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N-acetylcysteine Inhibits and Eradicates *Candida albicans* Biofilms

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Abstract N-acetylcysteine (NAC) is used in the treatment of chronic bronchitis that attributed to its mucus dissolving properties. Its ability to reduce biofilm formed by different types of bacteria was proven previously in many studies. Therefore we examined its effect on *C. albicans* biofilms by testing its effect alone and in combination with ketoconazole using Tissue culture plate assay method (TCP). NAC effects on *C. albicans* morphology and the texture of biofilms were determined using Scanning electron microscope (SEM). It was found that the inhibitory effect of NAC was concentration dependent. NAC reduced *C. albicans* adherence by ≥32.8% while ketoconazole reduced adherence by ≥25% in comparison to control. Also, it showed higher disruptive effect (50-95%) than ketoconazole (22-80.7%) on mature biofilms. Using NAC and ketoconazole in combination, a significant inhibitory effect ($P<0.01$) on both adherence and mature biofilms (54-100%) was seen. NAC reduced the amount of biofilm mass in all tested *Candida* in concentrations at which their growth was not affected. NAC and ketoconazole combinations showed complete eradication to mature biofilms formed in most of the tested strains. NAC can inhibit *C. albicans* growth, inhibit dimorphism, which is an important step in biofilm formation, and change the texture of the formed biofilms, what makes NAC an interesting agent to be used as an inhibitor for biofilm formation by *C. albicans*.

Keywords: *antifungal, mature biofilm, mucolytics, adherence, SEM*

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1. Introduction

C*andida albicans* is the most prevalent candida species isolated from healthy and diseased individuals. Mycological studies have shown that *C. albicans* represents over 80% of isolates from all forms of human candidosis [1,2]. The transition of candida from a harmless commensal to a pathogenic organism is complex as it depends on both host and candidal factors [3]. Candida can undergo adherence and colonization to host surfaces or biomaterials surfaces used in medical devices as silicone and dental prosthesis as acrylic resin [4]. It can adhere by many adhesins to different types of human cells including phagocytic, epithelial and endothelial cells. Also, adhesion may be attributed to cell surface hydrophobicity. In addition, the hyphal formation is found to be significant in the pathogenicity of *C. albicans* and play an important role in the adherence process [2]. As Baille and Douglas [5] reported that yeast–hypha transition is necessary for full maturation of biofilm. Strains that are unable to grow as yeasts or to form hyphae can still form biofilm but are easily detachable.

Biofilms can develop on different surfaces on three stages: (i) attachment and colonization of yeast cells to surfaces that mediated by both nonspecific factors (cell surface hydrophobicity and electrostatic forces) and by specific adhesins on the fungal surface, such as serum proteins (fibrinogen and fibronectin) and salivary factors. Importantly, biofilm formation correlates with cell surface hydrophobicity; (ii) growth and proliferation of yeast cells for the formation of basal layer, and (iii) growth of pseudohyphae and the extensive production of extracellular matrix material. After maturation, detachment of yeast cells act as a foci for the spread of infection to other body sites [6,7]. On the other hand, biofilm forming cells show resistance against antimicrobials and host defense mechanisms which has a considerable impact on the treatment of biofilm-related infections [8,9].

Studying biofilms is increasing at a high and fast pace, particularly for bacterial biofilms, but being somewhat neglected for fungal biofilms. *C. albicans* biofilm share several properties with bacterial biofilms, including their structural heterogeneity, the presence of exopolymeric material and their decreased susceptibility to antimicrobials [10].

The current treatment options for fungal biofilm associated infections are not enough due to the increased tolerance of biofilms to antifungals. Resistance of *C. albicans* biofilms to the majority of antifungals were reported since the mid of 1990s [11]. Patients with devices containing fungal biofilms are rarely cured with mono-antifungal therapy and their devices need to be removed [12]. Some catheters as percutaneous vascular catheters

can be removed quickly while the removal of infected voice prostheses, heart valves, joint prostheses, central nervous system shunts and other implanted medical devices is problematic because these implants generally have a life-supportive function. So, successful treatments are urgently needed in clinical practice for retaining the implanted devices [13].

