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Candida biofilm perfusion using active fractions of *Acorus calamus*

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Key words
Acorus calamus, biofilm, perfusion, *Candida* sp.

1 SUMMARY

***Candida albicans* is a dimorphic and opportunistic fungal pathogen that causes superficial or systemic infections in immunocompromised individuals. Biofilms, in general are universal, complex, interdependent communities of surface associated microbes. *Candida albicans* cells adhere to surfaces of medical devices, encase itself inside extracellular polymer matrix, and form a biofilm. Candidal biofilm consists of a dense network of yeast cells, pseudohyphae, and hyphae. Biofilms possess unique developmental characteristics that are in stark contrast to the characteristics of free-floating planktonic cells, and biofilms are much more difficult to treat chemotherapeutically. The present study aimed to demonstrate the perfusive efficacy of an active fraction of the methanolic extract of the traditional medicinal plant *A. calamus* in biofilm of *Candida albicans* in comparison with conventional antifungal agents**.

2 INTRODUCTION

Candida albicans, a dimorphic and opportunistic fungal pathogen, causes superficial or systemic infections in immunocompromised individuals (Baillie & Douglas, 1998a; Alem & Douglas 2004; Al-Fattani & Douglas, 2004). Biofilms are universal, complex, interdependent communities of surface associated organisms (Baillie & Douglas 1998b; Baillie & Douglas, 2000; Douglas, 2002; Kuhn *et al*.a ,b, 2002). *Candida albicans* cells adhere to surfaces of implanted medical devices and encase themselves within an extracellular polymer matrix and form a biofilm (Douglas, 2002; Ramage & Bachmann, 2002). In general, the Candidal biofilm consists of a dense network of yeast cells, pseudohyphae and hyphae. Biofilms possess unique developmental characteristics that are in stark contrast to the characteristics of free-floating planktonic cells, and biofilms are much more difficult to treat chemotherapeutically (Ramage *et al*., 2001; Douglas, 2002; Kuhn *et al*., 2002a; Ramage & Bachmann, 2002; Donlan & Costerton, 2002; Samaranayae *et al*., 2004; Cocuaud *et al*., 2005). *C. albicans* has the capacity to switch from yeast morphology to a hyphal morphology; this transition is one of the pivotal biological processes required for biofilm formation. This yeast-to-mycelium conversion, a differentiation process is fundamental for opportunistic pathogens to thrive, disseminate, and initiate infections.

The established biofilms inside an implant device develop a unique character of resistance towards antifungal drugs. The biofilms of *C. albicans* are 30 to 2000 times more resistant than planktonic cells to clinically important antifungal drugs (Baillie & Douglas, 1998a; Crump & Collingnon, 2000; Ernst *et al*., 2001). The biofilms on implants and other surgical devices may lead to failure of devices and furthermore, these biofilms serve as a

source of recalcitrant infections (Baillie & Douglas, 2000). Hence, it is difficult to treat implant-mediated infection and the only chance is to remove the implant device, which may complicate the situation in case of major surgeries. The recent studies report that Candidal infections are placed third in catheter related infection, second highest in colonization-to-infection rate, and overall highest in crude mortality (Douglas, 2002). Since these biofilms exhibit higher resistance to antifungal therapy; there is need for new drug formulation.

There are only scarce reports on perfusion of drugs through fungal biofilms (Al-Fattani & Douglas, 2004; Samaranayakae *et al*., 2004; Subha & Gnanamani, 2008) especially with reference to plant derived products. The present study evaluated the efficacy of the active fractions of *Acorus calamus* on the biofilms of *Candida* spp.

3 MATERIALS AND METHODS

3.1 Organisms: Perfusion studies were carried out with clinical isolates of *Candida albicans* (CLCA 0590, *SRMC*, Chennai, India), and *Candida tropicalis* (CLCT 0610, General Hospital, Chennai, India). To assess perfusion, we used an indicator organism, *Candida dubliniensis* (CLDS 0710, VHS, Chennai, India).

