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Antibiofilm activity of *Streptomyces toxytricini* Fz94 against *Candida albicans* ATCC 10231

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Abstract

Candida albicans is a significant cause of morbidity and mortality in immunocompromised patients worldwide. Biofilm formation by *Candida* species is a significant virulence factor for disease pathogenesis. Keeping in view the importance of *Streptomyces*' metabolites, the present study was initiated during the bioprospecting programme of Egyptian *Streptomyces* carried by the authors since 2013. Native *Streptomyces* isolates were recovered from soil samples collected from different governorates. Antifungal activity of forty isolates of *Streptomyces* were performed against planktonic (free cells) of *C. albicans* ATCC 10231 and resistant clinical *Candida* isolates. *Streptomyces* isolates showed high inhibition activity against free cells of *Candida* were further assayed against biofilm of *C. albicans* reference strain. The most active *Streptomyces* sp. (no.6) was identified phenotypically, biochemically and by using 16S rRNA. The 16S rRNA sequences obtained were compared with those deposited in the GenBank Database and registered with accession number KM052378 as *S. toxytricini* Fz94. Screening of *S. toxytricini* Fz94 extract capability in prevention and destruction of *C. albicans* reference strain biolfilm was assessed by resazurin dye adopted technique. In the pre-exposure scheme, the lowest concentration of 5 gL^{-1} showed biofilm viability inhibition of 92% after 120 min, while Ketoconazole® gave 90 % inhibition at concentration of 2 gL^{-1}. In post exposure, the concentration of *S. toxytricini* Fz94 extract 7gL^{-1} caused 82 % inhibition of biofilms viability after 120 min, while Ketoconazole did not show any destruction capability. The cytotoxicity of *S. toxytricini* Fz94 crude extract results showed that it was nontoxic at 10 gL^{-1}. *S. toxytricini* Fz94 is maintained in the Fungarium of Arab Society for Fungal Conservation (ASFC) with accession number FSCU-2017-1110.

Key words– Bioprospecting – Egypt – Ketoconazole – *Streptomyces toxytricini* Fz94 –resazurin.

Introduction

Microorganisms growing in a biofilm are associated with chronic and recurrent human infections (Sutherland 2001). The most important feature of biofilm growth is the high resistance to antimicrobial agents that can be up to 1000-fold greater than that of planktonic cells (Amorena *et al*. 1999; Saginur *et al*. 2006; Hassan *et al*. 2011). The preferred life-style for most of microorganisms is biofilm rather than planktonic cells (Douglas, 2003). The consequences of mature biofilm development result in structural and phenotypic changes that has numerous

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benefits to microbial cells that may include coherent attachment to surfaces, colonization of host tissues, the expression or enhancement of virulence traits, protection from stresses, sequestering of nutrients, enhanced cell-to-cell communication, dispersal, and survival during harsh conditions (Harding *et al*., 2009). However, *Candida albicans* is an important nosocomial pathogen in immunocompromised patients that has a high morbidity and mortality rate (Wisplinghoff *et al*., 2004, Mensa *et al*., 2008,). This is due to many of these *C. albicans* are implant-associated infections, owing in part to its greater capacity to form biofilms that are found on the surfaces of devices including central venous catheters, catheters, prosthetic heart valves, endotracheal tubes, pacemakers and joint replacements that cause bloodstream infection (Douglas, 2002, Chandra *et al*., 2005, Sardi *et al*., 2013).

To study *Candida* biofilms, several microtiter plate-based assays have been used recently to determine biofilm mass (Sherry *et al*., 2014), microbial physiological activity (Van den Driessche, *et al*., 2014, Noumi *et al*., 2010; De Logu *et al*., 2005, LaFleur *et al*., 2006), and extracellular matrix (Peeters *et al*., 2008). Their final results are based either on absorbance or fluorescence intensity at a certain wavelength, which means that fast and quantitative analyses are obtained with a microplate reader (Azevedo *et al*., 2009). These assays also show a broad applicability and reproducibility for many microorganisms (Peeters *et al*., 2008).

Recently, several microtiter plate-based assays have been used to study the *Candida* biofilms e.g. determination of mass (Sherry *et al*., 2014), microbial physiological activity (De Logu *et al*., 2005; LaFleur *et al*., 2006; Noumi *et al*., 2010; Van den Driessche *et al*., 2014), and extracellular matrix (Peeters *et al*., 2008). Their final results are based either on absorbance or fluorescence intensity at a certain wavelength, which means that fast and quantitative analyses are obtained with a simple microplate reader (Azevedo *et al*., 2009). These assays also show a broad applicability and a high repeatability for many microorganisms (Peeters *et al*., 2008)**.**

The aim of this study was to evaluate the inhibitory activity of *S. toxytricini* Fz94 against planktonic cells of both reference and clinical isolates of *C. albicans in vitro* Moreover, its activity against biofilm of reference *C. albicans* in both prevention and destruction modes in comparison with standard antifungals. Finally, evaluation the cytotoxicity of *S. toxytricini* Fz94 against Human Lung (HL) epithelial cells was assessed.

Materials and methods

Microorganisms

Isolation of *Streptomyces*

Taxa of *Streptomyces* were isolated from subsurface (5-7 cm) soil samples collected from different governorates in Egypt from January 2013 till December 2015 using soil dilution-plate method (Kuster and Williams 1964) on starch casein agar media (Shirling and Gottlieb 1966).

Isolation of clinical *Candida*

Clinical samples (urine, sputum, and pus, vaginal and pleural fluids) were collected from Kasr Elini Hospital, Egypt for isolation of clinical *Candida* using standard Mycology methods (Odds and Bernarets 1994). *Candida* spp. were inoculated on CHROM agar and incubated at 37°C for 24 hrs. *C. albicans* was identified by type and colour of the colonies on CHROM agar media as light green (Devi and Maheshwari 2014).

Reference *C. albicans*

The reference *C. albicans* was obtained from American Type Culture Collection (ATCC) under reference number of 10231.

