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INHIBITORY ACTION OF TOPICAL ANTIFUNGAL CREAMS AGAINST *Candida albicans* BIOFILM

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Abstract: Candidiasis is an infection caused by Candida fungi, especially *Candida albicans,* that generates high healthcare costs worldwide. The susceptibility of *C. albicans* biofilm towards existing topical antifungal creams need to be further investigated. This study evaluates the effects of econazole nitrate, miconazole nitrate, ketoconazole and tolnaftate-based antifungal creams against *C. albicans* biofilm. *C. albicans* ATCC MYA-2876 biofilm was developed in microplate assay in the absence and presence of antifungal creams. The concentration of all antifungal creams was evaluated in the range between 156.25µg/ml and 5000µg/ml. Biofilm biomass and biofilm viability were measured using crystal violet assay and resazurin assay respectively. Results demonstrated that all antifungal creams effectively inhibited *C. albicans* biofilm. Treatment with miconazole nitrate-based antifungal cream significantly ($p<0.05$) reduced biofilm biomass and biofilm viability at all test concentrations. Correlation between biofilm biomass and biofilm viability in the presence of miconazole nitrate-based antifungal cream was also found to be significant ($p<0.05$). The findings of the present study suggest that the most efficient agent against *C. albicans* biofilm is miconazole nitrate-based antifungal cream.

Keywords: *Candida albicans,* candidiasis, biofilm, antifungal cream.

Introduction

Candida species cause a wide spectrum of fungal infections (Hani *et al*., 2015). It can be found in the oral cavity, gastrointestinal tract, vagina and mucosal skin, and may lead to non-fatal illnesses and persistent infections in humans. *C. albicans* remains a major clinical problem causing infections among all of *Candida* species (Silva *et al.,* 2017). According to Madhavan *et al.* (2018), fungal diseases and infections are mainly caused by *C. albicans* biofilm formation, which is one of the notorious virulence factors of Candida. The role of biofilm formation as an important virulence factor of *C. albicans* has also been mentioned by other works (Mohandas & Ballal 2011; Nasution 2013)

Biofilm is an assembly of microorganisms which attached to biotic and abiotic surfaces (Hasan *et al.,* 2009). It is a heterogeneous microbial community that is surrounded by a self-produced extracellular polymeric matrix (Mahat *et al.,* 2012; Yaacob *et al.,* 2021). Essential proteins and changes in whole-cell proteome expression are known to associate with biofilm formation (Yahya *et al.,* 2017; Othman & Yahya 2019). The biofilm formed by *C. albicans* involves several stages of growth namely the formation of round budding cells, oval pseudohyphal cells and cylindrical hyphal cells that are surrounded by the extracellular matrix. Candida biofilm formation leads to high rates of morbidity and mortalitys especially in hospitalized patients (Serrano-fujarte *et al.,* 2015). The extracellular matrix of *C. albicans* mainly comprised of carbohydrates, polyssacharides, proteins, lipids and extracellular DNA (eDNA). It functions as a protective shield for microbial/ fungal cell growth and serves as a barrier against the host's immune cells. Furthermore, the extracellular matrix of *C. albicans* provides

the adhesive strength and stiffness that allow the microorganisms to aggregate and enhance the microcolonies formation (Lopez *et al.,* 2014; Nunes *et al.,* 2020). In recent years, the fungal biofilm has become more resistant due to the widespread use and misuse of antifungals. This causes the resistance of *C. albicans* towards antifungal agents, which become a threat to public health and contribute to treatment failure and persistent infection costing more than $2.6 billion annually in the United States alone (Wilson *et al.,* 2002; Nunes *et al.*, 2020). Regular assessment of antifungal efficacy needs considerable attention. The objective of this present study is to evaluate the effects of topical antifungal creams econazole nitrate, miconazole nitrate, ketoconazole and tolnaftate against *C. albicans* biofilm.