Prior to biofilm studies, all strains were identified and characterized (CHROM agar, Germ tube) and maintained on Sabouraud Dextrose Agar slants (SDA) at 4ºC. For biofilm studies, a loopfull of the selected *Candida* sp. was inoculated in yeast nitrogen base (YNB) medium with 50mM dextrose and incubated at 37ºC for 24h. Cells harvested (centrifugation at 10000 X g at 4ºC), were washed twice with 0.15M phosphate buffered- saline (PBS; pH 7.2), and adjusted to an optical density of 0.5 at 600 nm (Cary 100 UV –Visible spectrophotometer). Standardized cell suspension of $3X10^7$ cells/ml was used as inoculum for biofilm development.

3.2 Antifungal drugs: Rhizomes of *Acorus calamus L*. were obtained from the medicinal plants farms in South India, and voucher specimen deposited in the Plant Anatomy Research Centre, Chennai. The crude methanolic extract was fractionated using column chromatography and the active fractions AF1 and AF2 (based on its antifungal activity) was used for the present study. Ketoconazole and Amphothericin B were used as representatives of standard drugs.

3.3 Preparation of medium with antifungal compounds: Two antifungal agents, Ketoconazole and Amphotericin B, commonly used to treat oropharyngeal and systemic candidiasis were used for the present study. Both agents were tested at 2mg/ml concentration (*below this concentration, no antifungal activity was observed even to the planktonic cells, results not shown*). A stock solution of the respective concentration of drugs in dimethyl sulphoxide (Amphotericin B), dimethyl formamide (Ketoconazole) was prepared prior to use, filtered through sterile filtration unit (Tarson Ltd, India) and added to molten SDA containing 500 mM dextrose (Samaranayakae *et al*., 2004; Subha & Gnanamani, 2008).

3.4 Development of biofilm and antifungal perfusion studies: Biofilm was developed on cellulose membrane filter (diameter 20mm, pore size 12μm; Millipore, USA) with slight modifications as described by Samaranayakae *et al.* (2004). Initially, UV sterilized cellulose membrane (Base Membrane-BM), was aseptically placed on anSDA (containing 500 mM dextrose) plate. Subsequently, 50μl ($3X10^7$ cells /ml) of inoculum of selected strains were individually gently deposited on the surface of the cellulose membrane and incubated at 37°C for 1 h (initial adherence period) and the incubation period extended to 48 h (biofilm development period). During this period, after every 10 hours, the cellulose membrane (BM) with growing biofilm was manually lifted and re-positioned to a fresh location on the SDA plate.

3.5 Antifungal perfusion through *Candida* spp. biofilm: After 48 h, cellulose membrane (BM) with fully-grown biofilm was removed and placed on the agar containing the test compounds - (Ketoconazole, Amphothericin B, active fractions of *A. calamus* AF1 and AF2 individually at 2mg/ml concentration), with biofilm facing outwards. Subsequently, a plain cellulose membrane of 12 mm diameter with 0.2μm pore size (Millipore, USA) was placed over BM (Base Membrane) to form the intermediate membrane (IM) and above that, we placed a sterile blank antibiotic disk (AB) (9mm diameter; Hi- media) moistened with 10 μl of 0.15M PBS (pH 7.2). The whole assembly of membranes

(BM-IM-AB) was incubated at 37 ºC for 6h. A similar membrane setup; BM-IM-AB, devoid of biofilm was used as control (I). In addition, another control (control II) was maintained with the same membrane system (with biofilm) was placed in the SDA agar without antifungal compounds incorporated (Figure 1). The experiments wre conducted in triplicates on different occasions.