Identification of the bioactive *Streptomyces* isolate

Phenotypic characterization

The *Streptomyces* strain was inoculated on Starch Casein Agar (SCA) medium, incubated at 30 °C for 5 days and the macro-morphology of strains including colors of mature sporulating aerial and substrate mycelia were monitored. However, the micro-morphology of the strain including the form of the sporophores was examined under light microscope. Furthermore, the morphology of the spores and the spore chain was investigated by Scanning Electron Microscope (SEM) (JEOL, JSM-5910, Japan). For the SEM, A plug of sporulating *Sterptomyces* sp. 6 incubated for 7 days at 28 C on SCA medium was removed and fixed in glutaraldehyde vapor (2% v/v) at room temperature (RT) for 3 h. Then dehydrated through a series of ethanol solution (50, 60, 70, 80 and 95%, 15 min each; twice with 100% ethanol, 30 min/ time). Ethanol was substituted with acetone and subjected to critical-point dryer (CPD7510, Critical Point Drying/ Apparatus, Polaron, Rang) and then sputter coating with gold using a Gold Sputter (SPI-Module TM Sputter Coater, SPI Supplies, Division of Structure Probe Inc., USA) and observed by SEM (Hosny *et al*. 2014).

Physiological and Biochemical Characterization

Physiological characterization including melanin production was detected using International Streptomyces Project (ISP) included ISP-1, ISP-6 and ISP-7 media according to the method reported by Shirling and Gottlieb (1966), and carbon sources utilization was conducted as described in MacFaddin (2000) using each one of the nine sugars including D-glucose, D-sucrose, D-sorbitol, D-mannitol, L-arabinose, meso-inositol, L-rhamnose, D-melibiose and D-amygdalin at a concentration of 0.5%. The biochemical tests including starch and gelatin hydrolysis, Voges-Proskauer, citrate utilization, indole, and H_2S production were carried out according to MacFaddin (2000). Also, casein hydrolysis was performed as reported in Shirling and Gottlieb (1966) and urea hydrolysis was conducted as described by Chrisensen (1946). However, the results of biochemical and physiological properties of these isolates were confirmed by API® biochemical identification system (bioMerieux, France).

Molecular identification

DNA extraction

The extraction of genomic DNA was performed according to the protocol recommended for the DNA purification of Gene JET™ genomic DNA purification kit (Thermo Fisher Scientific) as follows: *Streptomyces* cells were harvested up to $2x10^9$ in a 1.5 or 2 mL micro centrifuge tube, then the culture was centrifuged for 10 min at 5000 ×g and the supernatant was discarded. Eventually, the genomic DNA of lysed bacterial cells was precipitated with 20µL of Proteinase K Solution and purified using ethanol 70%.

PCR amplification and Sequencing of 16S rRNA gene

PCR amplification was performed using Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific) in 50 µL of reaction system containing (25µL) Maxima Hot Start PCR master mix (2X), (1 µL) 20 µM 16S rRNA forward primer (5'-AGA GTT TGA TCC TGG CTC AG -3'), (1 µL) 20 µM 16S rRNA reverse primer (5' -GGT TAC CTT GTT ACG ACT T-3') (Lane *et al*.,1985; James, 2010), (5µL) DNA template, (18µL) water, nuclease- free. The initial denaturing was at 95^{O}C for 10 min; and then 35 cycle 95^{O}C for 30 s; 65 ^{O}C for 60 s; 72 ^{O}C for 90 s; and final extension at 72^{O}C for 10 min. Purification of PCR product was using Gene JET™ PCR Purification kit. Finally, the sequencing of the PCR product was done by ABI 3730xl DNA sequencer (GATC, Germany). For phylogenetic analysis, the determined sequences were compared with the sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank data base (www.ncbi.nlm.nih.gov) by BLAST search.

Phylogenetic analysis

The alignment was manually adjusted and then the phylogenetic tree was constructed by using neighbour-joining (Saito and Nei 1987) in the MEGA program version 6 (Tamura *et al*. 2013). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses based on 1000 resamplings (Felsenstien 1985).

Primary and secondary screening of *Streptomyces* isolates against reference strain of *C. albicans*

The primary and secondary screening were carried out against *C. albicans* (ATCC 10231) using cut plug method (Pridham *et al*. 1956) and agar well diffusion method (Kavanagh 1972) respectively after incubation at 37 °C for 24 h.

Screening for antifungal activity of *Streptomyces* isolates against clinical *C. albicans* isolates

A suspension of each of the clinical isolates plated onto CHROMagar *Candida* equivalent to 0.5 McFarland Standard was inoculated on Mueller–Hinton agar to which 2% glucose and 0.5 g mL^{-1} methylene blue dye was added (MH-GMB) using a sterile cotton-swab, removing excess fluid by pressing against the tube according to Tan and Peterson (2005) technique.

The culture filtrates of active strains (1 mg of crude extract/disk) were tested against clinical *Candida* isolates following National Committee for Clinical Laboratory Standards (NCCLS 2004). Different controls were involved. Positive antifungal control discs e.g. (ketoconazole 10 µg, fluconazole 25 µg, amphoteracin B 20 µg and nystatin 100 units) and negative non-antifungal control discs e.g. (metronidazole 5 µg, polymyxin B 300 units and fusidic acid 10 µg) (Oxoid) were also evaluated. Measuring of the IZ was performed after 18-24 h.

Screening of the antifungally active *Streptomyces* sp. against reference *C. albicans* biofilm using resazurin method

Preparation of the reference *C. albicans*

Fresh cultures of reference *C. albicans* were inoculated in YPD broth and incubated overnight at 30ºC in orbital shaker of 200 rpm. Cells were harvested at 4000 rpm for 5 min, washed in sterile Phosphate-buffered saline (PBS), centrifuged, resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS) (pH 7.0) to be counted by Bürker counting chamber and adjusted to a cellular density of 1×10^6 cells/mL for biofilm formation in 96-well microtitre plate (Ramage, 2001).