Material and Methods

Preparation of Test Microorganism

Candida albicans strain MYA-2876 was obtained from Microbiology Laboratory at Faculty of Applied Sciences, UiTM Shah Alam and was grown in Potato Dextrose Broth. The fungal suspension was adjusted to optical density (OD) of 0.7 at 600 nm.

Preparation of Topical Antifungal Creams

The topical antifungal creams in semi-solid form were purchased from pharmacy outlets. Their concentrations were prepared in the range between 156.25µg/ml and 5000µg/ml (Bojsen *et al*., 2014). All the topical antifungal creams were diluted by using sterile distilled water and stirred under low heat to fully dissolve the mixture (Alsterholm *et al.,* 2010).

Biofilm Formation in 96 wells-Microplate

C. albicans was grown in a 96-well microplate and incubated overnight at 37°C for 24 hours (Mahmoudabadi *et al.,* 2014). The wells of test groups were loaded with 150 µl of overnight fungal inoculum and 50 µl of diluted topical antifungal creams at different test concentrations. The wells of positive control were loaded with 150 µl of overnight fungal inoculum and 50 µl of intellectual property (IP)-protected antibiofilm cocktail. The wells of negative control were loaded with 200 µl of overnight fungal inoculum.

Crystal Violet Assay

Determination of biofilm biomass was performed as previously reported (Yahya *et al.,* 2018). After incubation for 24 hours, the growth medium was discarded and microplate was rinsed using sterile distilled water three times. Biofilm was heat-fixed for 30 minutes, stained with 0.5% w/v crystal violet (Sigma, USA) for 15 minutes and destained by using sterile distilled water (Guo *et al.,* 2019). A volume of 200 µl of absolute ethanol was added in the wells of microtiter plate to solubilize the stained biofilm (Sabaeifard *et al.,* 2014). The absorbance of biofilm biomass was quantified at 600 nm by using Epoch microplate reader (BioTek Instruments Inc., USA).

Resazurin Assay

Determination of biofilm viability was performed as previously reported (Yahya *et al.,* 2018). After 24 hours of incubation, the growth medium was discarded and the microplate was rinsed using sterile distilled water three times. A volume of 150 µl of phosphate buffer saline

Table 1: Active ingredient in each topical antifungal cream

Type of Topical Antifungal Creams	Active Ingredient
Topical antifungal cream A	Econazole nitrate
Topical antifungal cream B	Miconazole nitrate
Topical antifungal cream C	Ketoconazole
Topical antifungal cream D	Tolnaftate

and 50 µl of 0.02% resazurin solution (Sigma, USA) were added into the microplate wells. A color change from blue to pink was observed after incubation between 24 hours to 48 hours (Coban *et al.,* 2012). The suspensions in the wells of microtiter plate were measured at 570 nm by using Epoch microplate reader (BioTek Instruments Inc., USA).

Statistical Analysis

The data from triplicate crystal violet assay and resazurin assay were expressed as mean ± standard deviation. Independent T-test was used to analyze the significant difference between control and test groups with $p < 0.05$ considered as significant. The Pearson correlation coefficient test was used to determine the strength of association between all antimicrobial susceptibility profiles. The half-maximum inhibitory concentration (IC_{50}) values for the inhibition study of *C. albicans* biofilm were calculated using Quest Graph™ IC50 Calculator (AAT Bioquest 2019).

Results and Discussion

Biofilm Biomass

Figure 1 shows the biomass of *C. albicans* biofilm in the presence of topical antifungal creams. Treatment with econazole nitrate, miconazole nitrate, ketoconazole and tolnaftate-based antifungal creams inhibited the biomass of *C. albicans* biofilm. At all test concentrations of econazole nitrate, miconazole nitrate and ketoconazole-based antifungal creams, the biomass of *C. albicans* biofilm was significantly ($p<0.05$) inhibited.