3.6 Evaluation of antifungal perfusion using AB disk: Semi quantitative assessment of antifungal diffusion through biofilm (BM-IM-AB assembly) was made as per the following procedure. After specified incubation time (6h), plain antibiotic disk (AB) was placed on top of the membrane assembly (controls as well as treated) removed and placed on RPMI agar plate (Hi Media), and spread plated with 100 µl of *C. dubliniensis* cells, (24 h grown *C. dubliniensis* diluted with 0.15M PBS (pH 7.2) to have an optical density of 0.05 (at 520 nm). The RPMI plates were then incubated at 37ºC for 24 h. Diameters of the growth inhibition zones were measured using zone-measuring scale (Hi-media Ltd., India).

3.7 Quantitative measurement of biofilm growth: Growth of biofilm adhered to the membranes (BM and IM) was quantified by measuring OD, dry weight of biofilm biomass, viable cell count and metabolic activities as per the procedure summarized below.

3.7.1 Growth determination by OD measurement: Biofilm adhering on cellulose membrane (BM) and intermediate membrane (IM) was scrapped using cell scrapper. Subsequently, cellulose membrane and cell scrapper was rinsed with 0.15 M PBS (pH 7.2) and mixed with the scrapped biofilm. The combined extracts were vortex and the optical density read at 600nm. (Subha & Gnanamani, 2008).

3.7.2 Cell viability test: Cell viability of biofilm adhering on BM and IM was evaluated by serially diluting (10^{-1} to 10^{-7}) the supernatant obtained from growth measurement studies. An aliquot (100µL) of serially diluted supernatant (10^{-4}, 10^{-5}, and 10^{-6}) was spread-plated on SDA plate, incubated for 24h at 37 º C, and assessed for viability (Subha & Gnanamani, 2008).

3.7.3 Dry weight measurements: For dry weight measurements, the biofilm adhering on BM and IM was scrapped with a cell scrapper and the resulting solution was filtered through a 0.22µm-pore size. The millipore filter was dried at 50º C for 24 h and weighed (MacCourtie & Douglas, 1984; Subha & Gnanamani, 2008).

3.7.4 Metabolic activity assay: Metabolic activity of viable cells was quantified using XTT (2,3- bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenyl amino)carbonyl]-2H-tetrazoliumhydroxide) reduction assay (MacCourtier & Douglas, 1984; Gilfillan *et al*., 1998; Hawser *et al*., 2002). Cellulose membrane with biofilms was transferred to a new 6- well plate, containing 3 ml of PBS, 50µl of XTT solution (1 mg/ml in PBS; Sigma, USA) and 4 µl of menadione solution (1mM in acetone; Sigma, USA) and incubated for 4 h at 37º C. The reduced XTT formazan product was made cell free (centrifuged at 6000g, 5 min) and read at 492nm. Membranes of control samples were also subjected to XTT assay.

3.8 Scanning Electron Microscopy: Biofilms formed on cellulose membrane were processed for scanning electron microscopy (Samaranayakae *et al*., 2004), with slight modifications in the procedure. Briefly, biofilms were fixed in 4% formaldehyde (v/v) and 1% glutaraldehyde (v/v) in PBS overnight and rinsed with 0.1M potassium phosphate buffer (pH 7; 2 times, 3min each), treated with 1% osmium tetraoxide for 30 min. They were further dehydrated with a series of ethanol washes (70% for 10min, 95% for 10min and 100% for 20min) and air dried in a desiccator prior to sputter coating with gold–palladium (40%, 60%) and observed under scanning electron microscope in high vacuum 20kV. The images were processed using soft imaging viewer software and Adobe Photoshop CS 2 (Adobe Systems Inc., San Jose, California, USA).

4 RESULTS

4.1 Antifungal susceptibility studies: The present study demonstrate the efficacy of the active fractions of traditional plant *Acorus calamus* in controlling biofilm development in *Candida albicans* and *C. tropicalis* and compared their efficiency with two standard antifungal agents, Ketoconazole and Amphothericin B. The two standard antifungal agents reduced the growth of the biofilm in both *C. albicans* and *C. tropicalis*, but did not cause complete death. of the biofilm. Biofilms development

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measured in terms of growth OD, CFU, dry weight and XTT showed significant correlation between the parameters (Figures 2-5).

