

Inhibition of *Candida albicans* cell growth and biofilm formation by a bioactive extract produced by soil *Streptomyces* strain GCAL-25

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Abstract: Resistance to antifungal agents is a major public health concern since multidrug resistant (MDR) strains of *Candida albicans* have caused severe infections among immunosuppressed, diabetic and other hospital patients. This study focused on evaluating the effects of a bioactive extract (BEx) produced by a novel *Streptomyces* species on *C. albicans* cell germination and biofilm formation. Agar disk diffusion assays were used to select a streptomycete with inhibitory activity over *C. albicans* cells. Thereafter, minimal inhibition concentration (MIC) and time-kill values were obtained for the BEx prepared from the isolate GCAL-25. Also, the effects of BEx on biofilm formation were analyzed. Results showed that the GCAL-25 isolate from the *Streptomyces* genus displayed inhibitory activity on *C. albicans*. A paper disk soaked with BEx showed an inhibitory halo around confluent growing cells of *C. albicans*. The calculated MIC values for BEx indicated that *C. albicans* was three times more susceptible to BEx than the control fungicide, amphotericin B (AmpB). Time-kill studies with $\frac{1}{2}$ x and 1xMIC of BEx showed severe negative effects on cell viability, suggesting a strong fungicidal activity. In addition, an important reduction of *C. albicans* biofilm formation was observed. The BEx from *Streptomyces* sp. GCAL-25 altered yeast-to-hyphae transitions and induced abnormal cell morphology (e.g. cell shrinkage), including impairments of cell membrane integrity with negative effects on biofilm formation.

Key words: *Candida albicans*; biofilm; metabolites; antifungal; *Streptomyces*

INTRODUCTION

Candidiasis is a fungal infection caused by certain species of the *Candida* genus that normally coexist with the autochthonous human microbiome. However, this yeast can be the cause of opportunistic infections on the skin, in mucous membranes or of other systemic illnesses, particularly in patients affected by immunodeficiency disorders [1]. The fluconazole-resistant *C. albicans* is the *Candida* yeast most frequently involved in diseases and is considered as a microorganism posing a serious hazard to humans, according to the Centers for Disease Control and Prevention (CDC) of the US Department of Health and Human Services [2]. A high incidence of candidiasis has been reported in hospitals, particularly in neonatal intensive care units, in HIV units and in organ transplant units [3-5].

C. albicans planktonic cells are susceptible to commercial antifungal drugs; however, their medical impact depends on their ability to successfully form a biofilm [6]. *Candida* biofilms are frequently associated with indwelling medical devices (e.g. dental implants, catheters, heart valves and vascular bypass grafts) that function as substrates and as a physical support for biofilm growth [6,7]. Biofilms are capable of withstanding exposure to every available drug type at concentrations up to 1000 times higher than those that are effective against non-biofilm planktonic cells [8]. The mechanisms of resistance appear to be multifactorial and similar to those with planktonic antifungal resistance (decreased concentration of effective drugs, drug target modifications and metabolic bypasses), as well as those specific to the biofilm environment (production of an extracellular matrix) [9].

The excessive use of antifungal drugs can trigger resistance mechanisms similar to those elucidated in *Candida* at the molecular level. Unfortunately, a number of novel resistance patterns, as well as the emergence of multidrug resistant (MDR) *Candida* strains, has recently been observed. Specific resistance mechanisms may result in cross-resistance to other similar drugs [10]. Sterol $\Delta^{5,6}$ desaturase (encoded by *ERG3*) is a key enzyme in the ergosterol biosynthesis pathway, and *erg3* mutants of *Candida* are capable of circumventing the inhibitory action of polyenes and azoles [11]. Along with the appearance of strains of *Candida* displaying novel resistance mechanisms, alternative therapeutic approaches and new antifungal agents also need to be identified. *Streptomyces* bacteria possess a complex metabolic network involved in the regulation and production of antibiotics, antifungals and cell growth inhibitors [12]. Compounds produced by actinobacteria species show inhibitory activity against planktonic *C. albicans* cells: AmpB from *S. nodosus*, nystatin from *S. noursei*, bahamaolides from marine *Streptomyces* sp. and mycangimycin from *Streptomyces* sp. [13-16]. Based on the need for new antifungal agents, the aim of this work was to select and characterize streptomycete strains preserved at the Germplasm Bank of actinomycetes with anticandidal activity against *C. albicans*, and to study the effects of the BEx produced by a novel *Streptomyces* isolate on *C. albicans* cell germination and biofilm formation.