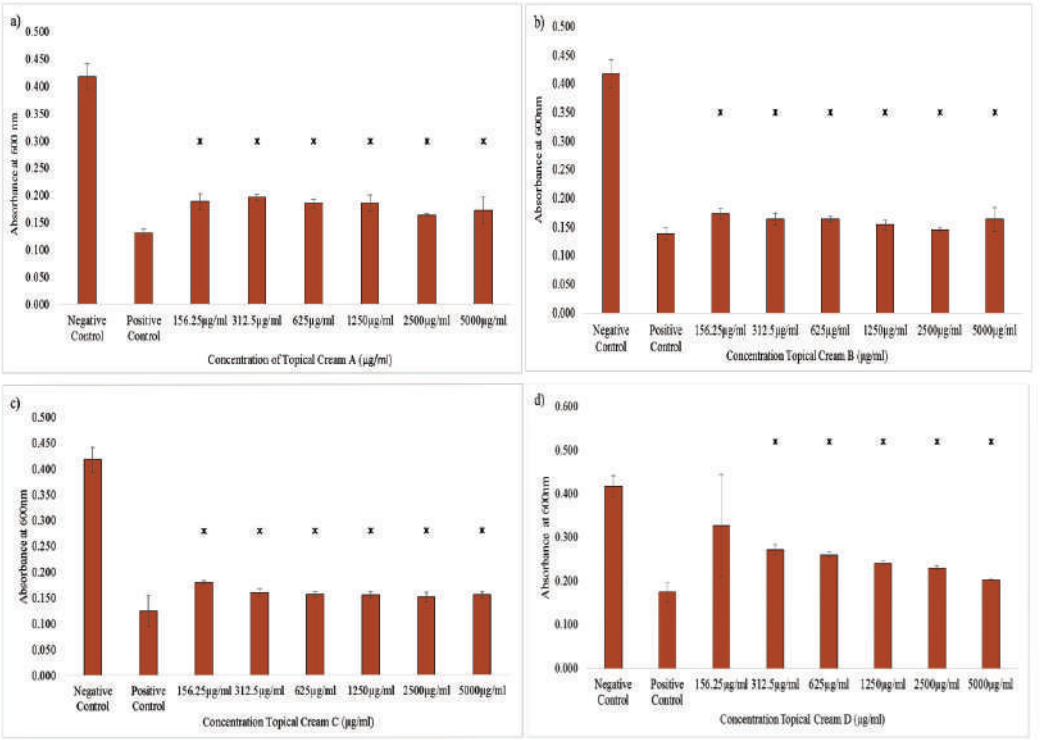

Figure 1: Effects of topical antifungal creams on the biomass of *C. albicans* biofilm. a) econazole nitrate-based topical antifungal cream; b) miconazole nitrate-based topical antifungal cream; c) ketoconazole-based topical antifungal cream; d) tolnaftate-based topical antifungal cream. Each bar represents mean ± standard deviation, n = 3. Positive control: fungal inoculum mixed with IP-protected antibiofilm cocktail. Negative control: fungal inoculum mixed with fresh broth. Significant differences ($p < 0.05$) when compared with negative control group are shown by *

Biofilm Viability

Figure 2 shows the viability of *C. albicans* biofilm in the presence of topical antifungal creams. Treatment with econazole nitrate, miconazole nitrate, ketoconazole and tolnaftate-based antifungal creams effectively inhibited the viability of *C. albicans* biofilm. At all test concentrations of miconazole nitrate-based antifungal cream, the viability of *C. albicans* biofilm was significantly ($p<0.05$) inhibited.

Correlations Between Biofilm Biomass and Biofilm Viability

Figure 3 shows the correlations between biomass and viability of *C. albicans* biofilm in the presence of topical antifungal creams. The correlation coefficient values of econazole nitrate, miconazole nitrate, ketoconazole and tolnaftate-based antifungal creams were 0.191, 0.766, 0.573 and 0.249 respectively. Only the correlation shown by *C. albicans* biofilm treated with miconazole nitrate-based antifungal creams was significant ($p<0.05$).

IC_{50} Values

Table 2 displays IC_{50} values of topical antifungal creams. The IC_{50} values shown by econazole nitrate, miconazole nitrate, ketoconazole and tolnaftate-based antifungal creams were 43.42 µg/ml, 118.26 µg/ml, 99.93 µg/ml and 10240 µg/ml respectively.