4.2 Perfusion studies: Biofilm growth analyses in terms of OD, cell viability, dry weight and metabolic activity for both BM and IM of BM-IM- AB assembly placed on agar containing test compounds revealed that biofilms treated with Ketoconazole showed 7-19 % reduction in growth, and 82-94% of the cells are metabolically active. Cell survival of >80% was noted in Amphotericin B treated biofilm of base membrane with high metabolic activity of 82-93%. Results with intermediate membrane (IM) for Ketoconazole and Amphotericin B showed existence of more than 70% of live cells in the intermediate membrane and the metabolic activity analysis further confirms the existence of life in the intermediate membrane. Cell viability assays carried out with the base membrane as well as the intermediate membrane revealed that Ketoconazole and Amphothericin B are able to reduce the growth of the biofilm by a very narrow margin. The viable cell count tests further confirmed that complete killing of the cells in the biofilm did not occur. Higher cell viability was encountered in both BM as well as in IM. Similar observations were made for the biofilm of *C. tropicalis*.

The biofilms treated with active fractions of *A. calamus* AF1 and AF2 interestingly showed a remarkable difference in biofilm development. About 99.1% reduction in biofilm growth was observed and cell viability assessment showed complete killing of biofilm biomass at 2mg/ml concentrations of AF1 and AF2, individually.

4.3 Assessment of perfusion: Perfusion assessed by cell viability assays of intermediate membrane (IM) showed about 270 Colony Forming Units (CFU) per 100 µl observed with the membrane obtained from the control treatments, where no test compounds was incorporated, while 100-120 CFU per per 100 µl for IM was obtained from the Ketoconazole incorporated media and 220-225 CFU with Amphotericin B amended treatments. Experiments carried out for the inhibition studies with the top layer (AB- the plain antibiotic disk) of the perfusion experimental set up, demonstrated no inhibition zone by the disks obtained from Ketoconazole and Amphotericin B amended solid media. However, interestingly, a zone of 15 -18mm was exhibited by the AB disks of active fraction 1 and active fraction 2 incorporated experiments.

4.4 Scanning Electron Microscopy: *C. albicans* is capable of growth as budding yeast (round or oval cells, also known as blastospores), as pseudo-hyphae (chains of polarized cells) or as true hyphae (multinucleated filamentous cells). The ultra structure of biofilms exhibited dense cell network of yeasts, hyphae and pseudo-hyphae. In case of Amphothericin B and Ketoconazole there is not much reduction in cell numbers. Moreover the cells are intact with clear morphology, where as in the case of active fraction 1 and 2 amendments, we observed greater quantity of cell debris in addition to few shrunken and broken cells (Figure 6).

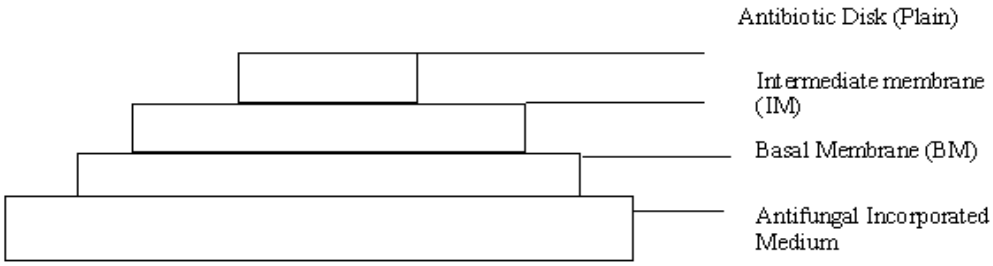

Figure 1: Experimental setup for biofilm penetration studies.