N-Acetyl-L-cysteine (NAC) is used in the treatment of chronic bronchitis, cancer, and paracetamol intoxication [14,15]. Also, it is thiol containing antioxidant. So, it may disrupt disulfide bonds in mucus inhibiting amino acid (cysteine) utilization [16,17]. In addition, it has antibacterial properties [18]. Many studies proved the anti-biofilm activity of NAC against bacterial biofilms [19,20]. So, we thought to study its effect on *Candida* biofilms. In this study we determined the effect of N-acetyl cysteine alone and in combination with ketoconazole on both adherence and preformed biofilms. Also we used scanning electron microscope to determine the morphological changes occurred for *C. albicans* cells and the changes in the intensity of biofilm formed on a surface model in the presence of the tested agents.

2. Methods

2.1. Microbial Strains

Ten *Candida albicans*, recently isolated from patients suffering from vaginal and oral infections were used in this study. The identification of organisms were based on the following: colony morphology, germ tube test, chlamydospores on Tween 80 cornmeal agar (Difco) and by the pattern of assimilation of a variety of carbon and nitrogen sources [21]. Biofilm production ability were tested using tissue culture plate assay (TCP) method [22].

2.2. Drugs

We have followed the guidelines of CLSI [23] for preparing of stock solution of ketoconazole (2 mg/ml) (Amriya, Egypt). Two mg of ketoconazole was dissolved in 1 mL of methanol according to manufacturer's instructions. Working solution concentrations were ranged from 0.025-12.8 µg/ml. For N-acetylcysteine (Sedico, Egypt), stock solution of 40 mg/ml was prepared by dissolving 4 gm of NAC in 100 ml of water. Working solution concentrations were ranged from 40-0.3125 mg/ml.

2.3. Determination of Minimum Inhibitory Concentrations (MIC) of Ketoconazole

Minimum inhibitory concentrations of ketoconazole were determined by agar dilution method [23].

2.4. Determination of Minimum Inhibitory Concentrations (MIC) of N-acetyl Cysteine

Microorganisms (0.5 ml) of 1.5×10^6 CFU/ml (0.5 Mcfarland turbidity) were plated in sterile petri dishes then 20 ml of sterile, molten and cooled (45°C) Muller Hinton agar media with the addition of methylene blue-glucose (to enhance zone diameter visualization) was added to all petri dishes. The plates then were rotated slowly to ensure uniform distribution of the microorganisms and then allowed to solidify on a flat surface. After solidification, four equidistant and circular wells of 10 mm diameter were carefully punched using a sterile cork bore.

Two fold serial dilutions were performed on the tested N-acetyl cysteine. Equal volumes of each dilution were applied separately to each well in three replicates using a micropipette. All plates were incubated overnight at 37°C, then collected and zones of inhibition that developed were measured. The average of the zones of inhibition was calculated. The minimum inhibitory concentration (MIC) was calculated by plotting the natural logarithm of the concentration of each dilution against the square of zones of inhibition. A regression line was drawn through the points. The antilogarithm of the intercept on the logarithm of concentration axis gave the MIC value [24].

2.5. Effect of the Tested Agents (ketoconazole and NAC) Each alone and in Combination on the Adherence and the Preformed Biofilm of *Candida albicans* on Plastic Surfaces

The TCP assay is most widely used and was considered as a standard test for the detection of biofilm formation [22]. Different materials have been used for the growth of biofilms *in-vitro*, including polystyrene, polyvinyl chloride, silicone elastomer and polymethyl methacrylate [25]. Although polystyrene is not used in the manufacture of indwelling medical device, it is widely used for testing biofilm production as it is considered as an excellent material for promoting adherence of cells [26]. So, a standardized method for biofilm formation based on polystyrene 96-well plates has been established [27].

2.5.1. Preparation of Inoculums

All strains were first streaked onto YEPD agar (1% yeast extract, 2% Bacto peptone, 2% D-glucose, 1.5% agar) then, incubated at 25°C for 48 h. A large loop of actively growing cells (for each strain) was transferred to sterile trypticase soy broth (TSB) (Difco Laboratories) containing 0.9% D-glucose. After incubation at 25°C for 24 h, the cells were centrifuged and washed twice with 0.5 ml PBS (phosphate buffer saline), followed by vortexing and centrifugation at 5000 *g* for 5 min. The washed cells were suspended in 1 ml TSB broth and adjusted to a final OD600 nm value of 1.0 with TSB broth. These cell suspensions were then used to grow biofilms.