There are several methods available for antibiofilm screening. Crystal violet assay stains and quantifies the negatively charged molecules

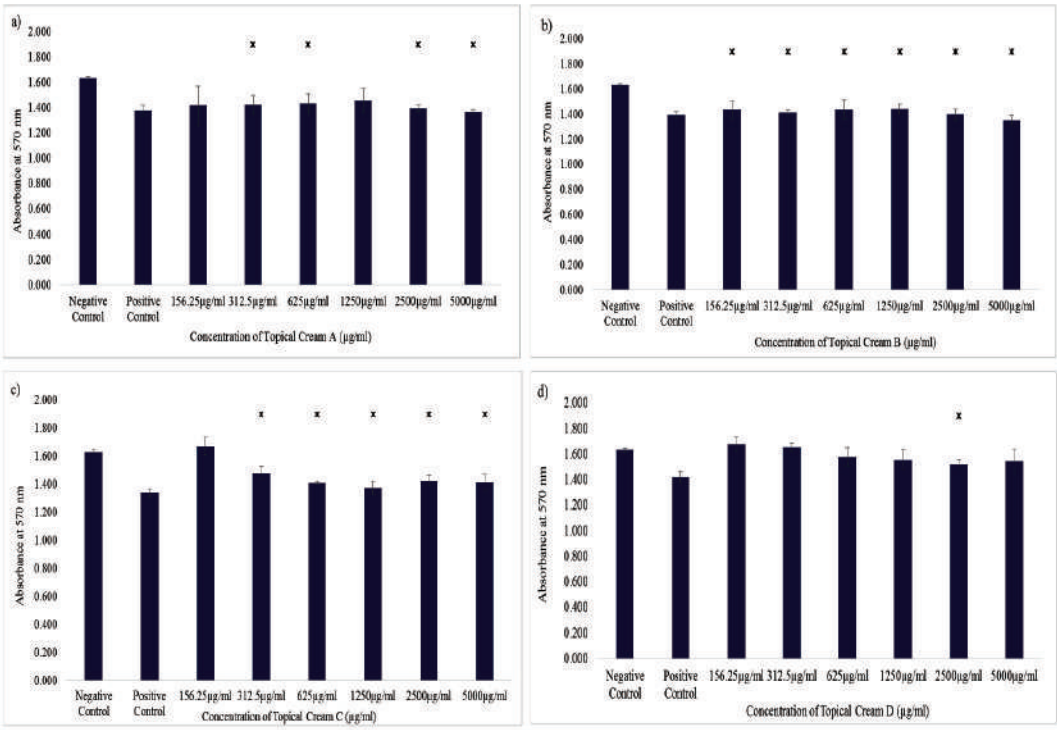

Figure 2: Effects of topical antifungal creams on the viability of *C. albicans* biofilm. a) econazole nitrate-based topical antifungal cream; b) miconazole nitrate-based topical antifungal cream; c) ketoconazole-based topical antifungal cream; d) tolnaftate-based topical antifungal cream. Each bar represents mean ± standard deviation, n = 3. Positive control: fungal inoculum mixed with IP-protected antibiofilm cocktail. Negative control: fungal inoculum mixed with fresh broth. Significant differences ($p < 0.05$) when compared with negative control group are shown by *