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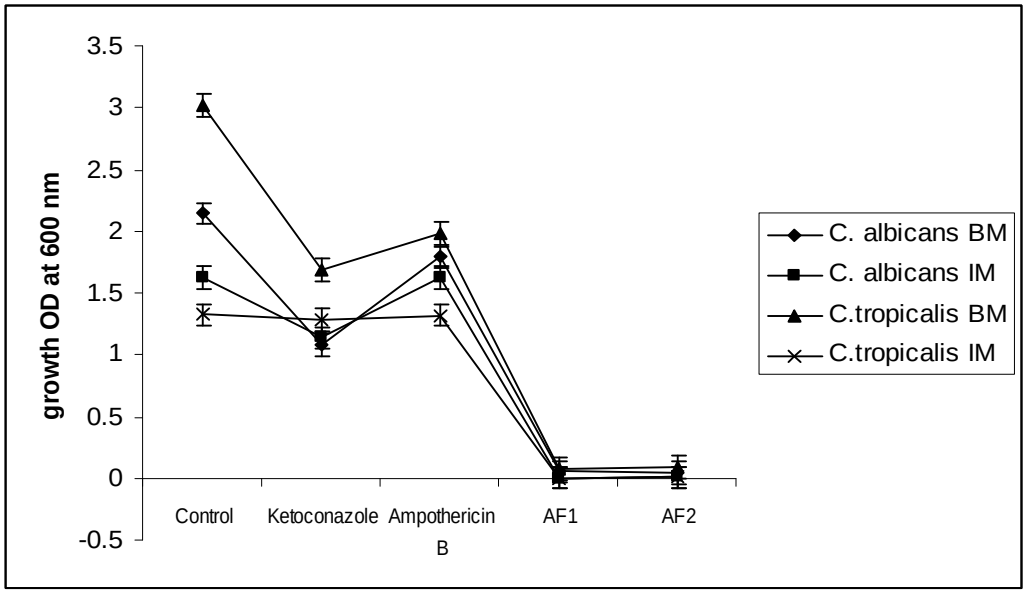

Figure 2: Growth of biofilms of different Candida sp. exposed to antifungal agents. Growth was determined by measuring optical density (OD) at 600 nm.

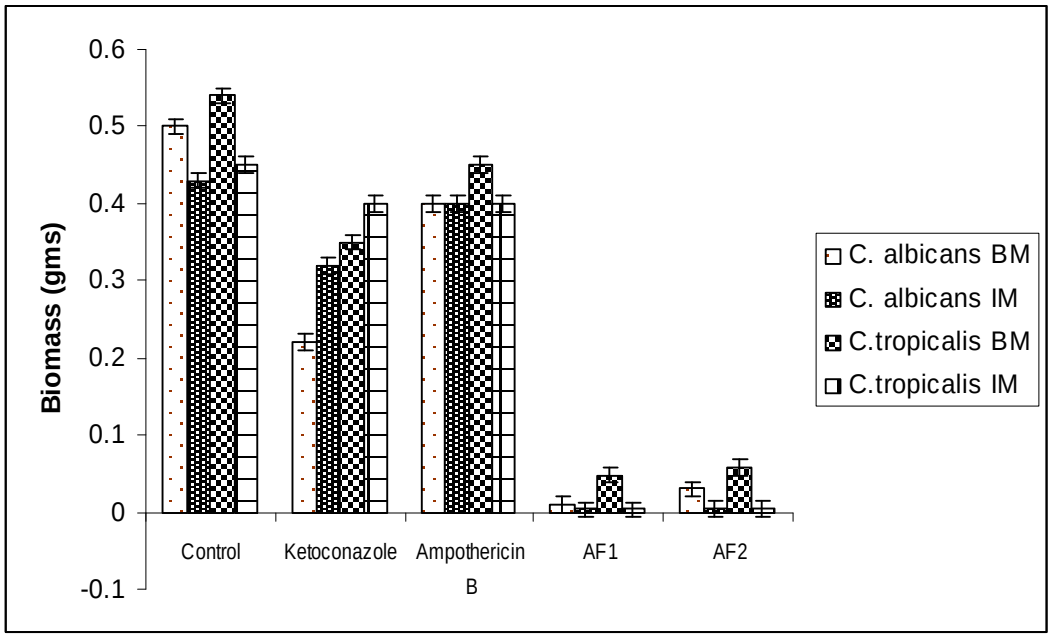

Figure 3: Growth of biofilms of different Candida sp. exposed to antifungal agents. Growth was determined by measuring biomass.

