the cell exhibited rupturing and aggregation of the cell membrane was observed. The cytoplasm lost its uniformity, resulting in the formation of a large vacuole around the nucleus characterized by an irregular wall and became connected to the cytoplasm and was attached to the cytoplasm. These alterations indicated substantial and extensive cell destruction (Fig. 5).

DISCUSSION

The present study aimed to identify novel natural antifungal agents derived from *Actinomycetes* against FLZ-resistant *C. albicans* species isolated from OPC in patients with head and neck cancer. Oral candidiasis is highly prevalent among these patients, particularly after

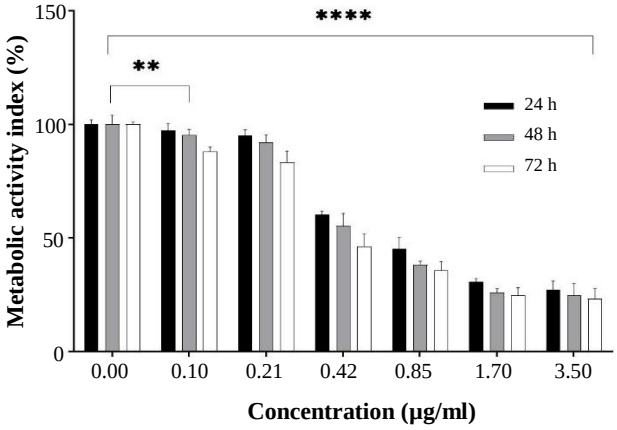

Fig. 4. Cellular metabolic activity (%) of human esophageal epithelial cells (KYSE-30), evaluated by MTT assay, after 24, 48, 72 h exposure to aqueous phase of strain 615 ($^{****}p < 0.0001$, $^{**}p < 0.005$).

radiotherapy. Radiation therapy may disrupt the balance between fungal pathogenic factors and the host's immune system[28]. Moreover, the extensive use of antifungals has led to an increasing resistance of clinical *Candida* isolates to certain antifungals, mainly FLZ, which contributes to treatment failures in candidiasis[4,18].

Actinomycetes are known as significant sources of bioactive compounds[29]. Indeed, nearly one-third of the 33,500 identified bioactive molecules derived from bacteria have been attributed to the *Actinomycetes* genus[30]. Studies have indicated that secondary metabolites from *Actinomycetes* exhibit unique properties, such as antimicrobial effects and enzyme inhibition[31]. Guo and colleagues have shown that the suspensions, culture supernatants, and cell lysates of oral *Actinomycetes* effectively inhibit the proliferation of *C. albicans*. This inhibition affected the adhesion, acid protease, and phospholipase activities, as well as biofilm development and hyphal formation of *Candida*[32]. Taechowisan et al. have reported that among the 330 *Actinomycetes* isolated from 36 plant species, the most prevalent group of isolates was the *Streptomyces* sp., which exhibited strong growth inhibitory effects on fungal species, including *Colletotrichum musae*, and *Fusarium oxysporum*[33]. Sarika et al. collected 28 soil samples from coal mine sites in Telangana, India. They found that the potential strains isolated from these samples belonged to the *Streptomyces* genus and represented antibacterial and antifungal activity[10].

In this study, we examined the effect of soil *Actinomycetes* on the proliferation of FLZ-resistant *C. albicans* species isolated from the oropharynx of patients with head and neck cancer. Our results showed a potent inhibitory effect of the aqueous phase of *Streptomyces* 615 sp. on FLZ-resistant *C. albicans* isolates at a very low concentration (0.0035 µg/ml). Furthermore, we observed a significant suppression of the *HWP1* gene expression after treatment with this compound. HWP1 is a well-known cell surface protein in *C. albicans*, which is expressed only on hyphae and has a crucial role in the adherence to oral epithelial cells. In addition, we found that *Streptomyces* sp. 615 significantly suppressed phospholipase and aspartyl proteinase activities, indicating strong efficacy in destroying biofilms formed by FLZ-resistant *C. albicans* isolates. *Candida* species produce virulence factors such as extracellular enzymes and biofilms, which help them colonize and invade host tissues. Since phospholipases hydrolyze glycerophospholipids in cell membranes to facilitate mucosal invasion, the bacterial extract showing inhibition of these enzymes may be valuable in preventing candidiasis[34-36]. Research has demonstrated that secreted aspartyl proteinases,

