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Efficacy of Crude Extract from *Streptomyces cellulosae* Against Biofilm-Related Genes of *Candida albicans*

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Abstract

Background: *Candida albicans* is notably pathogenic due to its ability to form biofilms that are resistant to conventional antifungal treatments.

Objectives: This study aims to explore the effectiveness of *Streptomyces cellulosae* extract in disrupting biofilm formation by targeting specific genes within *C. albicans*.

Methods: The study began by isolating *S. cellulosae* from soil and *C. albicans* from clinical specimens. *S. cellulosae* was then cultured and fermented to produce bioactive compounds. The ability of these extracts to inhibit *C. albicans* biofilm formation was tested using a crystal violet assay. Additionally, the effects of the *S. cellulosae* extracts on the expression of biofilm-related genes in *C. albicans* were evaluated using quantitative real-time PCR (qRT-PCR). The growth rates of *C. albicans* were also measured to determine the impact of the extracts.

Results: The crude extract of *S. cellulosae* significantly ($P < 0.05$) inhibited the formation of *C. albicans* biofilms at concentrations exceeding 0.5 μg/mL, with the inhibition becoming more pronounced at concentrations above 2.0 μg/mL. The qRT-PCR results showed significant changes in the expression of biofilm-related genes *ALS1*, *ALS3*, and *EFG1* at different extract concentrations ($P < 0.05$). The extract also significantly affected the expression of the HWPa and BRG1 genes.

Conclusions: The crude extract of Streptomyces cellulosae shows potential as a novel antibiofilm agent against *C. albicans*. This finding opens new avenues for research and potential therapeutic applications in combating biofilm-associated infections.

Keywords: Biofilm Formation, Candidiasis, Pathogenicity, *Streptomyces cellulosae*

1. Background

Candida albicans is an opportunistic fungal pathogen that colonizes various human body parts such as the oral cavity, throat, gastrointestinal tract, vagina, and skin in healthy individuals. It is a part of the body's normal flora. Under certain conditions, these opportunistic microorganisms can lead to infections (1, 2). Several virulence factors, including adhesin expression, hydrolytic enzyme production, the ability to switch from yeast to hyphae, and metabolic adaptability, enable *C. albicans* to proliferate and invade host tissues (3). The most recurrent and severe infections are often associated with the formation of biofilms on biological or artificial surfaces.

Biofilm formation by *C. albicans* significantly enhances its resistance to conventional antifungal treatments and the host's immune defenses, making infections difficult to eradicate (4). Key genes regulating biofilm formation include *BCR1*, *ALS1*, *ALS3*, *HWP1*, and *ECE1*, which are considered promising therapeutic targets. Previous research has shown that specific compounds can inhibit biofilm formation by suppressing these genes (5). *Streptomyces cellulosae*, a species of Actinobacteria known for producing bioactive compounds with antimicrobial properties, has shown potential against various pathogens, including fungi and harmful Gram-positive bacteria (1, 2). Notably, Mahmood et al. found that *S. cellulosae* produces compounds that inhibit biofilm formation by *Pseudomonas aeruginosa* (6).

2. Objectives

Given the rising number of infections and concerns about resistance, it is crucial to develop new strategies to combat biofilm-forming organisms like *C. albicans*. Therefore, this study aimed to evaluate the effectiveness of extracts from *S. cellulosae* in targeting genes associated with biofilm formation in *C. albicans*.

3. Methods

3.1. Isolation and Identification of Microorganisms

Pure cultures of *S. cellulosae* were sourced from the Charmo Research Center at Charmo University, Sulaimaniyah, Iraq. The bacterium was cultivated on Gause's medium after being extracted from soil and identified using colony morphology and 16S rDNA genomic sequencing. Sequence analysis confirmed that the bacteria matched those in the NBRC 13027 strain (6). Clinical samples for isolating *C. albicans* were collected from Hiwa Hematology/Oncology Hospital in Sulaymaniyah, Iraq. Samples were taken using swabs (7) from symptomatic leukemic patients' oral lesions, then inoculated on Sabouraud dextrose agar (SDA) and incubated at 37°C for 24 hours (8). Isolates were further cultivated on CHROM agar Candida and incubated at 37°C for 48 - 72 hours (9). Colony morphology, including color, size, and texture, was examined to identify the species (10).