5 DISCUSSION

In nature, most of the microorganisms predominately exist as biofilms, which is the foundation for the multi cellular type of life. Biofilms are of special interest because of their unique potentiality of resisting antifungal drugs (by unidentified mechanisms) which poses serious management challenges. Candida biofilm development is closely associated with generation of extracellular matrix and the matured biofilm exhibit a highly heterogeneous structure that depends on

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the topography of the substrate. Baillie and Douglas (1998a) observed 20 times of MIC of commonly used drugs such as Amphothericin B and Fluconazole were required to cause significant changes in the biofilm. Amphothericin B is the gold standard of the drugs used to treat the fungal infection; however, its effect on the biofilms is not yet proved. Previous studies (Samaranayake *et al*., 2004; Subha & Gnanamani, 2008) showed the low penetrability of Amphothericin B through biofilm is because of the large molecular size and the hydrophobic nature. During development, the biofilms encase themselves in the matrix, which may restrict the penetration of drugs. According to Samarnayakae et al. (2004), biofilms are known to regulate diffusion of antibiotics.

In vitro methodology used in the present study exhibit several advantages, i.e. (i) flexibility to investigate the antifungal resistance using simultaneous and parallel samples; (ii) Accessibility to both sides of biofilm after its removal from the membrane surface and (iii) the possibility of using these biofilms as primitive models of pseudomembranous candidal infections. Previous studies (Hawser & Douglas, 1995; Ramage *et al*., 2001; Douglas, 2002; Ramage *et al*., 2002; Garcı´a-Sa´nchez *et al*., 2004; Mukherjee & Chandra, 2005) suggested that the above mentioned characteristics of biofilm explain the increased rate of reoccurrence of infection, and finally ends up with killing only the outermost layer of the biofilm and leaving the remaining layers unexposed to antifungal agents. Results obtained from the present study reveals that the chosen antifungal agents could not inhibit cell growth, cell viability and the metabolic activity of the cells in the biofilm. In addition, they are not able to penetrate through the biofilm and thus live cells thrive even in the intermediate membrane (IM). Further, the topmost layer of the plain antibiotic disc (AB) exhibited no growth inhibition in the lawn of indicator organism, which further authenticates that the agents did not penetrate. Though penetration of drugs through the biofilm cannot be the only parameter to be considered to determine antifungal efficacy, in the case of standard drugs a reduction in growth was observed, but complete death did not occur, possibly due to the non-perfusive nature of the drug.

In the case of the active fractions AF1 and AF2 of *A. calamus*, the fractions killed the cells and were able to perfuse through the biofilm of BM and IM and could reach the top antibiotic disk (AB) of BM-IM-AB assembly, as evidenced by the inhibition zone exhibited by the AB disks on the lawn of the indicator organism. Hence, it is beyond doubt that these fractions possess higher antifungal activity and also have the potency to perfuse through the biofilms of *C. albicans* and *C. tropicalis*.