2.5.2. For Testing the Effect of Ketoconazole and N-Acetylcysteine on the Adherence of EACH STRAIN

100 µl of the suspension (OD600) was inoculated into individual wells of polystyrene 96-well plates (flat bottom; Nunc). TSB broth was used as a negative control. The plates were incubated at 25°C for 90 min (adhesion period). Supernatants including planktonic cells were discarded and wells were gently washed with PBS twice to remove any non-adherent cells. 100 µl of fresh TSB broth containing one of the following solutions: ketoconazole (MIC and 2x MIC), N-acetylcysteine (4 and 8 mg/ml) and Ketoconazole/N-acetylcysteine (MIC/4 mg/ml and 2x MIC /8 mg/ml) was added to each well. The plates were covered to prevent evaporation and incubated

at 25°C for 24 h. Liquid media containing the non adherent cells were discarded through two rounds of washing with 200 µl sterile PBS buffer. Adherent cells to the plastic surfaces were quantified using Crystal violet assay [28]. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard error was calculated.

2.5.3. The Effect of the Tested Agents on the Preformed Biofilms

100 µl of the suspension (OD600) was inoculated into individual wells of polystyrene 96-well plates (flat bottom; Nunc). The plates were incubated at 25°C for 48 h. After the incubation period, the supernatants from each well were aspirated and the wells washed twice with PBS without disturbing the biofilms at the bottom of the wells, then one of the following solutions: ketoconazole (MIC and 2xMIC), N-acetylcysteine (4 and 8 mg/ml) and Ketoconazole / N-acetylcysteine (MIC/4 mg/ml and 2xMIC /8 mg/ml) was added to each well. Normal saline without any agents was added to the control wells. The plates were incubated at 25°C for 24 h. Supernatants were discarded through two rounds of washing with 200 µl sterile PBS buffer. Cells adherent to the plastic surfaces were quantified using Crystal violet assay. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard error was calculated.

2.6. Scanning Electron Microscopy (SEM)

Polyurethane segments were used as a surface for testing the effect of the tested agents on biofilm formed by *C. albicans*. Polyurethane segments (1 cm length) were incubated in 5 ml of Trypticase soy broth (BBL, USA) that contained 5×10^6 cfu/ml of *C. albicans* for 90 min. To test the effect of the tested agents on fungal adherence to the polyurethane surfaces, One of the following solutions: Ketoconazole (MIC and 2x MIC), N-acetylcysteine (4 and 8 mg/ml) and ketoconazole/NAC (MIC/2 mg/ml and 2MIC/4 mg/ml) were added to each tube, normal saline was added to control tubes and incubated at 25°C for 24 h.

To test the effect of the tested agents on the preformed mature biofilms, polyurethane segments were incubated with *C. albicans* cultures at 25°C for 48 h. After incubation, stents washed twice with normal saline without disturbing the biofilms, Then placed in new test tubes containing TSB medium with Ketoconazole (MIC and 2x MIC), N-acetylcysteine (4 and 8 mg/ml) and Ketoconazole/NAC (MIC/2 mg/ml and 2MIC/4 mg/ml). Normal saline was added to control tubes and incubated for 24 h.

2.6.1. Scanning Electron microscope Examination

Polyurethane segments were fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco PBS (PH 7.2) for 1.5 h, rinsed with PBS, and then dehydrated through an ethanol series. Samples were dried and gold-palladium coated. SEM examinations were made on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan) (Soboh *et al.*, 1995).

2.7. Statistical Analysis

One-Way ANOVA as employed to evaluate any significant difference between the values obtained without the drug (controls) and the values obtained in the presence of different drug concentrations. Differences were done using SPSS version19 (SPSS Inc., Chicago, IL) for windows was used. Descriptive statistics were calculated. Kruskal-Wallis test was used to compare groups. A significant *P*-value was considered when it was less than 0.05.

3. Results

One hundred thirty-eight samples were collected from patients suffering from oral thrush and vaginities. Twenty three samples (16.7%) were positive for *Candida albicans.* As out of 99 vaginal swabs and 39 oropharyngeal swabs, 14 (14%) and 9 (23%) were positive for *Candida albicans*, respectively. Out of 23 *Candida albicans* isolates, 10 (43.5%) biofilm-producing strains were identified (7 from vaginal swabs and 3 from oropharyngeal swabs) (Figure 1). By examining polyurethane segments using SEM, It was found that *Candida* biofilm contains both the yeast and the hyphal forms (Figure 2).