MATERIALS AND METHODS

Strains, media and growth conditions

For this study, reference yeast cells of *C. albicans* were obtained from the American Type Culture Collection (ATCC 10231). Yeast cell suspensions (YS) at a 1×10^6 cells/mL density were prepared using an overnight culture grown on a medium of yeast extract peptone dextrose (YPD) broth at 30°C. One hundred μ L of the above suspension was used in agar plug diffusion, with 96-well plate microdilution and biofilm assays. The strains were conserved at the Southeast Unit of CIATEJ in the Germplasm Bank of Actinomycetes, which is secured at -80°C. The protocols for culture and storage conditions for the maintenance of *Streptomyces* are described in the Laboratory Maintenance of

Streptomyces Species [17], which describes streptomycetes grown at 29°C for the International *Streptomyces* Project (ISP) in agar media 2 during a 10- to 15-day period, and long-term storage as preservation stocks (PS). To perform agar plug assays and phenotypic characterization, a general inoculum (GI) of spores at 1.5×10^8 spores/mL was prepared for each isolate from the PS [18].

Screening of streptomycetes displaying anticandidal activity using the agar diffusion assay

Two microliters of GI from streptomycete strains were inoculated on ISP2 plates and incubated for 10 days at 29°C. Subsequently, a 5-mm-diameter agar section, measured at a 1.5-mm distance from the colony border of an individual streptomycete, was collected with a sterile cork borer. Five sections from the independent isolates were placed on YPD agar plates that were previously seeded with 100 μ L of YS. After 48 h of incubation at 29°C, the inhibition halo diameter was measured with a caliper. Two independent experiments were conducted, along with a replicate.

Phenotypic characterization of the GCAL-25 isolate

Morphological and biochemical characterization of the bioactive isolate was assessed according to Shirling and Gottlieb [19] with slight modifications. For biochemical characterization, 2 μ L of GI were inoculated in 24-well culture plates containing an ISP9 medium supplemented with different complex substrates, such as ISP2, ISP3, ISP9, Muller-Hinton (MH), nutrient agar (NA), potato dextrose agar (PDA), triple sugar iron agar (TSI), Luria-Bertani (LB) agar, trypticase yeast extract (TYE), lysine iron agar (LIA), Sabouraud dextrose agar (SDA) and Simmons citrate agar (SCA). Carbon source assimilation was maintained with 1% (w/v) D-glucose, D-arabinose, sucrose, D-xylose, myoinositol, L-rhamnose, glycerol and D-raffinose. Physiologically, growth was observed in the presence of sodium chloride concentrations (2 and 15%, w/v) which were added to the ISP2 agar. The differentiation steps, which included growth of mycelium substrate and spore production, were observed after a 14-day incubation period at 29°C.

Molecular identification

Identification of the GCAL-25 isolate was carried out according to Evangelista-Martínez [18], whereby the 16S rDNA gene was amplified using the oligonucleotides fD1: 5'-CCGAATTCGTCGACAA-CAGAGT-3', and rD1: 3'-CCCGGGATCCAAGCT-TAAGGA-5', and Promega® GoTaq DNA polymerase [20]. The reaction conditions were: once for 3 min at 94°C; 35 cycles of 45 s at 94°C; 45 s at 55°C; 1.4 min at 70°C; one cycle of 10 min at 72.2°C. The PCR product was directly sequenced at LANGEBIO (National Laboratory of Genomics for Biodiversity, CINVESTAV-Irapuato, Mexico), using Chromas software to assemble and trim the sequences. A 1243-bp fragment was analyzed for homology using BLASTN software. The 16S rDNA gene sequences from several strains of various genera were retrieved from the non-redundant GeneBank database <http://blast.ncbi.nlm.nih.gov/> [21]. Phylogenetic analysis was carried out at the phylogeny.fr website: <http://www.phylogeny.fr/version2.cgi/index.cgi>, with *Streptosporangium* sp. used as an outgroup. The partial sequence from *Streptomyces* sp. GCAL-25 was deposited in the GenBank database under the accession number KY327365.