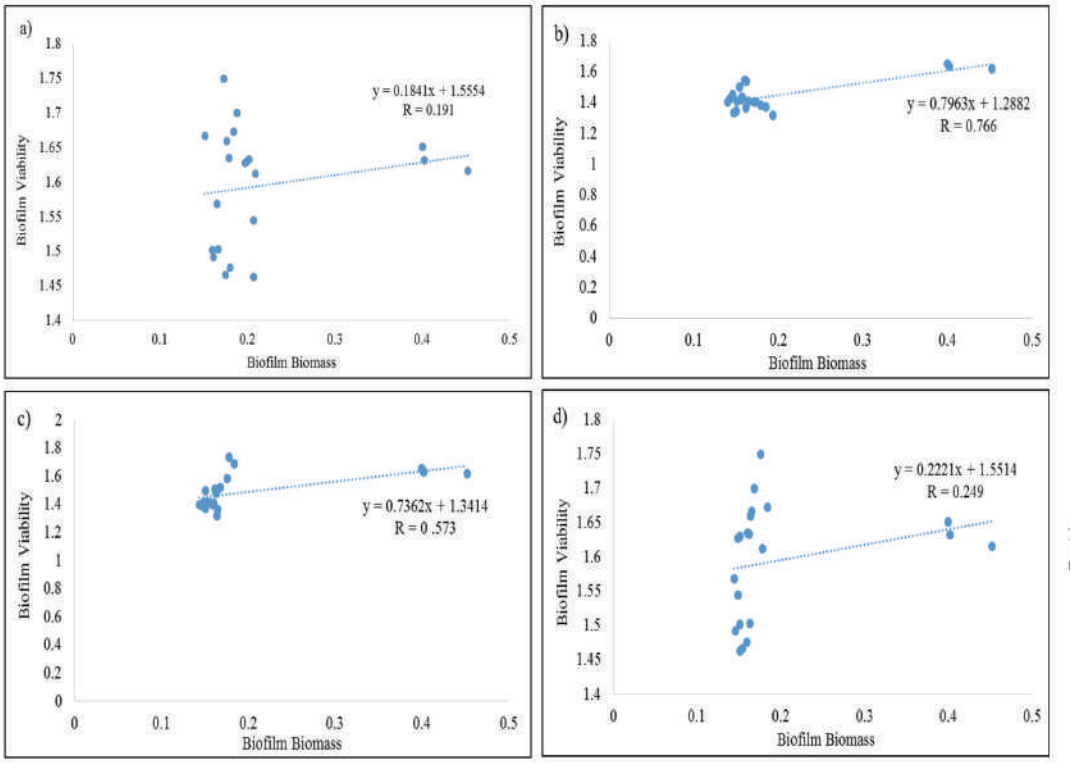

Figure 3: Correlations between biomass and viability of *C. albicans* biofilm in the presence of topical antifungal creams. a) econazole nitrate-based topical antifungal cream (coefficient correlation value: 0.191); b) miconazole nitrate-based topical antifungal cream (coefficient correlation value: 0.766); c) ketoconazole-based topical antifungal cream (coefficient correlation value: 0.573); d) tolnaftate-based topical antifungal cream (coefficient correlation value: 0.249)

Table 2: Half maximal inhibitory concentration (IC_{50}) values of topical antifungal creams

Antifungal Cream	**Active Compound**	**IC_{50}** (µg/ml)
A	Econazole nitrate	43.42
B	Miconazole nitrate	118.26
C	Ketoconazole	99.93
D	Tolnaftate	10240

within a biofilm, such as polysaccharides of the extracellular matrix, whilst resazurin assay measures the reduced form of resazurin namely pink and highly fluorescent resorufin. Both assay procedures are based on inexpensive reagents and have a simple absorbance endpoint reading. The present work used crystal violet assay and resazurin assay to determine biofilm biomass and biofilm viability respectively. Both methods are a straightforward, fast and effective method for primary antibiofilm screening (Skogman *et al.,* 2012).

Econazole nitrate is another imidazole derivative with fungistatic properties. It inhibits fungal cells by inhibiting the biosynthesis of ergosterol, thereby damaging the fungal cell wall membrane and increasing its permeability that leads to a substantial loss of essential

intracellular components. The present study demonstrated the efficacy of econazole nitrate-based antifungal cream against biomass and viability of *C. albicans* biofilm. The information on the inhibitory activity of econazole against fungal biofilm is still limited. However, econazole has been shown to inhibit the free floating form of *C. albicans* and also inhibit *Streptococcus mutans* biofilm formation (Qiu *et al.,* 2017).