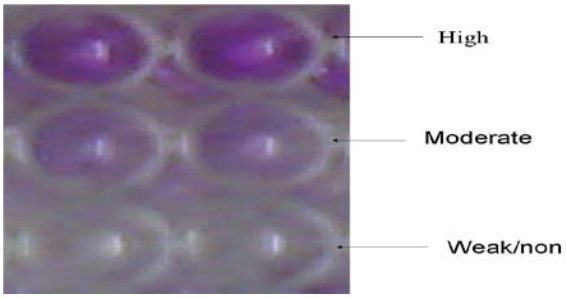
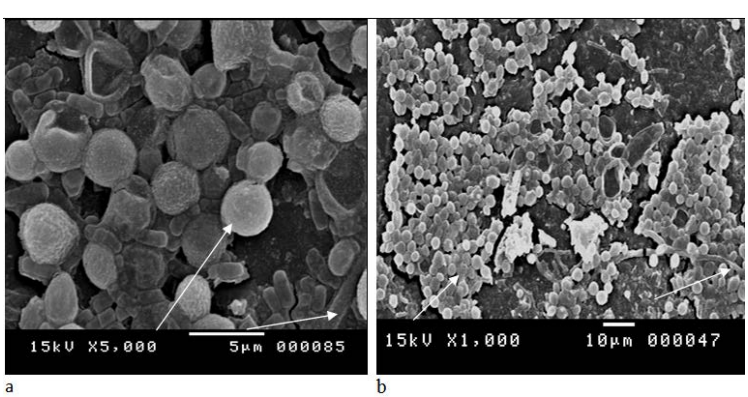


Figure 1. Screening of the extent of biofilm production by Tissue culture plate method (TCP): high, moderate and non slime producers differentiated with crystal violet staining in 96 well tissue culture plate



a b

Figure 2. SEM images of a mature (48 h) *C. albicans* biofilm. Arrows refers to *Candida* cells and hyphal and pseudohyphal elements in both images (a) 5,000X, *Bar represents 5µm* (b) 1,000X, *Bar represents 10µm*

3.2. Minimum Inhibitory Concentrations of Ketoconazole and NAC

All the tested *C. albicans* strains were sensitive to ketoconazole (9 strains showed MIC of ketoconazole of 0.4 µg/ml while one had MIC of 0.8 µg/ml). Nine strains showed MIC of NAC of 20 mg/ml but one showed MIC of 16.5 mg/ml.

3.3. The Effect of Ketoconazole and N-Acetylcysteine on *C. albicans* Adherence

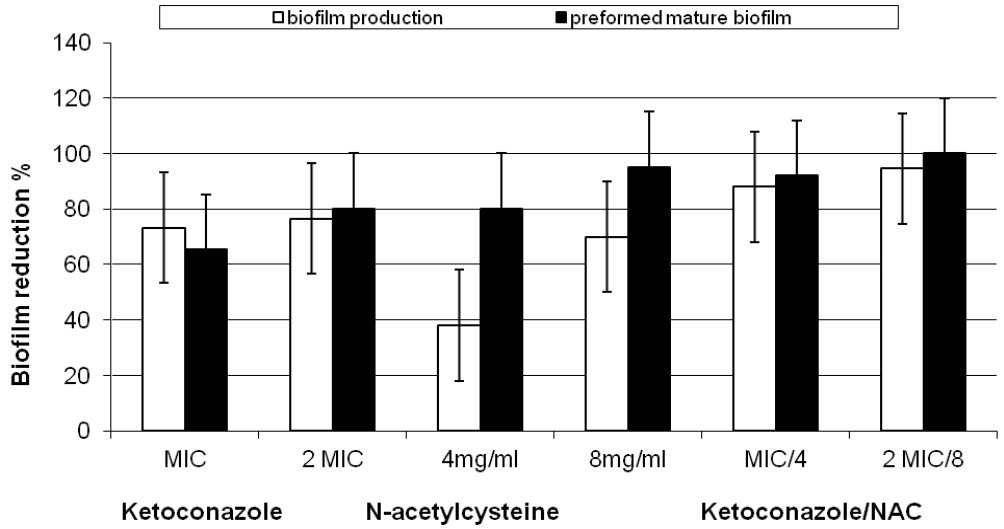


Figure 3. Effect of ketoconazole and N-acetylcysteine each alone and in combination on biofilm production and preformed biofilm formed by *C. albicans* strains. Most of the tested concentrations had higher inhibitory effect on preformed biofilm than biofilm production (adherence)

Table 1. Effects of ketoconazole and N-acetylcysteine each alone on biofilm production (adherence) by the tested *Candida albicans*