Preparation of BEx

Ten Petri dishes containing ISP2 agar media were inoculated with 100 µL of GI and homogeneously dispersed. After a 2-week incubation period at 29°C, spores and mycelia were discarded and the agar layer was scratched from each plate. Macerated agar was then placed in a sterilized bottle and the compounds were extracted overnight using 100 mL of 98% ethanol at 4°C. The solution from the ethanolic extract was centrifuged at 2500 x g for 10 min and the supernatant was evaporated at 45°C for 48 h. The precipitate obtained from the extract was dissolved in sterile distilled water until further use.

A disk diffusion test was carried out to assess the effectiveness of the BEx. Ten µL of the concentrated extract were placed on 5-mm-diameter filter paper disks and subsequently on Petri dishes containing a previously prepared confluent culture of *C. albicans* as mentioned above. After a 24-h incubation period

at 37°C, the resulting inhibitory halo was measured using a caliper.

Determination of the minimal inhibition concentration (MIC) by the microdilution method

MIC testing was carried out by the microdilution method using 96-well plates [22]. The plates were prepared by placing 100 µL of 2xYPD broth and 100 µL (1 mg/mL) of the BEx in the 1st row of wells. Two-fold serial dilutions of BEx were performed to obtain concentrations ranging from 25-0.12 µg. Finally, 100 µL of a *C. albicans* suspension containing 10⁶ cells/mL were added to the wells. Control wells were included in each microplate including: (i) a solution containing non-bioactive extract with and without cells; (ii) 125-0.06 µg from an AmpB solution (Sigma-Aldrich, 250 mg/mL); and (iii) 50-0.024 µg from an itraconazole solution (ITRA, 1 mg/mL). The contents of the wells were mixed, and the microplates were subsequently incubated at 37°C for 24 h. Microbial growth was measured at a 540-nm optical density (OD). The MIC was defined as the lowest concentration at which total inhibition of microbial growth was visually detected.

Time-kill studies of the BEx with *C. albicans* planktonic cells

A fresh *C. albicans* cell suspension was taken from an overnight culture grown on YPD broth medium at 37°C, which was adjusted to OD₅₄₀=1.0. Subsequently, a 10-mL aliquot of the culture was transferred to an Erlenmeyer flask and placed into an orbital shaker at 37°C, operating at 150 rpm. After 1 h of growth, 125 µg ITRA, 1xMIC of AmpB (10.9 µg/mL), ½xMIC, and 1xMIC of the BEx (3.9 and 7.8 µg/mL, respectively) were added. The growth's OD was monitored over 5 h. Cell viability was evaluated by directly counting the cells. A sample from each treatment was collected and submitted to serial dilutions with fresh YPD broth, 10 times. A 100-µL aliquot was seeded onto a YPD plate, and subsequently incubated during a 24-48 h period at 37°C to perform a colony count. The effects of the treatment on morphology were also observed at the end of the experiment using an Eclipse Ti Nikon® inverted microscope. Two independent

experiments were conducted along with a replicate. Impairments on planktonic cell morphology induced by the BEx were confirmed by placing 100 μL of *C. albicans* planktonic cells at a $10^6/\text{mL}$ density in 24-well microplates containing 125 μg ITRA, 1xMIC of AmpB, as well as $\frac{1}{2}\text{x}$ and 1xMIC of BEx. Then, after a 3-h incubation at 37°C (with no shaking), the effects on cell morphology were observed using an inverted microscope.

Biofilm inhibition assay

Biofilms were produced on commercially presterilized non-treated, flat-bottom 96-well microplates (Eppendorf® 0030 730.011) as described [23]. One hundred μL of a planktonic cell suspension (10^8 cells/mL) were transferred to each well and shaken at 60 rpm in an orbital shaker at 37°C for 2 h to allow yeast attachment to the bottom surfaces. Subsequently, non-attached cells were removed from each well and washed twice with phosphate-buffered saline (PBS) pH 7.4. One hundred μL of yeast nitrogen base broth (YNB) mixed with ITRA and AmpB (control), and the BEx ($\frac{1}{2}\text{x}$ and 1xMIC) was placed in wells. Biofilms were incubated at 37°C for 48 h, and for visual we used an inverted microscope. The inhibition of biofilm formation was determined by the crystal violet assay. The content of each well was removed and washed three times with PBS. Afterwards, 0.05% (w/v) of crystal violet solution were added to the wells and maintained for 20 min. Excess stain was carefully removed by rinsing the plate with PBS, and 200 μL of 95% ethanol were immediately added to the stained adherent cells. After 30 min of destaining, 100 μL of this solution were transferred to a new well for measurement with a microplate reader Elx808 (BioTek Instruments Inc.) at 540 nm. The percentage of biofilm inhibition was determined according to the formula:

$$\% \text{ Biofilm Formation} = \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control} - \text{Abs blank}} * 100$$

Statistical analysis

Data were expressed as means \pm standard deviation (SD) and compared by one-way ANOVA test and post hoc Dunnett test, $P=0.05$, using Prism7 GraphPad® software.