Optimization of reference *C. albicans* biofilm forming conditions

Different parameters affecting biofilm formation of *C. albicans* ATCC 10231 in flat-bottomed, 96- microtitre well polystyrene surface (Nunclon™ Δ surface, Nunc, Roskilde, Denmark) were studied. Parameters included duration of biofilm formation (18, 24 and 48 h) at 37 °C, inoculum size and resazurin contact time (20, 30, 45, 60, 90, 120 and 180 min). The optimal factors affecting biofilm formation were chosen based on the results of the statistical analysis.

Effect of extract against *C. albicans* biofilm

Prior-to-exposure (prevention mode)

The crude extracts were added in 2% concentration of total volume simultaneously with the *Candida* suspension in RPMI media. Ketoconazole (0.2 and 2 gL^{-1}) and metronidazole (1 and 10 gL^{-1}) were used as controls. Therefore, all compounds were dissolved in distilled water of 50 times of the desired concentration and were incubated under optimized conditions. After 24 h

incubation, the medium was discarded and non-adherent cells were removed and 200 µL of resazurin was added in each well.

Post-exposure (destruction mode)

After biofilm formation for 24 h, the planktonic suspension was removed by aspiration using multichannel automatic pipette and replaced by fresh RPMI 1640 medium along with 2 % of crude extract. Also, 2% of ketoconazole and metronidazole were used as standard controls. The plates were incubated for further 24 h and then the medium was removed, the wells were washed with PBS and 200 µL of resazurin was added in each well. Eventually, The fluorescence was measured using an excitation filter of 560 nm and an emission filter of 590 nm using Varioskan Multimode Plate Reader (Thermo Fisher Scientific, Vantaa, Finland) after optimum incubation time in both prevention and destruction modes.

Cytotoxic evaluation of antifungal compounds on Human Lung HL epithelial cells

It was done using resazurin method according to Karlsson *et al*. (2012). Human Lung epithelial HL cells (Kuo and Grayston 1990) were grown in RPMI 1640 medium supplemented with 7.5% fetal bovine serum (FBS), 2 mM L-glutamine and 20 µg/mL gentamycin. For the cell viability assays, the cells were seeded into 96-well plates at density 60.000 cells/ well and incubated overnight before starting the exposure. The extracts were diluted with cell culture medium to yield the indicated concentrations, the medium from the 96-wellplates with cells was removed and aliquots of 200 µL/ well of the samples were added in three replicates. The plates were incubated for 24 h after which the samples were removed and wells were washed with 100 µL PBS. After washing, 20 µM resazurin solution in 200 µL PBS was added into each well and the plate was incubated at 37 ˚C for 2 h. The fluorescent signal (excitation/emission 560/590 nm) resulting from the reduced form of resazurin was read with Varioskan Multimode Plate Reader (Thermo Fisher Scientific, Vantaa, Finland). The results were expressed as viability percentages normalized using a negative control.

Data processing and statistical analysis

Statistical parameters included signal window coefficient Z-factor, signal-to-noise (S/N), signal-to-background (S/B) and separation band were calculated according to Zhang *et al*., (1999) and Bollini *et al*. (2002). In all equations, SD_{min}, X_{min} and SD_{max}, X_{max} represent the standard deviations and means of the minimal (min) and maximal (max) signals, respectively. The means of the relative fluorescence units (RFU) measurements of negative (RPMI 1640 medium, minimal signal, min) and positive (untreated biofilms, maximal signal, max) control wells, respectively. The hit limits were used in the screening experiments to distinguish active from non-active compounds (Sandberg *et al*. 2009).

Results

Isolation of *Streptomyces* and Screening of antifungal activity against reference strain

Out of the forty *Streptomyces* isolates, *Streptomyces* sp. 6 isolated from soil of cattle farms in Dakahlya showed strong antifungal activity was selected for identification.

Identification of the active *Streptomyces* isolate

Phenotypic, physiological and biochemical characterization

Streptomyces sp. 6 has buff color of the aerial mycelium. The substrate mycelium is unpigmented and no diffusible pigment was produced (Table 1). The microscopic examination illustrated that the spore chain morphology is long straight spore shaped (Fig.1). *Streptomyces* sp. 6 utilized starch as a carbon source. *Streptomyces* sp. 6 utilized casein as a good nitrogen source and it was able to produce melanin. The biochemical characterization of *Streptomyces* sp.

6 revealed that it has the capability to produce different enzymes such as arginine dihydrolase, gelatinase, utilized citrate and produced acetoin (Voges-Proskaur).

Table 1 The phenotypic, physiological and biochemical characteristics of the bioactive *Streptomyces* sp.6

Phenotypic characteristics	
Spore morphology	Long straight spore chain
Aerial mycelium presence	Present
Color of aerial mycelium	Buff
Color of substrate mycelium	un-pigmented
Diffusible pigments	-
Physiological characteristics	
Melanin production	+
Carbon sources fermentation:	
D-Glucose	-
D-Mannitol	-
Meso-Inositol	-
D-Sorbitol	-
L-Rhamnose	-
D-Sucrose	-
D-Melibiose	-
D-Amygdalin	-
L-Arabinose	-
Biochemical characteristics	
Indole production	-
Voges Proskauer (acetoin) test	+
Citrate test	+
Hydrogen sulfide production	-
Hydrolysis of starch	+
Hydrolysis of gelatin	+
Hydrolysis of urea	-
Hydrolysis of casein	+

A plus sign (+), the isolate gave positive reaction for the test; a negative sign (-), the isolate gave negative reaction for the test. The isolate' metabolic activities was confirmed using API® strips.