Candida albicans	Ketoconazole			N-acetyle cystiene		
	Conc. (µg/ml)	Mean±S.E	% OF reduction	Conc. (mg/ml)	Mean±S.E	% OF reduction
	CTR	0.128±0.00				
	1 [a]	0.048±0.002*	62.50%	4	0.086±0.002*	32.80%
1	2 [b]	0.040±0.002*	68.75%	8	0.038±0.002*	70.30%
	CTR	0.150±0.002				
	1 [a]	0.040±0.001*	73.30%	4	0.093±0.001*	38%
2	2 [b]	0.035±0.001*	76.60%	8	0.045±0.001*	70%
	CTR	0.247±0.000				
	1 [a]	0.035±0.458*	85.80%	4	0.034±0.003*	86.25%
3	2 [b]	0.031±0.001*	87.40%	8	0.030±0.001*	87.80%
	CTR	0.134±0.003				
	1 [a]	0.083±0.001*	38.05%	4	0.055±0.005*	58.90%
4	2 [b]	0.065±0.001*	51.40%	8	0.025±0.006*	81.30%
	CTR	0.152±0.002				
	1 [a]	0.040±0.001*	73.60%	4	0.093±0.001*	38.80%
5	2 [b]	0.035±0.001*	76.90%	8	0.045±0.001*	70.30%
	CTR	0.137±0.002				
	1 [a]	0.092±0.002*	32.80%	4	0.064±0.005*	53.2%
6	2 [b]	0.065±0.001*	52.50%	8	0.037±0.002*	72.90%
	CTR	0.155±0.000				
	1 [a]	0.092±0.003*	40.60%	4	0.092±0.001*	40.60%
7	2 [b]	0.065±0.002*	58.06%	8	0.055±0.000*	64.50%
	CTR	0.124±0.000				
	1 [a]	0.093±0.003*	25%	4	0.083±0.001*	33.06%
8	2 [b]	0.079±0.002*	36.20%	8	0.070±0.000*	43.50%
	CTR	0.125±0.000				
	1 [a]	0.085±0.002*	32%	4	0.062±0.001*	50.40%
9	2 [b]	0.071±0.000*	43.20%	8	0.053±0.003*	57.60%
	CTR	0.135±0.000				
	1 [a]	0.085±0.002*	40%	4	0.062±0.001*	54.07%
10	2 [b]	0.071±0.001*	47.40%	8	0.053±0.003*	60.70%

CTR: without drug (control).
a: At MIC. b: At 2 MIC. * P<0.05: Significant value, compared to controls.
** P<0.01: Significant value, compared to controls, Ketoconazole group and NAC group.
Mean±S.E.M= *Mean values ± Standard error of means of 3 experiment.*

Table 2. Effects of ketoconazole and N-acetylcysteine combinations on biofilm production (adherence) by the tested *Candida albicans*

Candida albicans	Ketoconazole/N-acetyle systiene		
	Conc.	Mean±S.E	% of reduction
1	CTR	0.128±0.00	
	1\4[a]	0.042±0.001**	67.10%
	2\8[b]	0.016±0.001**	87.50%
2	CTR	0.150±0.002	
	1\4 [a]	0.018±0.001**	88%
	2\8 [b]	0.008±0.003**	94.60%
3	CTR	0.247±0.000	
	1\4 [a]	0.009±0.001**	96.30%
	2\8 [b]	0.000±0.000**	100%
4	CTR	0.134±0.003	
	1\4 [a]	0.025±0.001**	81.30%
	2\8 [b]	0.002±0.006**	94.70%
5	CTR	0.152±0.002	
	1\4 [a]	0.018±0.001**	88.10%
	2\8 [b]	0.008±0.003**	94.70%
6	CTR	0.137±0.002	
	1\4 [a]	0.052±0.003**	62.04%
	2\8 [b]	0.042±0.002**	69.30%
7	CTR	0.155±0.000	
	1\4 [a]	0.041±0.001**	73.50%
	2\8 [b]	0.037±0.002**	76.10%
8	CTR	0.124±0.000	
	1\4 [a]	0.053±0.001**	57.20%
	2\8 [b]	0.043±0.001**	65.30%
9	CTR	0.125±0.000	
	1\4 [a]	0.042±0.001**	65.80%
	2\8 [b]	0.033±0.000**	73.60%
10	CTR	0.135±0.000	
	1\4 [a]	0.042±0.001**	68.80%
	2\8 [b]	0.033±0.000**	75.50%

CTR: without drug (control).
a: MIC/2 mg/ml; b: 2X MIC/4 mg/ml; * P<0.05: Significant value, compared to controls.
** P<0.01: Significant value, compared to controls, Ketoconazole group and NAC group. Mean±S.E.M= *Mean values ± Standard error of means of 3 experiment.*