3.2. Antibiofilm Formation by Streptomyces cellulosae

Initially, *S. cellulosae* was cultured using the batch culture method (11), and 100 µL of bacterial spore stock (1×10^8 spores/mL) was added to 100 mL of tryptic soy broth (TSB) (12). The cultures were maintained in a shaking incubator (LSI-5004M, Indonesia) at 30°C for 48 hours at 150 rpm. Subsequently, TSB was inoculated with a 5% v/v inoculum and cultured for 120 hours at 37°C (13). The bioactive compounds from the broth culture were then extracted. The culture filtrate was mixed with ethyl acetate (1:1, v/v) and vigorously shaken for 20 minutes. Using a rotary evaporator (Heidolph, GmbH, Germany), the ethyl acetate layer was separated from the liquid phase and concentrated by evaporation at 40°C until dry. The resultant residue was dissolved in pure methanol to produce a reddish-brown crude extract and stored at -20° C until needed.

3.3. Biofilm Formation by Candida albicans

Biofilm development was evaluated using 96-well microplates (14). Briefly, *C. albicans* isolates (McFarland 1 $\times 10^8$ CFU/mL) were cultured overnight in SDA broth at 37°C (12). After incubation for 24 hours at 37°C in 2.0 mL of yeast extract peptone dextrose (YPD) broth, tubes were diluted (1:20) with YPD supplemented with 1% glucose and incubated for another 24 hours at 37°C (15). The optical density (OD) was then measured at 630 nm. Each test was conducted in triplicate. Subsequently, the OD of each strain (ODs) was compared to that of the negative control (ODnc). The reference strain of *C. albicans* (ATCC 90028) served as the positive control for biofilm formation, whereas the negative control comprised solely of broth media without *C. albicans*. Strains were categorized based on their biofilm-forming ability as none (ODs = ODnc), weak (ODnc < ODs < 2ODnc), moderate (2ODnc < ODs < 4ODnc), and strong (4ODnc < ODs) (15).

3.4. Antibiofilm Activity of Streptomyces cellulosaeagainst Candida albicans

The antibiofilm efficacy of Streptomyces isolates against *C. albicans* was determined using a crystal violet assay (16, 17). The previously formed *C. albicans* biofilms in 96-well microplates were treated with 0.5, 1, 2, 3, and 4 µg/mL of the *S. cellulosae* antibiofilm extract (100 µL) for 48 hours at 37°C. Subsequently, the medium was discarded, and the wells were washed with sterile phosphate-buffered saline (PBS), inverted for blotting, and air-dried. Each well then received 200 µL of methanol for fixation and was incubated for 15 minutes at 25°C. After discarding the methanol, the plates were air-dried for 45 minutes. Next, 200 µL of a 0.1% w/v crystal violet solution was added to each well, and the plate was incubated for 20 minutes at 25°C. Following two washes with distilled water, 200 µL of an acetone: Ethanol (20:80 v/v) mixture was added to each well and incubated for 15 minutes at 25°C to stain the biofilm (18). Biofilm production was quantified using a plate reader by transferring 100 µL from each well to a new, sterile microplate and measuring the OD at 630 nm. Each test was repeated three times (15).

3.5. Quantitative Real-time PCR Analysis of Candida albicans Biofilm-Specific Genes

A 96-well polystyrene microtiter plate (Iwaki), flat-bottomed and pre-sterilized, was used to culture a standard cell culture of *C. albicans* (1.0 mL). After incubating with agitation at 37°C for 1.5 hours, the liquid was discarded and the wells were rinsed twice with PBS. Each well was then replenished with 1.0 mL of fresh YPD

medium containing 3.0 mg/mL of Streptomyces crude extract and incubated for 24 hours at 37°C. Post-incubation, the supernatant was removed and the wells were washed twice with PBS. Total RNA from *C. albicans* biofilms was isolated using the SV Total RNA Isolation System (Biolab), and 2.0 mg of RNA was reverse-transcribed using Superscript II (Invitrogen). The resulting cDNAs were PCR-amplified, with sequencing confirming product specificity. quantitative real-time PCR (qRT-PCR) primers were designed specifically for the *C. albicans* biofilm-associated genes *ALS1*, *ALS3*, *BRG1*, *EFG1*, *HSP9*, *HWP1*, and *ACT1* (Table 1).