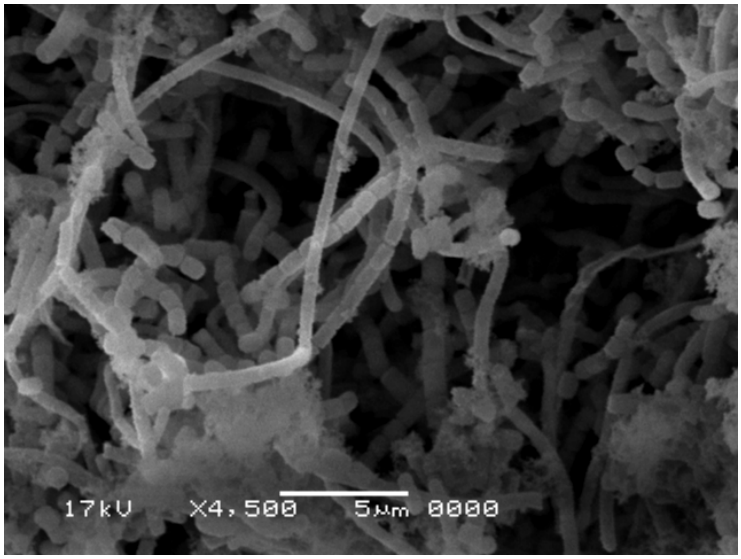

Fig. 1- Scanning electron microscope (SEM) micrograph of *Streptomyces* sp. 6 showing long straight spore chains.

Molecular identification and phylogenetic taxonomy

Streptomyces sp. 6 exhibited a maximum similarity level 97% to *Streptomyces toxytricini.* It was identified as *Streptomyces toxytricini* Fz94 in the Genbank databse under accession no. of KM052378. The phylogenetic tree of *S. toxytricini* Fz94 is shown in figure.2

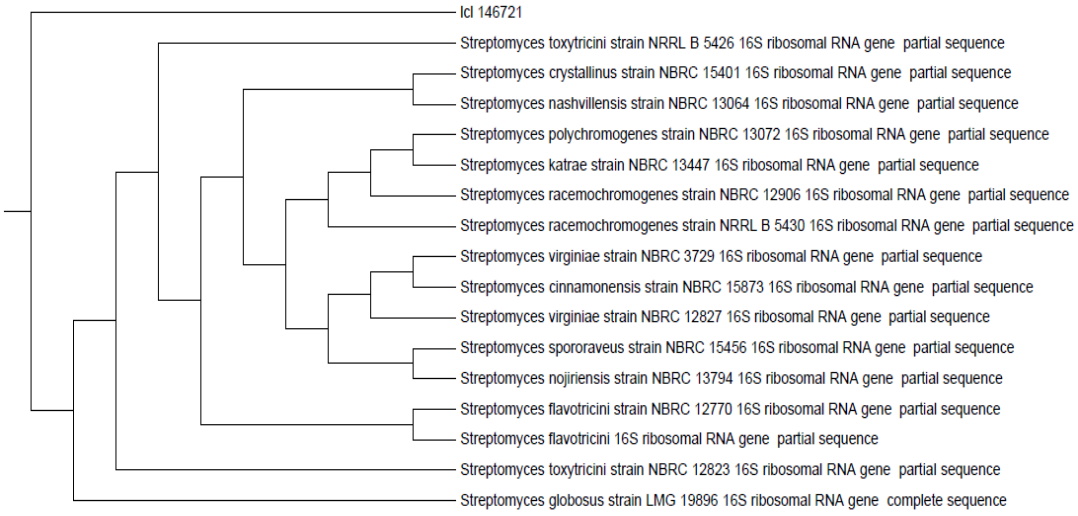

Fig. 2- The phylogenetic tree of *S. toxytricini* Fz94 and other known *Streptomyces* spp.

Screening of the activity of *S. toxytricini* Fz94 against planktonic and biofilm of *C. albicans*

Primary and secondary screening of *S. toxytricini* Fz94 against reference strain of *C. albicans*

S. toxytricini Fz94 showed potent antifungal activity against *C. albicans* ATCC 10231 in both cut plug agar and agar well diffusion methods.

Screening of *S. toxytricini* Fz94 against clinical *C. albicans* isolates

In vitro antifungal susceptibilities of these isolates showed that almost all the clinical *C. albicans* were resistant to metronidazole and polymyxin B (93.3% & 86.6%) respectively and to a lesser extend resistant to fluconazole, nystatin and amphotericin B (60, 53.3 and 40%) respectively. On the other hand, 100% of clinical *C. albicans* isolates were susceptible to both crude extract *S. toxytricini* Fz94 and ketoconazole.

Resazurin test of antifungal activity of *S. toxytricini* Fz94 on biofilm forming reference *C. albicans*

Optimization of *C. albicans* biofilm forming conditions

The optimal conditions for *C. albicans* ATCC 10231 biofilm formation, such as (volume of bacterial inoculum, growing time, static or shaking cultivation) and staining procedures (resazurin incubation time, elimination of washing steps). It was found that the optimal conditions for biofilm formation are: using 200µL of 1.0×10^6 cells per mL as inoculums for 24 h under shaking or static conditions. The resazurin assay optimal conditions were: incubation of resazurin for 90 and 120 min.

Effect of extract against *C. albicans* biofilm

Prior-to-exposure (prevention mode)

In the pre-exposure scheme, at the lowest concentration of the extract of *S. toxytricini* Fz94 (5 gL^{-1}) a 92% inhibition of biofilm viability after 120 min was detected, which is more potent than ketoconazole that gave 70 and 90% at concentrations of 0.2 and 2 gL^{-1} respectively. However, metronidazole that caused 25 and 30 % at concentrations of 1 and 10 gL^{-1}(Fig. 3).