RESULTS

Detection, characterization and identification of streptomycetes displaying anticandidal activity

Sixty streptomycete isolates that have been preserved at the Germplasm Bank of Actinomycetes at CIATEJ were randomly selected to screen for novel species that exhibit anticandidal activity. The diffusible substances produced by streptomycetes against *C. albicans* growth were measured using the agar diffusion method, with the GCAL-25 isolate exhibiting an inhibitory halo of 8.74 ± 0.3 mm (Fig. 1).

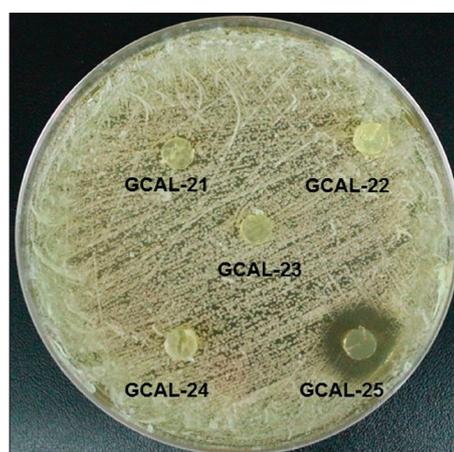


Fig. 1. Primary screening for anti-*Candida* streptomycete species using the agar disk diffusion method.

The colonies of the GCAL-25 isolate showed a yellow-colored substrate mycelium, whereas the aerial mycelium was a white or cream color, with a sporulating aerial hyphae morphology observed as a spiral type [24]. Additionally, some biochemical activity, physiological traits and culture characteristics were observed (Tables 1 and 2). For instance, GCAL-25 glucose fermentation was without gas and hydrogen sulfide production (in TSI), it did not enable synthesis of lysine decarboxylase enzymes and hydrogen sulfide (in LIA); also, it uses citrate as a carbon source because citrate permease is expressed, which is useful for the tricarboxylic acid cycle (in SCA). Interestingly, the strain exhibited tolerance when grown in the presence of different sodium chloride concentrations (6%).

With regards to molecular identification, the contig sequence of the GCAL-25 isolate 16S rDNA was

Table 1. Phenotypic characterization of the selected *Streptomyces* sp. GCAL-25.

CHARACTERISTICS	
Morphological	
Color of aerial mycelium	White to cream
Color of substrate mycelium	Yellow-colored
Gram's reaction	Positive
Biochemical	
Carbon source utilization	Growth*
None	+
D-glucose	++
D-arabinose	+
Sucrose	+
D-xylose	+++
Myo-Inositol	+
α -L-rhamnose	+
Glycerol	+++
D-raffinose	+
Physiological	
Sodium chloride growth	1-6% (w/v)

+++ , excellent; ++ , moderate; + , poor; - , not detected

Table 2. Culture characteristics of *Streptomyces* sp. GCAL-25 on different media.

Medium	Growth	Spore production
ISP9	+	-
ISP2	+++	++
ISP3	+++	-
MH	+++	+
NA	+++	-
PDA	+++	+++
TSI	+++	++
LB	+++	+
TYE	++	-
LIA	+++	+++
SDA	+++	+
SCA	+	-

+++ , excellent; ++ , moderate; + , poor; - , not detected

Abbreviations: MH – Muller Hinton; NA – nutritive agar; PDA – potato dextrose agar; TSI – triple sugar iron; LB – Luria Bertani agar; TYE – trypticase yeast extract; LIA – lysine iron agar; SDA – Sabouraud dextrose agar; SCA – Simmons citrate agar; ISP – international *Streptomyces* project medium 2, 3, and 9

analyzed with BLAST, and it exhibited a very high similarity level (>98%) with other sequences from the *Streptomyces* species. Based on the neighbor-joining method, a phylogenetic tree was constructed, showing that this isolate is in close proximity to a branch that includes some antimicrobial compound-producing species [25-27]. This cluster is primarily represented by a