The RT-PCR mixture (20 µL) included 10 µL of Luna Probe One-Step Reaction mix (No ROX) (2 ×), 1.0 µL of Luna WarmStart RT Enzyme Mix (20 m ×), 0.8 µL of primers, 0.4 µL of Probe (10 µL), template RNA, and 20 µL of nuclease-free water. The Roche LightCycler® was utilized for qRT-PCR, with each run comprising a 10-minute pre-incubation at 95°C, followed by 40 cycles of 10 seconds of denaturation at 95°C and 30 seconds of annealing/extension at 60°C. ACT1 served as the reference housekeeping gene. Each concentration of crude extract was tested in at least three experimental repeats.

3.6. Growth Rate Measurements

Candida albicans cells were cultivated overnight in SDA at 37°C with shaking at 200 rpm. After obtaining two cell counts, 20 mL of fresh SDA was inoculated with the cells at a density of 1×10^6 cells/mL. Spectrophotometric readings were taken hourly, with the cultures shaken at 200 rpm and maintained at 37°C. Growth rates were monitored over three days for various initial cultures.

3.7. Statistical Analysis

Statistical analyses of the qRT-PCR results were performed using Microsoft Excel and Graph Pad PRISM 8. The average Ct values for the genes *ALS1*, *ALS3*, *BRG1*, *EFG1*, *HSP9*, *HWP1*, and *ACT1* were presented as mean ± standard deviation (SD) before and after treatment with the crude extract. A P-value of < 0.05 was considered statistically significant.

4. Results

4.1. Antibiofilm Activity of Streptomyces cellulosae Crude Extract

The study demonstrated that the crude extract from *S. cellulosae* inhibited biofilm formation by *C. albicans* at various concentrations (0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL).

4.2. Gene Regulation of Biofilm Formation in Candida albicans

The efficacy of *S. cellulosae* crude extract against *C. albicans* showed that concentrations above 0.5 µg/mL notably impeded biofilm formation by the *Candida* isolates. Additionally, dosages greater than 2.0 µg/mL significantly inhibited *Candida* biofilm production ($P < 0.05$).

4.3. Antibiofilm Activity of Streptomyces cellulosae Crude Extract on the Expression of Biofilm-Related Genes in Candida albicans

Quantitative real-time PCR analysis revealed the impact of the crude extract on the expression levels of biofilm-associated genes in *C. albicans* at concentrations ranging from 0.5 to 4.0 µg/mL. Gene expression was normalized against *ACT1*, used as the reference gene. The expression of *ALS1* and *ALS3* genes decreased significantly at concentrations of 0.5 and 4.0 µg/mL, and at 2.0 and 4.0 µg/mL respectively, compared to the control group (CG) which received no treatment ($P < 0.02$). Additionally, the expression levels of *ALS1* and *ALS3* increased significantly at a concentration of 3.0 µg/mL compared to CG ($P < 0.03$) (Figure 1).

Furthermore, the expression of *EFG1* was markedly higher after treatment with the crude extract at 0.5 and 3.0 µg/mL compared to CG ($P < 0.05$) and decreased progressively at concentrations of 1.0, 2.0, and 4.0 µg/mL. Conversely, the *HSP9* gene maintained normal expression levels following treatment with the crude extract (Figure 2). The expression of both *HWPa* and *BRG1* genes was notably affected at concentrations of 0.5 and 3.0 µg/mL. Notably, a significant reduction in the expression of *HWPa* and *BRG1* genes was observed at concentrations of 2.0 and 4.0 µg/mL when the *C. albicans* isolate was treated with the crude extract (Figure 3).