Table 2 Antifungal activity of crude extract of *S. toxytricini* Fz94 and reference antibiotics against clinical *C. albicans* isolates

Clinical *C. albicans* no.	**Source**	**Positive control (Antifungal discs)**				**Negative Control (Non-antifungal discs)**			***S. toxytricini* Fz94**
		KET	**FCA**	**AB**	**NS**	**MTZ**	**PB**	**FD**	
*C.albicans*01	Urine	S	R	R	I	R	R	R	S
*C.albicans*02	Urine	S	I	R	S	R	R	R	S
*C.albicans*03	Urine	S	R	R	I	I	R	R	S
*C.albicans*05	Urine	S	R	S	I	R	R	R	S
*C.albicans*06	Urine	S	R	S		R	R	R	S
*C.albicans*04	Sputum	S	R	I	S	R	I	R	S
*C.albicans*07	Sputum	S	S	I	R	R	R	R	S
*C.albicans*09	Sputum	S	S	I	R	R	R	R	S
*C.albicans*11	Sputum	S	S	R	R	R	R	R	S
*C.albicans*12	Sputum	S	S	R	R	R	R	R	S
*C.albicans*13	Sputum	S	S	R	R	R	R	R	S
*C.albicans*08	Pus	S	R	S	R	R	R	R	S
*C.albicans*10	vaginal swab	S	R	S	I	R	R	R	S
*C.albicans*14	vaginal swab	S	R	S	R	R	R	R	S
*C.albicans*15	pleural fluid	S	R	S	R	R	I	R	S

Abbreviations R, Resistant; I, Intermediate; S, Sensitive;
KET, Ketoconazole; FCA, Fluconazole; AB, Amphoteracin B; NS, Nystatin; MTZ, Metronidazole; PB, Polymxin B; FD, Fucidic acid.

Post-exposure (destruction mode)

In post exposure, the extract of *S. toxytricini* Fz94 (7 gL^{-1}) caused 82 % inhibition of biofilms viability after 120 min, Metronidazole caused inhibition of 80% at concentration of 1 gL^{-1} and wasn't active at higher concentration, while ketoconazole didn't show any destruction ability.

Cytotoxic evaluation of crude extract of *S. toxytricini* Fz94 on Human Lung (HL) epithelial cells

To further characterize the biological activity of the crude extract of *S. toxytricini* Fz94, it was evaluated for its effect on human epithelial cells of *S. toxytricini* Fz94 was nontoxic at 10 gL^{-1} (>85% viability), while it showed toxicity at 20 gL^{-1} (15% viability).

Discussion

Biofilm formation is one of the most important virulent factor of *Candida* spp. that has led to high prevalence of *Candida* infections concomitant with high morbidity and mortality rate in hospitalized patients (Pfaller and Diekema 2007; Favero *et al*. 2011; Vinitha and Mamatha 2011). *C. albicans* ATCC 10231, the target test strain in this study, has been a widely used as reference strain for antifungal (Atta 2010; Saurav and Kannabiran 2012) and biofilm research (De Logu *et al.* 2005; Palmeira-de-Oliveiraa *et al.* 2012). However, the aim of this study was screening of antifungal agents from genus *Streptomyces* against reference strain and clinical

isolates of *C.albicnas* and thereafter, evaluates the potential of this antifungal crude extract in prior to exposure and post exposure of reference strain of *C.albicans*.

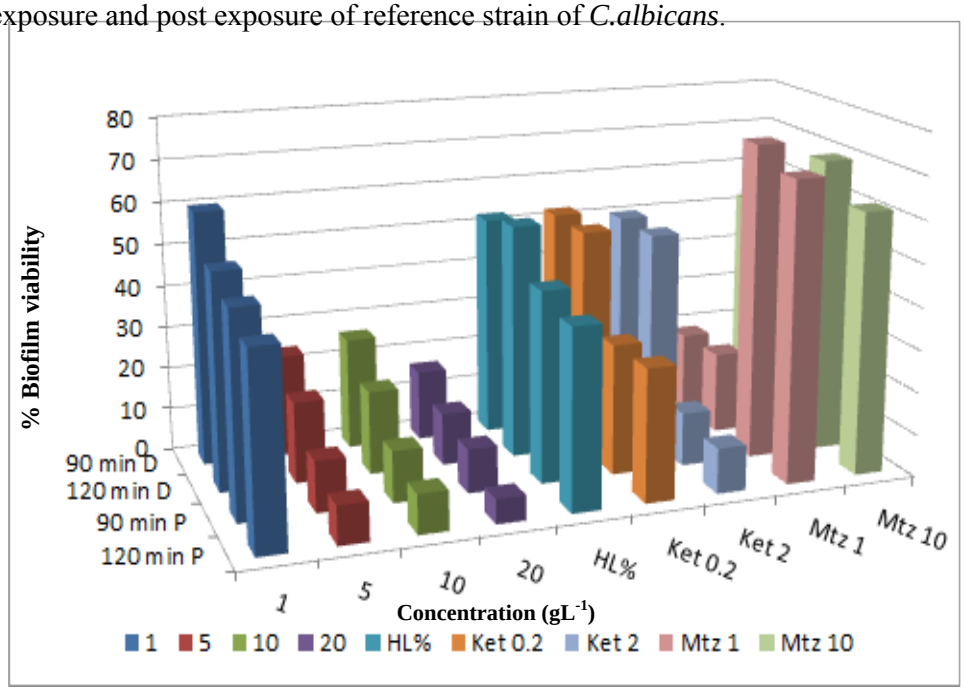

Fig. 3- Comparison of percent control (viability) of biofilm using different concentrations of *S. toxytricini* Fz94 extract in prevention (P) and destruction (D) modes after resazurin incubation times 90 and 120 min. Abbreviations: Ket, ketoconazole; Mtz, metronidazole; HL%, hit limit percent. Positive hits found are those scoring below the hit limit. Fluorescence was measured at $\lambda_{excitation}$=570 nm and $\lambda_{emission}$=590 nm.