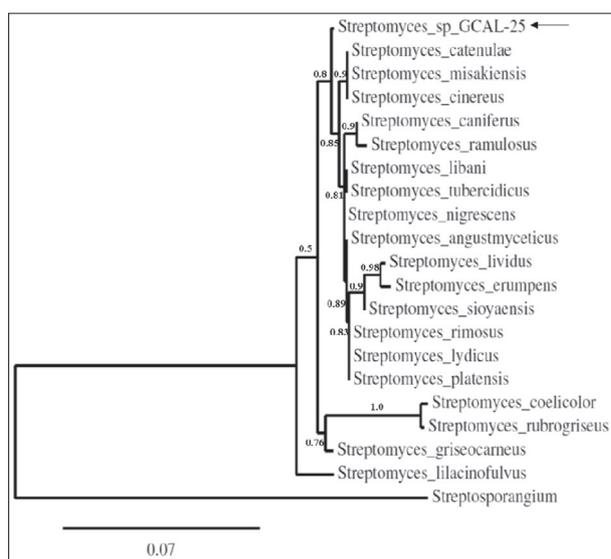


Fig. 2. Phylogenetic relationship based on the 16S rRNA gene between *Streptomyces* sp. GCAL-25 with closely related genus members. The tree was constructed by the neighbor-joining method using a partial 16S rDNA sequence (1243 bp). The numbers at the nodes are percentages that indicate the levels of bootstrap support (n=1000 resamplings). Except for the sequence determined in this study, all 16S rDNA sequences were retrieved from GenBank. The scale bar corresponds to 0.07 nucleotide substitutions per site. The accession numbers for the sequences used are as follows: *Streptomyces nigrescens* DSM 40276 (HG794417.1), *Streptomyces platensis* JCM 4662 (NR_024761.1), *Streptomyces sioyaensis* NRRL B-5408 (NR_043498.1), *Streptomyces angustmyceticus* NBRC 3935 (AB184818.1), *Streptomyces erumpens* 13667D (EU741210.1), *Streptomyces cinereus* BS35 (KR063215.1), *Streptomyces lydicus* S3521 (JN180217.1), *Streptomyces griseocarneus* NBRC 12776 (AB184135.1), *Streptomyces libani* DSM 40555 (NR_117952.1), *Streptomyces tubercidicus* AS 4.1414 (FJ406112.1), *Streptomyces caniferus* AS 4.1588 (FJ406113.1), *Streptomyces coelicolor* A3(2) (AL939110.1), *Streptomyces rubrogriseus* 173513 (EU593560.1), *Streptomyces ramulosus* DSM 40100 (KC954558.1), *Streptomyces lilacinofulvus* NBRC 13677 (AB184458.2). *Streptosporangium* sp. PA147 was used as the outgroup (AF223347.1).

soil species possessing antimicrobial activity, but none have an inhibitory effect on *C. albicans* growth (Fig. 2).

Determination of the minimal inhibitory concentration (MIC) of BEX on *Candida albicans*

The previously selected *Streptomyces* isolate was subjected to a secondary evaluation, where the bioactive extract was evaluated. A paper disk soaked with BEX and deposited on a confluent lawn of *C. albicans* cells showed an inhibitory halo of 11.4 ± 1.0 mm. To rule out inhibition mediated by hydrolytic enzymes, an

Table 3. Minimum inhibitory concentration (MIC) of the BEx against planktonic cells of *C. albicans*.

Component	MIC ($\mu\text{g/mL}$)
AmpB	6.83 \pm 1.95
GCAL-25	1.95 \pm 0.78
GCAL-25 H*	2.34 \pm 0.90

*H – heated

aliquot of the fermentation extract was heated at 95°C for 10 min to inactivate all enzymes. An inhibitory halo similar to the non-heated extract was observed (10.9 \pm 1.2 mm). MIC evaluation for both extracts showed that similar concentrations prevented yeast growth and confirmed the non-proteinaceous nature of the BEx. BEx was better than AmpB for the inhibition of *C. albicans* growth (Table 3). The GCAL-25 isolate produced one or several thermostable non-protein molecules that displayed inhibitory activity against *C. albicans*. Considering the previous results, MIC values were used for subsequent experiments.