5. Discussion

Candida is the fourth most common cause of hospital-acquired infections (26) and a leading contributor to biofilm formation associated with medical interventions, drug use, and implants (27). Consequently, this study focused on the local impact of these biofilms. Our findings showed that concentrations of the crude extract above 0.5 µg/mL significantly inhibited *Candida* biofilm formation, with even more pronounced effects observed at concentrations exceeding 2.0 µg/mL. Furthermore, the study revealed that the expression levels of the *ALS1* and *ALS3* genes

Table 1. Primers Used in RT-PCR to Detect the Biofilm Formation-Related Genes in *Candida albicans*

Genes	Sequence	Reference
ALS1	F: GACTAGTGAACCAACAAATACCAGA	(19)
	R: CCAGAAGAAACAGCAGGTGA	
ALS3	F: CCAAGTGTTCCAACAACTGAA	(20)
	R: GAACCGGTTGTTGCTATGGT	
EFG1	F: TATGCCCCAGCAAACAACTG	(21)
	R: TTGTTGTCCTGCTGTCTGTC	
hsp90	F: GCTTTAAGTGCTGGTGCTGACGTT	(22)
	R: TGGTACCACGACCCAATCTTTCGT	
HWP1	F: GACCGTCTACCTGTGGGACAGT	(23)
	R: GCTCAACTTATTGCTATCGCTTATTACA	
BGR1	F: ACGATCAACCATTAGTGGAGG	(24)
	R: GAAGAAGTAGGTGTAGATGATCCAC	
ACT1	F: TTTCATCTTCTGTATCAGAGGAACTTATTT	(25)
	R: ATGGGATGAATCATCAAACAAGAG	

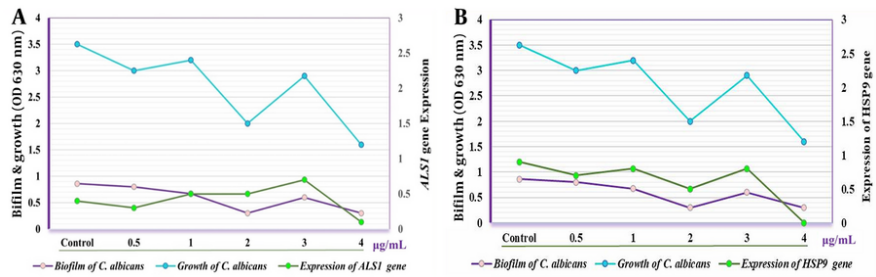

Figure 1. Quantitative real-time PCR (qRT-PCR) analysis of expression of biofilm-specific genes (ALS1 and HSP9)

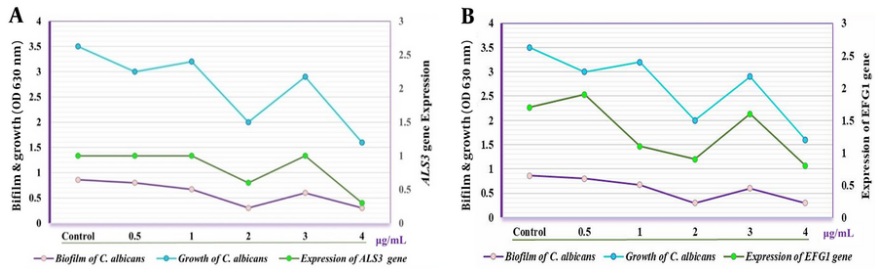

Figure 2. Quantitative real-time PCR (qRT-PCR) analysis of expression of biofilm-specific genes (ALS3 and EFG1)

varied under different concentrations of the crude *Streptomyces* extract, generally decreasing at most concentrations but increasing at 3.0 μg/mL. Research by Deng et al. highlighted the influence of ALS3 gene expression on the biofilm formation capabilities of *C. albicans*, suggesting that manipulating this gene's expression could aid in developing both therapeutic and preventive strategies (28), a finding that