The screening studies of the forty *Streptomyces* isolates resulted in a potent *Streptomyces* sp. 6. that has been identified using classical methods including morphological, physiological, biochemical characterization using 16S rRNA as *S. toxytricini* (strain fz94) and was deposited in the GenBank nucleotide sequence database under accession numbers of KM052378. Nowadays, detection and classification of *Streptomyces* is also commonly performed by molecular approaches (Locatelli *et al*. 2002). However, *S. toxytricini* was recorded to produce pancreatic lipase inhibitor which is lipstatin of which the antiobesity drug orlistat is a derivative (Barbier and Schneider 1987; Weibel *et al.* 1987). Also, it has antifungal activity against *C. albicans* (Abdel Azeiz *et al*. 2016).

In these screening studies, *S. toxytricini* fz94 showed strong antifungal activity against *C. albicans* ATCC 10231 and all the clinical *Candida* isolates which was similar to ketoconazole. This result was in accordance with Ng *et al*. (2000) who reported the same activity of ketoconazole against clinical *Candida* isolates. It was selected for evaluation for its anti-biofilm activity against *C. albicans* ATCC 10231 using resazurin method. However, In recent years, biofilm quantification assays were based on microtiter plates using several probes such as XTT which was the most commonly used method (Ramage *et al*. 2011), crystal violet (CV) (Peeters *et al*. 2008), and resazurin to a lesser extent in case of *Candida* biofilms (Driessche *et al*. 2014). Resazurin assay has been proven to be simple, fast, cost-effective and suitable for high-throughput method for biofilm quantification (Sandberg *et al*. 2009). Therefore, it was essential to optimize the biofilm formation and resazurin incubation time before evaluation of anti-biofilm activity of the crude extract. The optimal biofilm formation according to statistical

performance detected using inoculum volume of 200 µL of 1.0 × 10^6 cells per mL cellular density of *C. albicans* concentration for 24 h at 37°C, 100 rpm which is in accordance with Ramage *et al*. (2001) results. Because resazurin is nontoxic, microorganisms continue duplicating during resazurin incubation. As such, baseline fluorescence rises proportionally with incubation; the best result was achieved after adding the resazurin by 120 min. Therefore, the fluorescence was measured after 90 and 120 min for more convenience. In the pre-exposure scheme, at the lowest concentration of *S. toxytricini* Fz94 (5 gL^{-1}) extract caused 92% inhibition of biofilm viability after 120 min.Whereas, ketoconazole caused 70 and 90% biofilm inhibition at concentrations of 0.2 and 2 gL^{-1} respectively. However, metronidazole caused 25 and 30 % at concentrations of 1 and 10 gL^{-1}. In post exposure, the extract of *S. toxytricini* Fz94 (7 gL^{-1}) caused 82 % inhibition of biofilms viability after 120 min. While ketoconazole didn't show any destruction ability. Results showed that the antifungal produced by *S. toxytricini* fz94 had anti-*Candida* biofilm activities in both prevention and destruction modes. However, search in the literature revealed that there is few literatures regarding anti-*Candida* biofilm from *Streptomyces* spp. such as Pierce *et al.* (2009) and Rajalakshmi *et al*. (2014) only in prevention mode.

It was important to study the effect of the bioactive extract on cell lines because of the predicted cytotoxicity of the antifungal metabolites. However, cytotoxicity was carried out against normal Human Lung epithelial HL cells (Kuo and Grayston 1990) using resazurin method (Karlsson *et al*. 2012). The crude extract of *S. toxytricini* fz94 showed that it was nontoxic at 10 gL^{-1} (>85% viability), while it showed toxicity at 20 gL^{-1} (15% viability).

Conclusion

Based on the data available out of this study is that *S. toxytricini* fz94 extract may represent effective and safe anti- *Candida* biofilm at concentrations in prevention and destruction modes using resazurin method. It was similar to ketoconazole against clinical *Candida* isolates and in prevention of *Candida* biofilm but crude extract of *S. toxytricini was* more potent than ketoconazole in destruction of *C.albicans* biofilm. Additionally, more chemical studies will be needed to isolate and identify the active principles of the extract *S. toxytricini* evaluated in the present study.

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Conflict of Interest

The authors do not have any conflicts of interest.

References

Abdel Azeiz A. Z., Hanafi D. K. and Hasanein S. E. 2016. Identification of a new antifungal oligoacetal derivative produced by *Streptomyces toxytricini* against *Candida albicans*. Natural Product Research 30(16):1816-23.

Amorena B., Gracia E., Oteiza C., Pérez M., Alabart J. and Hernández-Yago J. 1999. Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. Journal of Antimicrobial Chemotherapy 44: 43-55.

Atta H. M. 2010. Production, purification, physico-chemical characteristics and biological activities of antifungal antibiotic produced by *Streptomyces antibioticus*, AZ-Z710. American-Eurasian Journal of Scientific Research 5: 39-49.

Augustine N., Wilson P. A., Kerkar S., Thomas S. 2012. Arctic actinomycetes as potential inhibitors of *Vibrio cholerae* biofilm. Current Microbiology 64:338-342.

Azevedo N. F., Lopes S. P., Keevil C. W., Pereira M. O., Vieira M. J. (2009) Time to "go large" on biofilm research: advantages of an omics approach. Biotechnology Letters 31(4): 477-485.

Bakkiyaraj D. and Pandian S. K. 2010. *In vitro* and in vivo antibiofilm activity of a coral associated actinomycete against drug resistant *Staphylococcus aureus* biofilms. Biofouling 26: 711–717.

Balasubramanian S., Othman E. M., Kampik D., Stopper H., Hentschel U., Ziebuhr W., Oelschlaeger T. A. and Abdelmohsen U. R. 2017. Marine sponge-derived *Streptomyces* sp. SBT343 extract inhibits Staphylococcal biofilm formation. Frontiers in Microbiology 8(236): 1-14.

Barbier P. and Schneider F. 1987. Syntheses of tetrahydrolipstatin and absolute configuration of tetrahydrolipstatin and lipstatin. Helvetica Chimica Acta 70(1): 196-202

Bollini S., Herbst J. J., Gaughan G. T., Verdoorn T. A., Ditta J., Dubowchik G. M., Vinitsky A. 2002. High-throughput fluorescence polarization method for identification of FKBP12 ligands. Journal of Biomolecular Screening 7: 526-30.