Effects of BEx on growth

Kill curve experiments were analyzed to assess the effects of BEx on planktonic cell cultures *in vitro* (Fig. 3). After the addition of BEx at 1/2x or 1xMIC levels to *C. albicans* cultures, an inhibitory effect was observed on growth that persisted throughout the 5-h experiment. Similar inhibitory results were obtained after treatment with AmpB (1 MIC); however, cells exposed to the fungistatic compound itraconazole

(62.5 $\mu\text{g/mL}$) grew in a manner similar to the control cells (Fig. 3A). Results from the kill curves suggested that the BEx obtained from *Streptomyces* sp. GCAL-25 exerted a fungicidal activity on *C. albicans*. The fungicidal properties displayed by the bioactive extract were further confirmed by measuring cell viability after the kill curve experiment was conducted for 5 h. As shown in Fig. 3B, a statistically significant decrease in colony forming units (CFU/mL, $P=0.5$) was observed when cultures were exposed to the BEx at 1/2x and 1xMIC levels, similarly to AmpB. In a separate experiment using the same treatments without shaking, drastic morphological alterations were observed on planktonic cells (Fig. 4). In comparison to the control cells (Fig. 4A), ITRA impaired yeast cell germination, although it did not affect its viability (Fig. 4B). AmpB killed the cells (Fig. 4C). Similar to AmpB, when the cells were exposed to the BEx, severe effects were observed on cell viability and morphology (Fig. 4D and 4E, 1/2x and 1xMIC, respectively). It is clear that the cells' membrane integrity was seriously impaired, as evidenced by the collapsed or shrunken cell morphologies that disrupt the morphological transitions from yeast to hyphal germination [28,29].

Inhibition of biofilm formation in the presence of the BEx

In addition to the loss of cell viability and the presence of severe cell alterations, the BEx inhibited cellular

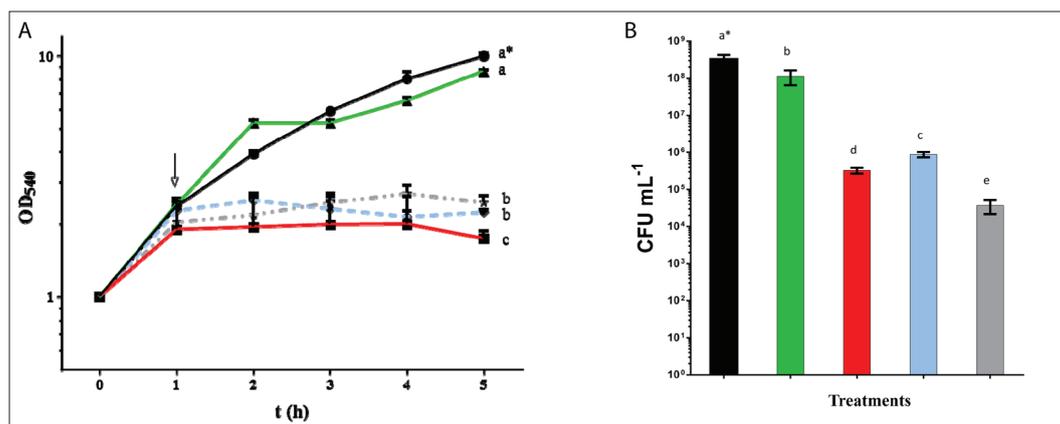


Fig. 3. Effect of *Streptomyces* sp. GCAL-25 BEx on *C. albicans* growth and viability. **A** – Time-kill curve of growth after the addition of the BEx (1 h). **B** – Colony counts of *C. albicans* cells after 5 h of exposure to the BEx. Treatments were: ■ – control; ■ – ITRA (62.5 $\mu\text{g/mL}$); ■ – AmpB (1xMIC); ■ – GCAL-25 BEx (1/2xMIC); ■ – GCAL-25 BEx (1xMIC). The assays were performed in duplicate. *Means with the same letter are not significantly different from each other ($P>0.05$ ANOVA followed by Dunnett test). Error lines represent \pm standard deviation of the mean.