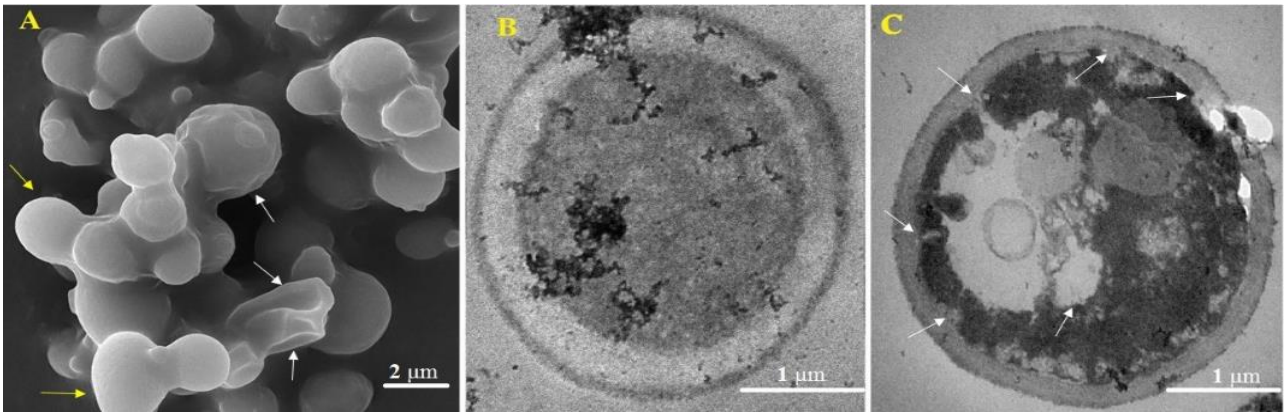

Fig. 5. SEM and TEM images of FLZ-resistant *C. albicans* isolates cells after exposure to aqueous phase of strain 615. (A) Effected (white arrows) and unaffected (yellow arrows) *C. albicans* cells; (B) non-treated cell; (C) cells treated with MIC concentration; ruptured cell membrane and large vacuole with irregular wall are indicated by white arrows.

specifically SAP1, SAP2, and SAP3, play a significant role in causing tissue damage and invading oral epithelium and cutaneous epidermis[3,37]. These proteins also contribute to the ability of *C. albicans* to cause mucosal and disseminated infections. Our findings showed a reduction in the expression of the *SAP1* and *SAP2* genes after treatment with bacterial extract. Notably, the decrease in expression was greater for *SAP1* than *SAP2*.

Ergosterol is a major amphipathic lipid found in the plasma membrane of fungi. Based on our literature survey, a primary target for many antifungal agents is the enzymes involving in the ergosterol biosynthetic pathway. The main classes of these antifungals are polyenes and azoles. Polyenes, such as amphotericin B, strongly bind to ergosterol molecules, damaging the plasma membrane and causing leakage of intracellular ions. The most important azole is FLZ, which acts in the late phases of the biosynthesis pathway and inhibits ergosterol production by blocking the cytochrome P450 (Erg11p), responsible for the 14α demethylation of lanosterol[4,38]. Herein, we observed a decrease in ergosterol content following treatment with the bacterial extract. Gene expression analysis showed a slight suppression in the expression of *ERG11* gene. However, the decrease in ergosterol does not appear to be related to the expression level of *ERG11*, suggesting that this reduction may be caused by effects on other targets in the ergosterol biosynthesis pathway. Moreover, changes in ergosterol content could arise from other factors, such as availability of iron, which has been shown to affect the sterol composition in certain fungi, influencing their growth and survival[39].