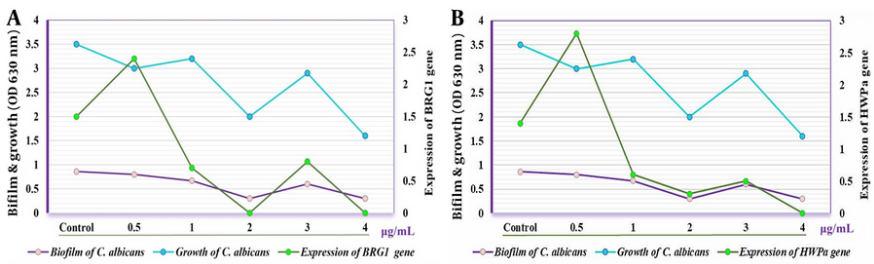

Figure 3. Quantitative real-time PCR (qRT-PCR) analysis of expression of biofilm-specific gene (BRG1 and HWPa)

corroborates our results. Additionally, Martorano-Fernandes et al. demonstrated that gene expression levels, including those of the *ALS1*, *ALS3*, and *HWPa* genes, could vary based on the environmental conditions and substances present, noting that increased gene expression could exacerbate pathogenic conditions (29).

Zarimeidani et al. explored the minimum inhibitory concentrations (MIC) of caprylic acid/nanoencapsulated caprylic acid against *C. albicans*, ranging from 625 - 400 to 1.3-50 µL/mL. Their study utilized qRT-PCR to measure the expression level of the *EFG1* gene, finding the highest activity of caprylic acid at 450 and 500 µg/mL, with the most potent activity for nanoencapsulated versions at 6.2 and 3.1 µg/mL (30). Another innovative approach involves combining inhibitors of Histone Deacetylase (HDAC) and Heat Shock Protein 90 (HSP90) to enhance the efficacy of fluconazole against resistant strains of *C. albicans*. This pharmacological strategy was shown to reduce gene expression related to drug resistance and mitigate major pathogenic factors, presenting a contrast to our findings where gene expression varied based on treatment concentration and gene targeted (31). These differences could stem from variations in experimental methods and the specific genes analyzed.

Antimicrobial peptides (AMPs) have emerged as a promising approach to treat *Candida* infections. Samot and Rouabhia investigated the antimicrobial effects of Dermaseptin S4 (DS4) against *C. albicans*, focusing on its impact on the growth, morphological alterations, biofilm formation, and gene expression of the fungus. DS4 halted the growth of *C. albicans* at concentrations of 32 and < 16 µg/mL. It appears to obstruct the yeast-to-hyphae transition and limit biofilm development by reducing biofilm mass weight. Significantly, Dermaseptin also reduced the expression of the HWP1 and EAP1 genes (32). The polymorphic nature of *C. albicans*, which can grow in both yeast-like and filamentous forms, makes the transition between these states crucial for its pathogenicity. *EFG1* and *BRG1* are important transcriptional regulators that influence the growth and morphological transformations of this fungus, impacting its pathogenicity and biofilm formation (33-35). Biofilms are microbial communities that form structures by attaching to surfaces, and can include combinations of bacterial or fungal species (36). The inhibitory effect of *S. cellulosae* on biofilm synthesis was also observed in Iraq by Mahmood et al., supporting the current investigation that demonstrates *S. cellulosae*'s ability to produce metabolites with antibacterial and antibiofilm properties (6)

5.1. Conclusions

It is concluded that the crude extract of *S. cellulose* has potential as a novel antibiofilm agent against *C. albicans* by suppressing the expression of some involved genes, offering a promising avenue for future research and potential therapeutic applications.

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Footnotes

Authors' Contribution: CHF, conceptualization, methodology, writing the original manuscript; KIM, methodology, data analysis, study registration; KAM, supervision, validation, resources, writing the original manuscript; SKR, supervision, edition and manuscript revision. All authors have agreed to submit the manuscript to this journal.

Conflict of Interests Statement: There are no conflicts of interests.

Data Availability: No new data were created or analyzed in this study. Data sharing does not apply to this article.

Ethical Approval: The Scientific and Ethical Committees of the College of Medicine, University of Sulaimani, Iraq, approved this study protocol (No. 5, on March 18, 2022). All experiments were conducted in accordance with the Declaration of Helsinki.

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Informed Consent: Written informed consent was obtained from patients to collect their samples after securing permission from hospital authorities.

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