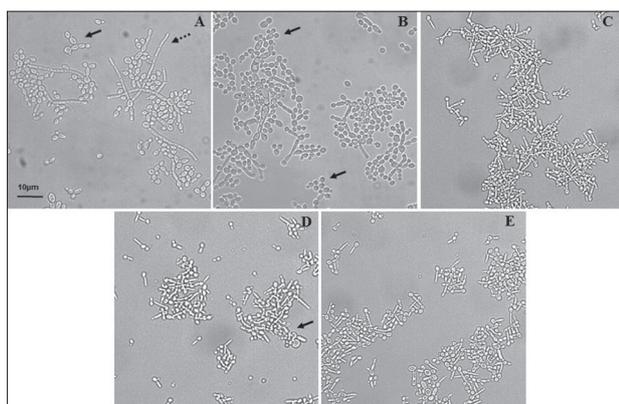


Fig. 4. Inhibition of yeast-to-hyphae transition of *C. albicans* by *Streptomyces* sp. GCAL-25 BEx. Yeast cells were incubated at the indicated treatment for 3 h without shaking. The final concentrations were: A – control; B – ITRA (125 µg); C – 1xMIC AmpB; D – ½xMIC BEx; E – 1xMIC BEx. Morphologic effects were monitored under inverted microscope and images were recorded at 20X magnification. Scale bar=10 µm. Filled arrow shows pseudohyphal formation; dotted arrow indicated hyphal germination. These experiments were repeated four times with similar results.

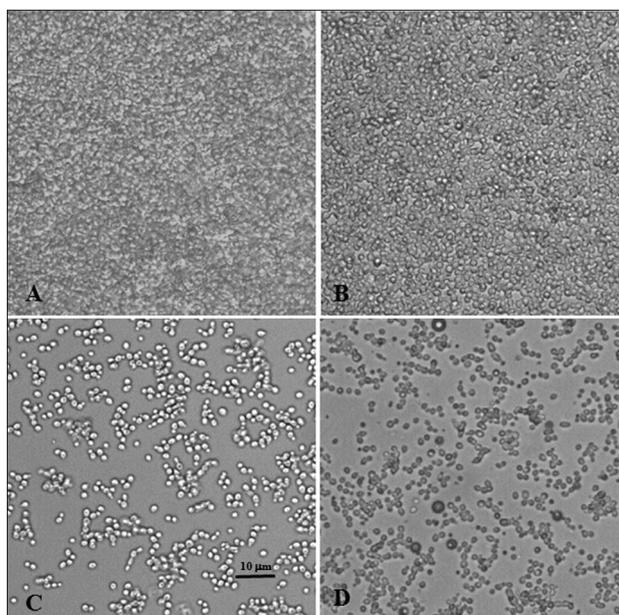


Fig. 5. Inhibition of *C. albicans* biofilm formation by *Streptomyces* sp. GCAL-25 BEx. Morphologic effects were evaluated after 48 h of treatments: A – control; B – ITRA (125 µg); C – 1xMIC AmpB; D – 1xMIC BEx. Effects on biofilm development were observed under inverted microscope and images were recorded at 20X magnification. Scale bar=10 µm. These experiments were performed three times with similar results.

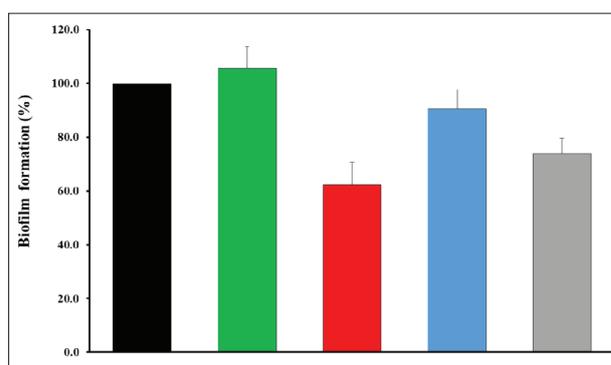


Fig. 6. Effect of *Streptomyces* sp. GCAL-25 BEx on *C. albicans* biofilm formation. Treatments were: ■ – control; ■ – ITRA (62.5 µg·ml⁻¹); ■ – AmpB (1 MIC); ■ – GCAL-25 extract (1/2xMIC); ■ – GCAL-25 extract (1xMIC). The assays were performed in triplicate. *Means with the same letter are not significantly different from each other ($P>0.05$). Error lines represent \pm standard deviation of the mean.