In fungal cells, the balance between ergosterol content and reactive oxygen species production is significant for cellular health. High levels of reactive oxygen species can lead to oxidative stress, which affects the synthesis or function of ergosterol[39]. Evidence has indicated that changes in ergosterol levels due to environmental stressors (e.g. nutrient limitation or antifungal exposure) might trigger compensatory mechanisms involving in efflux pumps as a part of the broader stress response[40]. While there was no direct relationship between ergosterol content, iron metabolism or ROS production and efflux pumps, interconnected pathways exist where changes in one may influence the others within fungal biology and related systems. Further research is needed to clarify these interactions more definitively. To examine the cytotoxic effect of strain 615 extract, MTT test was conducted on KYSE-30 esophageal epithelial cells. The results showed that after 72 hours of incubation, only 24% of the cells remained alive. This low survival rate could be attributed to the ability of *Actinomycetes* to produce a wide variety of secondary metabolites. The presence of multiple compounds in the extract could affect the cytotoxicity results; however, the active ingredient may show different results. Moreover, it is suggested that the extracts from this bacterium might be used in designing topical antifungals such as mouthwashes, to reduce the cytotoxic effects in clinical settings for head and neck cancer patients suffering from oral candidiasis.

Studies have measured the cytotoxic effect of *Actinomycetes* in the human cancer cell lines. Qui et al.[41] examined several bacterial extracts and reported that one of the bacterial compound inhibited the growth of tumor cell lines KYSE30 and KYSE180 at IC_{50} values of 15.92 and 30.77 μM. Benko et al.[42] demonstrated that for human cells, the IC_{50} values were 0.553 mg/l for itraconazole, 1.24 mg/l for saperconazole, 2.58 mg/l for clotrimazole, 5.33 mg/l for miconazole, 6.17 mg/l for econazole, 6.27 mg/l for ketoconazole, and 8.38mg/l for oxiconazole. In this study, we also found that a bacterial metabolite strongly inhibited the FLZ-resistant strain of *C. albicans*. TEM and SEM revealed changes in the cell wall after exposure to the bacterial extract, as well as significant damage to the plasma membrane. The decrease in ergosterol content suggests that a compound of the bacterial metabolite may act by targeting plasma membrane ergosterol.

Our results showed that the bacterial aqueous phase had a significantly greater effect on resistant strains compared to sensitive strains. One of the most important mechanisms of resistance is changes in the ergosterol biosynthesis pathway and the function of efflux pumps[43]. Although there was only a slight reduction in the expression of *ERG11* gene, it seemed that the bacterial extract affected other genes within the ergosterol biosynthesis pathway. Likewise, the resistance mechanism of this strain of *C. albicans* involves in the increased expression of ABC transporters, and interaction with these metabolites likely inhibit their function, leading to decreased fungal growth. However, additional studies are required to identify the active metabolites and assess their clinical applicability. Due to the complexity of bacterial extracts and the existence of multiple compounds in crude extract, we had some limitations in our study. This complexity can complicate the identification and isolation of active ingredients responsible for therapeutic effects. Additionally, some compounds produced by *Actinomycetes* may exhibit toxicity or cause adverse side effects on humans or other organisms[44]. Therefore, necessary safety tests should be performed before using these extracts for therapeutic purposes.

CONCLUSION

The aqueous extract of *S. monomycini* strain 615 strongly inhibits drug-resistant *C. albicans* growth and pathogenicity, shows low cytotoxicity, and requires no hazardous solvents, making it a promising source for new antifungal drugs. However, it is necessary to investigate different fractions of aqueous extracts to identify effective metabolite(s) in the control of drug-resistant pathogens.

DECLARATIONS

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The authors did not use any artificial intelligence-assisted technologies in the production of the current work.

Ethical approval

All the experimental procedures in this study were approved by the Research Ethics Committee of Pasteur Institute of Iran, Tehran (ethical code: IR.PII.REC.1398.052).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

MK: carried out the experiments and writing the manuscript; ZJ: designed and directed the project, planning and supervised the work, analyzed the results, and wrote the manuscript; AS: analyzed the data and contributed to sample preparation.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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