Chandra J., Patel J. D., Li J., Zhou G., Mukherjee P. K., McCormick T. S., Anderson J. M., Ghannoum M. A. 2005. Modification of surface properties of biomaterials influences the ability of *Candida albicans* to form biofilms. Applied and Environmental Microbiology 71(12): 8795-8801.

Christenswen B. 1946. Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other and from *Salmonella* and *Shigella* types. Journal of Bacteriology 52: 461.

De Logu A., Saddi M., Cardia M. C., Borgna R., Sanna C., Saddi B. and Maccioni, E. 2005. *In vitro* activity of 2-cyclohexylidenhydrazo-4-phenyl-thiazole compared with those of amphotericin B and fluconazole against clinical isolates of *Candida* spp. and fluconazole-resistant *Candida albicans*. Journal of Antimicrobial Chemotherapy 55: 692-698.

Devi L. S. and Maheshwari M. 2014. Speciation of *Candida* Species isolated from clinical specimens by using Chrom agar and conventional methods. International Journal of Scientific and Research Publications 4(3): 1-5.

Douglas L. J. 2002. Medical importance of biofilms in *Candida* infections. Revista iberoamericana de micologia. 19: 139–143.

Douglas L. J. 2003. *Candida* biofilms and their role in infection. Trends in microbiology 11: 30-36.

Driessche V. F., Rigole P., Brackman G., Coenye T. 2014. Optimization of resazurin-based viability staining for quantification of microbial biofilms. Journal of Microbiological Methods 98: 31-34.

Favero D., Franca, E. J., Furlaneto-Maia, L., Quesada R. M., Furlaneto, M. C. 2011. Production of haemolytic factor by clinical isolates of *Candida tropicalis*. Mycoses 54:816–20.

Felsenstien J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Harding M. W., Marques L. L., Howard R. J. and Olson M. E. 2009. Can filamentous fungi form biofilms? Trends in Microbiology 17(11).

Hassan A., Usman J., Kaleem F., Omair M., Khalid A. and Iqbal M. 2011. Evaluation of different detection methods of biofilm formation in the clinical isolates. Brazilian Journal of Infectious Diseases 15:305-11.

Hosny A.M. S., Sheir D. H., Abdelallah N. A., Amin D. H. M., El-Diwany A. I., Abdelwahed N. A. M., El-Beih A. A., Fallarero A. and Vuorela P. M. 2015. Current Science International 4(3): 259-263.

James G. 2010. Universal Bacterial Identification by PCR and DNA Sequencing of 16S rRNA Gene. In: Schuller M., Sloots P. T., James S. G., Halliday L. C. and Carter, W. J. I., Eds., PCR for Clinical Microbiology, Springer, Dordrecht 209-214.

Kämpfer and Peter. 2006. "The Family *Streptomycetaceae,* Part I: Taxonomy". In Martin D., Stanley F., Eugene R., Karl-Heinz S., Erko S. The Prokaryotes. pp. 538-604.

Karlsson D., Fallarero A., Brunhofer, G., Mayer C., Prakash O., Mohan C. G., Vuorela P. and Erker T. 2012. The exploration of thienothiazines as selective butyrylcholinesterase inhibitors. European Journal of Pharmaceutical Sciences 47:190-205.

Kavanagh. 1972. Analytical microbiology, 2, Academic Press, New York.

Khan ST. 2011. Streptomyces associated with a marine sponge Haliclona sp.; biosynthetic genes for secondary metabolites and products. Environmental Microbiology 13:391-403.

Kim Y-G., Lee J-H., Kim C-J., Lee J-C., Ju Y. J., Cho M. H, Lee J. 2012. Antibiofilm activity of *Streptomyces* sp. BFI 230 and *Kribbella* sp. BFI 1562 against *Pseudomonas aeruginosa*. Applied Microbiology Biotechnology 96: 1607–1617.

Kuo C. C. and Grayston J. T. 1990. A sensitive cell line, HL cells, for isolation and propagation of *Chlamydia pneumoniae* strain TWAR. International journal of infectious diseases 162: 755-758.

Kuster E. and Williams S. T. 1964. Selection of media for isolation of Streptomycetes. Nature, London 202: 928-929.

LaFleur M. D., Kumamoto C. A. and Lewis K. 2006. *Candida albicans* Biofilms Produce Antifungal-Tolerant Persister Cells. Antimicrobial Agents and Chemotherapy 50 (11): 3839-3846.

Lam K. S. 2006. Discovery of novel metabolites from marine actinomycetes. Current Opinion in Microbiology 9: 245-251.

Lane D. J., Pace B., Olsen G. J., Stahl D. A., Sogin M. L. and Pace N. R. 1985. Rapid determination of 16S ribosomal RNA sequence for phylogenetic analyses. Proceedings of the National Academy of Sciences of the United States of America 82: 6955 -6959.

Locatelli L., Tarnawski S., Hamelin J., Rossi P., Aragno M. and Fromin N. 2002. Specific PCR amplification for the genus Pseudomonas targeting the 3 half of 16S rDNA and the whole 16S- 23S rDNA spacer. Systematic and Applied Microbiology. 25: 220-227.

MacFaddin J. F. 2000. Biochemical tests for the identification of medical bacteria, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Mensa J., Pitart C. and Marco F. 2008. Treatment of critically ill patients with candidemia. International journal of antimicrobial agents 32(Suppl. 2):S93-7.

NCCLS 2004. Reference method for antifungal disk diffusion susceptibility testing of yeasts; approved guideline. NCCLS document M44-A. National Committee for Clinical Laboratory Standards, Wayne.

Ng K.P., Saw T. L., Na S. L., Soo-Hoo T. S. 2000. Systemic *Candida* infection of University Hospital 1997-1999: the distribution of *Candida* biotypes and antifungal susceptibility patterns. Mycopathologia, 149(3):141-146.