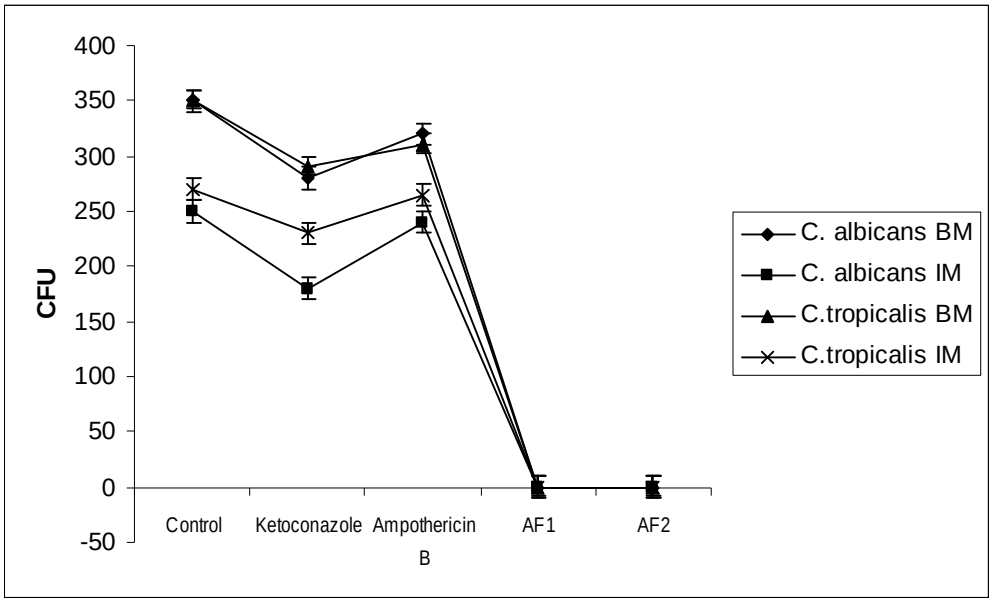

Figure 4: Growth of biofilms of different Candida sp. exposed to antifungal agents. Growth was determined by cell viability on Sabouraud's dextrose agar media.

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6 CONCLUSION

The resistance exhibited by the Candida biofilms is a challenge to continued use of the existing antifungal agents, more so because the mechanisms of biofilm resistance to these antimicrobial agents is not yet fully explained. Hence, there is continuous need for new drug discovery and formulation. In this study, the antifungal efficacy of two active fractions of *Acorus calamus* was well established. From the perfusion studies, it is evident that unlike the standard drugs, these active fractions were able to perfuse through biofilm layers leading to cell death. The one long-standing hypothesis for the resistance of bacterial biofilms is that the matrix material restricts drug penetration by forming a reaction-diffusion barrier and thus, it is a fact that only the surface layers of a biofilm are exposed to a lethal dose of antibiotics. Al Fattani and Douglas suggest that the Fungal biofilms, also possess the extra cellular matrix like bacteria which serves as cross barrier in limiting the antifungal drugs. Thus, future antifungal agents should be able to cross the barriers and seep through the biofilm matrix, destroying the biofilm layers and lysing the live cells in the bottom layer. This would reduce the frequency of recurrent infections. .