transitions from yeast to hyphal germination and enhanced pseudohyphae formation in planktonic cell cultures. Thus, the ability of the BEx to decrease or inhibit biofilm formation by *C. albicans* was tested *in vitro*. Fig. 5 provides evidence for the inhibition of biofilm formation that correlated with the addition of the BEx (1xMIC, Fig. 5D), which is in contrast to the presence of mature biofilm in *C. albicans* communities completely coated within an extracellular matrix that were observed in control and after the ITRA treatment (Fig. 5A and B). Similar inhibitory effects on biofilm formation were observed when AmpB was used as the control (Fig. 5C). These results suggest a strong effect of the *Streptomyces* sp. GCAL-25 extract on *C. albicans* biofilm formation, since it lacked hyphal organization and consisted mostly of yeast cells. The percentage of biofilm inhibition was calculated for the BEx using the crystal violet staining method (Fig. 6). After a 48-h incubation, ½x and 1xMIC of the BEx quantitatively reduced the transition from yeast cells to mature biofilm formation by 10-30%, in comparison with ITRA and control assays. Similarly, AmpB reduced biofilm formation to 60%. A statistically significant difference was detected among the BEx of AmpB and ITRA ($P=0.05$ in all pairwise comparisons). No differences were detected between the mean values of the biofilm-forming ability of *C. albicans* under ITRA and the control treatments.

DISCUSSION

The effects of the BEx on *C. albicans* cells were similar to the morphological damage caused by AmpB and other fungicidal compounds. In this work, the bioactive extract displayed a fungicidal effect on planktonic cells. It affected the yeast-to-hyphae transitions, increased pseudohyphal development, and induced an abnormal cell morphology, including impaired cell membrane integrity, such as collapsed or shrunken cell morphologies. Considering that the yeast-to-hyphae differentiation process is a virulence factor during a *C. albicans* infection, the inhibition of hyphal growth induced by the BEx is an important finding. *C. albicans* hyphae development and growth represent an important step during the onset of biofilm formation, as they mediate pathogen dissemination, invasion and penetration into host tissues, as well as its evasion from the immune cell elimination [30,31]. Furthermore, the increased numbers of pseudohyphae induced by the GCAL-25 extract is a significant and important finding, which could be correlated with the pathogen's attenuated virulence. Mutant *C. albicans* strains characterized by defective hyphae formation produce pseudohyphae with attenuated virulence [32,33].

Different compounds have been tested for their ability to impair biofilm formation *in vitro*, such as chitosan, as an alternative therapeutic strategy to target fungal biofilms in medical devices [34], heterocyclic compounds synthesized from thiazolidinedione and succinimide molecules for therapeutic purposes [35], the glycolipid biosurfactant (Sophorolipid) produced by some species of *Starmerella* yeast displaying inhibitory activity against *C. albicans* biofilm formation and hyphal growth [36], and the anticandidal activities of urauchimycins A and B produced by *Streptomyces* sp. TD025, which show antifungal activity similar to that of nystatin. A *Streptomyces* species belonging to the *S. violaceusniger* clade, produced metabolites that damage and kill *Candida* cells through shrinkage and loss of cytosolic material [37]. Another metabolite extract from the *S. chrestomycticus* ADP4 strain produced anticandidal compounds that are effective against a number of *Candida* species. The aforementioned extract showed a marked inhibitory effect on the attachment and conversion into the hyphal state and also caused *Candida* cell surface disruption [38].

During the maturation phase, the biofilm displayed a hazy appearance due to increased extracellular material accumulation during the incubation period. Accordingly, it was difficult to focus on the basal yeast cell communities covered by the matrix, as was previously observed. The BEx produced by *Streptomyces* sp. GCAL-25 affected the transition process from the early to the mature phases. In the well treatments with $\frac{1}{2}x$ and $1xMIC$ of BEx, only blastospores were observed, and no extracellular material could be detected, which is characteristic of an early biofilm stage [39]. Biofilm formation occurs in a sequential process, which includes adherence of yeast cells to the substrate, proliferation of the yeast cells, formation of hyphal cells, accumulation of extracellular matrix material and dispersion of yeast cells from the mature biofilm [40]. The present study presents evidence that this sequential process of biofilm formation was affected by the BEx, which interrupted the proliferation of yeast cells and the formation of hyphal cells.

The metabolites produced by the *Streptomyces* sp. GCAL-25 strain damaged planktonic *C. albicans* cells and killed and/or damaged precursor cells to hyphal growth and biofilm formation. These findings confirm the importance of *Streptomyces* as sources of potent anticandidal metabolites. Additional studies are required to purify the specific compound(s) that display antibiofilm activity, to elucidate the structure of the active metabolites and to study the particular mechanism for inhibition of biofilm formation.

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Conflict of interest disclosure: The authors declare that they have no competing of interest.

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