Noumi E., Snoussi M., Hentati H., Mahdouani K., del Castillo L., Valentin E., Sentandreu R., Bakhrouf A. 2010. Adhesive properties and hydrolytic enzymes of oral *Candida albicans* strains. Mycopathologia 169(4): 269-278.

Odds, F. C. and Bernaerts, R. 1994. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. Journal of Clinical Microbiology 32:1923-1929.

Ômura S., Ikeda H., Ishikawa J., Hanamoto A., Takahashi C., Shinose M., Takahashi Y., Horikawa H., Nakazawa H., Osonoe T., Kikuchi H., Shiba T., Sakaki Y. and Hattori M. 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*:

Deducing the ability of producing secondary metabolites. Proceedings of the National Academy of Sciences of the United States of America 98: 12215-12220.

Patzer S. I. and Volkmar B. 2010. Gene cluster involved in the biosynthesis of griseobactin, a catechol-peptide siderophore of *Streptomyces* sp. ATCC 700974. Journal of bacteriology 192:426–35.

Peeters E., Nelis H. J. and Coenye T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of Microbiological Methods 72(2): 157-165.

Pfaller M. A. and Diekema D. J. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clinical Microbiology Reviews 20:133–63.

Pierce C. G., Thomas D. P. and Lo´pez-Ribot J. L. 2009. Effect of tunicamycin on *Candida albicans* biofilm formation and maintenance. Journal of Antimicrobial Chemotherapy 63: 473-479.

Pridham T. G., Lindenfelser L. A. and Shotwell O. L. 1956. Antibiotics against plant disease: I. Laboratory and green house survey. Phytopathology 46: 568–75.

Rajalakshmi M., Raaghavi J., Poffe M. F., Srinivasan P. and Priyadarisini B. V. 2014. Effect of crude Fatty acid extract of *Streptomyces* sps on biofilm forming *Candida albicans* MTCC. Research Journal of Recent Sciences 3: 283-290.

Ramage G., Jose A., Coco B., Rajendran R., Rautemaa R., Murray C., Lappin D. F. and Bagg J. 2011. Commercial mouthwashes are more effective than azole antifungals against *Candida albicans* biofilms *in vitro*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology 111(4): 456-460.

Ramage G., Walle K. V., Wickes B. L. and Lo´pez-Ribot J. L. 2001. Standardized method for *In Vitro* antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrobial Agents and Chemotherapy 45(9): 2475-2479.

Saginur R., Stdenis M., Ferris W., Aaron S., Chan F., Lee C. and Ramotar K. 2006. Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. Antimicrobial Agents and Chemotherapy 50: 55–61.

Saito N. and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

Sandberg M. E., Schellmann D., Brunhofer G., Erker T., Busygin I., Leino R., Vuorela P. M. and Fallarero A. 2009. Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. Journal of Microbiological Methods 78: 104–106.

Sardi J. C., Scorzoni L., Bernardi T., Fusco-Almeida A. M., Mendes Giannini MJ. 2013. *Candida* species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. Journal of Medical Microbiology 62 (Pt 1):10-24.

Saurav, K. and Kannabiran, K. 2012. *In vitro* activity of 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp. against fungal and bacterial human pathogens. Revista Iberoamericana de Micología 29: 29-33.

Sherry L., Rajendran R., Lappin D. F., Borghi E., Perdoni F., Falleni M., Tosi D., Smith K., Williams C., Jones B., Nile C. J., Ramage G. 2014. Biofilms formed by *Candida albicans* bloodstream isolates display phenotypic and transcriptional heterogeneity that are associated with resistance and pathogenicity. BMC Microbiology 14:182.

Shirling E. B. and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* sp. International Journal of *Systematic* Bacteriology 16: 313-340.

Srivastava V. and Dubey A. K. 2016. Anti-biofilm activity of the metabolites of *Streptomyces chrestomyceticus* strain ADP4 against *Candida albicans*. Journal of Bioscience and Bioengineering 122(4):434-40

Sutherland I. W. 2001. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147:3-9.

Tamura K., Stecher G., Peterson D., Filipski A. and Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 28(10): 2731-2739.

Tan G. L. and Peterson E. M. 2005. CHROMagar *Candida* Medium for direct susceptibility testing of yeast from blood cultures. Journal of Clinical Microbiology 43(41): 727-1731.

Tanaka Y. and Omura S. 1990. Metabolism and products of actinomycetes-an introduction. Actinomycetologica 4: 13-14.

Vinitha M. and Mamatha B. 2011. Distribution of *Candida* Species in different clinical samples and their virulence; biofilm formation, proteinase and phospholipase production; a study on hospitalized patients in Sourthern India. Journal of Global Infectious Diseases 3:4-8.

Watve M., Tickoo R., Jog M., Bhole B. 2001. "How many antibiotics are produced by the genus *Streptomyces* ?". Archives of Microbiology 176(5): 386–90.

Weibel E., Hadvary P., Hochuli E., Kupfer E., Lengsfeld H. 1987. "Lipstatin, an inhibitor of pancreatic lipase, produced by *Streptomyces toxytricini*. I. Producing organism, fermentation, isolation and biological activity". Journal of Antibiotics (Tokyo) 40(8): 1081-5.

Wisplinghoff H., Bischoff T., Tallent S. M., Seifert H., Wenzel R. P., Edmond M. B. 2004. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases 39:309-17.

You J., Xue X., Cao L., Lu X., Wang J., Zhang L. and Zhou S. 2007. Inhibition of Vibrio biofilm formation by a marine actinomycete strain A66. Applied Microbiology Biotechnology 76: 1137-1144.

Younis K. M., Usup G. and Ahmad A. 2016. Secondary metabolites produced by marine streptomyces as antibiofilm and quorum-sensing inhibitor of uropathogen *Proteus mirabilis*. Environmental Science and Pollution Research 23:4756–4767.

Zhang J., Chung T. and Oldenburg K. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. Journal of Biomolecular Screening, 4: 67-73.