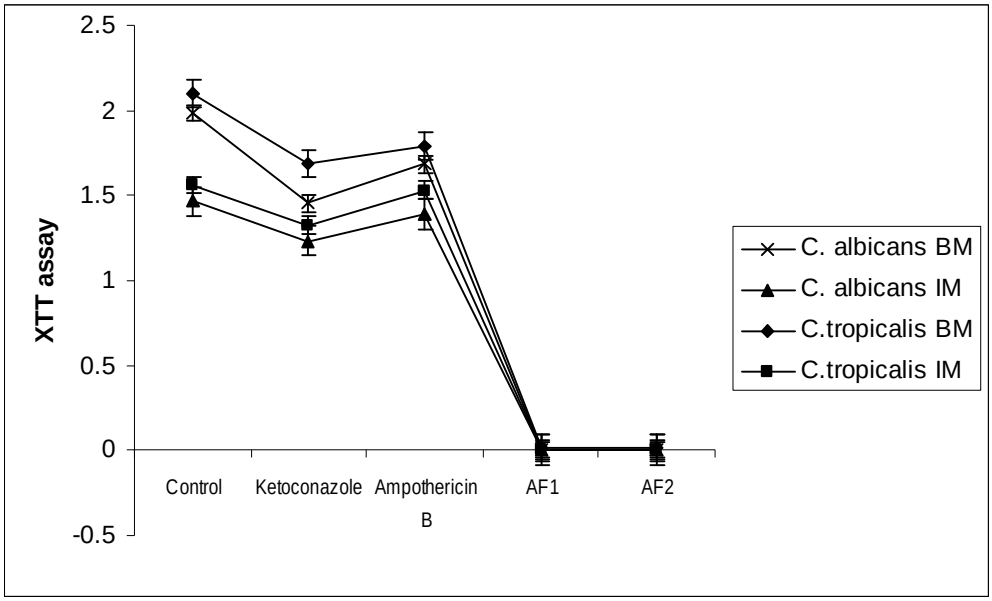

Figure 5: Growth of biofilms of different Candida sp. exposed to antifungal agents. Growth was determined by measuring metabolic activity.

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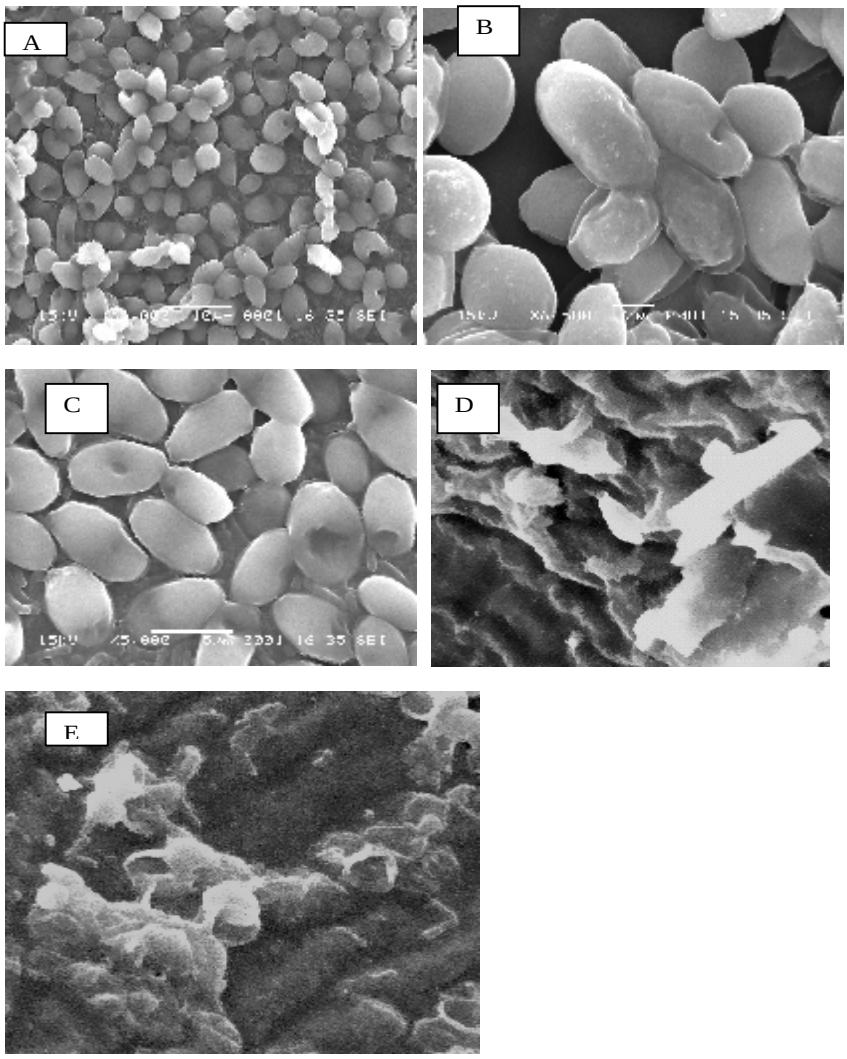

Figure 6: Scanning Electron Micrograph of biofilms of *Candida albicans* CLCA0510. A: Control; B: with Ketoconazole- treatment; C: with Amphothericin B - treatment, D and E are treated with active fractions of Acorus calamus (AF1 and AF2), respectively.

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