Miconazole nitrate is an antifungal medication in the imidazole family and is commonly used to treat yeast infections of the skin and vagina. It is known to inhibit the fungal enzyme 14α-sterol demethylase, resulting in a reduced production of ergosterol. In the present study, miconazole nitrate-based antifungal cream was found to effectively inhibit biomass and viability of *C. albicans* biofilm. In 2010, Vandenbosch *et al.,* demonstrated significant reduction in viable cells and significant accumulation of reactive oxygen species in *C. albicans* biofilm following the treatment with miconazole.

Ketoconazole is a nonsteroidal imidazole that is used in both oral and topical forms. Similar to other azole antifungal agents, ketoconazole inhibits the enzyme cytochrome P450 14α-demethylase involved in the sterol biosynthesis pathway. Herein, ketoconazole-based antifungal cream inhibited biomass and viability of *C. albicans* biofilm. A similar finding has been reported by (Abd *et al.,* 2014). They demonstrated that treatment with ketoconazole reduced biofilm biomass of *C. albicans* by between 22% and 80.7%.

Tolnaftate is a synthetic thiocarbamate used as an antifungal agent. The molecular mechanism underlying its fungicidal action is not completely understood. However, it may inhibit squalene epoxidase, an important enzyme in the ergosterol biosynthetic pathway. In the present study, tolnaftate-based antifungal cream inhibited biomass and viability of *C. albicans* biofilm. Antifungal activity of tolnaftate has been elucidated since 1986 (Ryder *et al.,* 1986). However, to our knowledge, the present study provides the first evidence of the antibiofilm activity of tolnaftate against *C. albicans*.

Biomass, viability and extracellular matrix represent the important pathogenic characteristics of microbial biofilms causing a wide spectrum of disease. Insightful elucidation of these pathogenic characteristic of microbial biofilm can ease the biofilm control strategies. The present work showed a significant correlation between biomass and viability of *C. albicans* biofilm in the presence of miconazole nitrate-based antifungal cream. There is still a lack of data available on the correlation between antifungal susceptibility profiles. Nonetheless, Yahya *et al.* (2018) showed a significant correlation between biomass and extracellular matrix of bacterial biofilm in the presence of antimicrobial. The synergistic inhibition of multiple pathogenic characteristics contributes to successful biofilm control (Skogman *et al.,* 2012).

The half maximal inhibitory concentration (IC_{50}) is defined as a measure of the potency of a chemical substance to inhibit a specific biochemical function *in vitro*. This measure is commonly used in evaluation of pharmaceutical drugs that block a biochemical reaction by binding to and blocking the active site or the allosteric site on a receptor. The present study suggests that the best antibiofilm agent is the miconazole nitrate-based antifungal cream because i) it significantly ($p<0.05$) inhibited the biomass and viability of *C. albicans* biofilm at all test concentrations and ii) showed a low IC_{50} value. Emele *et al.* (2000) demonstrated that the antifungal activity of miconazole nitrate in patients with vaginal candidiasis was comparable to econazole but they did not investigate their inhibitory actions against Candida biofilm.

In the past decade, little information is available about the antibiofilm activities of antifungal creams used herein. Only miconazole nitrate-based and ketoconazole-based antifungal creams have been tested against *C. albicans* biofilm (Vandenbosch *et al.,* 2010; Abd *et al.,* 2014, Tits *et al.,* 2020). However, their inhibitory actions against both biomass and viability of *C.*

albicans biofilm are not addressed sufficiently. The present study provides the first evidence on i) the antibiofilm activities of econazole nitrate-based and tolnaftate-based antifungal creams against *C. albicans*, and ii) the synergistic inhibition of multiple pathogenic characteristics of *C. albicans* biofilm by miconazole nitrate-based antifungal creams.

Conclusion

The present study demonstrates that all the topical antifungal creams effectively inhibited the biomass and viability of *C. albicans* biofilm. Among all tested antifungal creams, miconazole nitrate-based antifungal creams may be the most efficient agent against *C. albicans* biofilm.

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