The inhibitory effects of ketoconazole and N-acetylcysteine were found to be concentration dependent. Ketoconazole at MIC inhibited biofilm synthesis by ≥ 25%. At 2xMIC, reduction of biofilm synthesis was ≥36.2%. In this study we used two concentrations that were lower than MICs of NAC to test its effect on the adherence and mature biofilms of tested strains without affecting their growth. N-acetylcysteine showed a significant inhibitory effect ($p<0.05$) on adherence and more than that observed by ketoconazole. As reduction of biofilm synthesis was ≥32.8% in a concentration of 4 mg/ml, at 8 mg/ml, biofilm synthesis was reduced by ≥43.5% in all tested strains. Ketoconazole/N-acetylcysteine combinations were found to have the highest inhibitory effect on adherence ($p<0.01$) (54-100%) in comparison to controls (Table 1 and Table 2) (Figure 3)

3.4. Disruption of Preformed Mature Biofilms

Ketoconazole and N-acetylcysteine were found to have significant inhibitory effects ($p<0.05$) on preformed mature biofilm. Results showed that N-acetylcysteine has a higher disruptive effect on mature biofilms than ketoconazole. As N-acetylcysteine showed a reduction in the optical density that ranged from 50 to 95.2% while ketoconazole showed a reduction ranged from 22 to 80.7% in comparison to controls. On the other hand, ketoconazole combined to N-acetylcysteine showed the highest ability to disrupt preformed biofilms ($p<0.01$) (54.07-100%, in comparison to controls) (Table 3 and Table 4) (Figure 3 and Figure 4). Our results revealed that ketoconazole/N-acetylcysteine combination showed the highest ability to reduce biofilm production and to disrupt preformed mature biofilms.

Table 3. Effects of ketoconazole and N-acetylcysteine each alone on preformed biofilm by the tested *Candida albicans*

Candida albicans	Ketoconazole			N-acetyle cystiene		
	Conc.(µg/ml)	Mean±S.E	% of reduction	Conc.(mg/ml)	Mean±S.E	% of reduction
1	CTR	0.128±0.000				
	1[a]	0.049±0.003*	61.70%	4	0.033±0.001*	74.20%
	2[b]	0.030±0.000*	76.50%	8	0.030±0.000*	76.50%
2	CTR	0.150±0.000				
	1 [a]	0.052±0.001*	65.30%	4	0.030±0.000*	80%
	2 [b]	0.030±0.000*	80.00%	8	0.008±0.000*	95%
3	CTR	0.247±0.000				
	1 [a]	0.084±0.000*	65.90%	4	0.038±0.001*	84.60%
	2 [b]	0.048±0.000*	80.50%	8	0.018±0.000*	77.32%
4	CTR	0.134±0.001				
	1 [a]	0.085±0.000*	36.50%	4	0.020±0.000*	85.07%
	2 [b]	0.065±0.000*	51.40%	8	0.015±0.000*	88.80%
5	CTR	0.152±0.000				
	1 [a]	0.102±0.001*	32.80%	4	0.022±0.000*	85.50%
	2 [b]	0.081±0.065	46.70%	8	0.018±0.000*	88.10%
6	CTR	0.137±0.000				
	1 [a]	0.098±0.000	28.40%	4	0.044±0.001*	67.80%
	2 [b]	0.048±0.000*	64.90%	8	0.033±0.03*	76.60%
7	CTR	0.155±0.001				
	1 [a]	0.082±0.000*	47.09%	4	0.045±0.000*	70.90%
	2 [b]	0.043±0.000*	72.20%	8	0.029±0.000*	81.20%
8	CTR	0.124±0.000				
	1 [a]	0.084±0.011	32%	4	0.062±0.000	50.00%
	2 [b]	0.061±0.000*	50.80%	8	0.034±0.000*	72.50%
9	CTR	0.125±0.000				
	1 [a]	0.097±0.000	22%	4	0.048±0.030*	61.60%
	2 [b]	0.031±0.000*	75.20%	8	0.006±0.000*	95.20%
10	CTR	0.135±0.000				
	1 [a]	0.027±0.000*	80%	4	0.037±0.004*	72.50%
	2 [b]	0.026±0.000*	80.70%	8	0.025±0.000*	81.40%

CTR: without drug (control).
a: At MIC. b: At 2 MIC. * P<0.05: Significant value, compared to controls.
** P<0.01: Significant value, compared to controls, Ketoconazole group and NAC group. Mean±S.E.M=*Mean values±Standard error of means of 3 experiment*

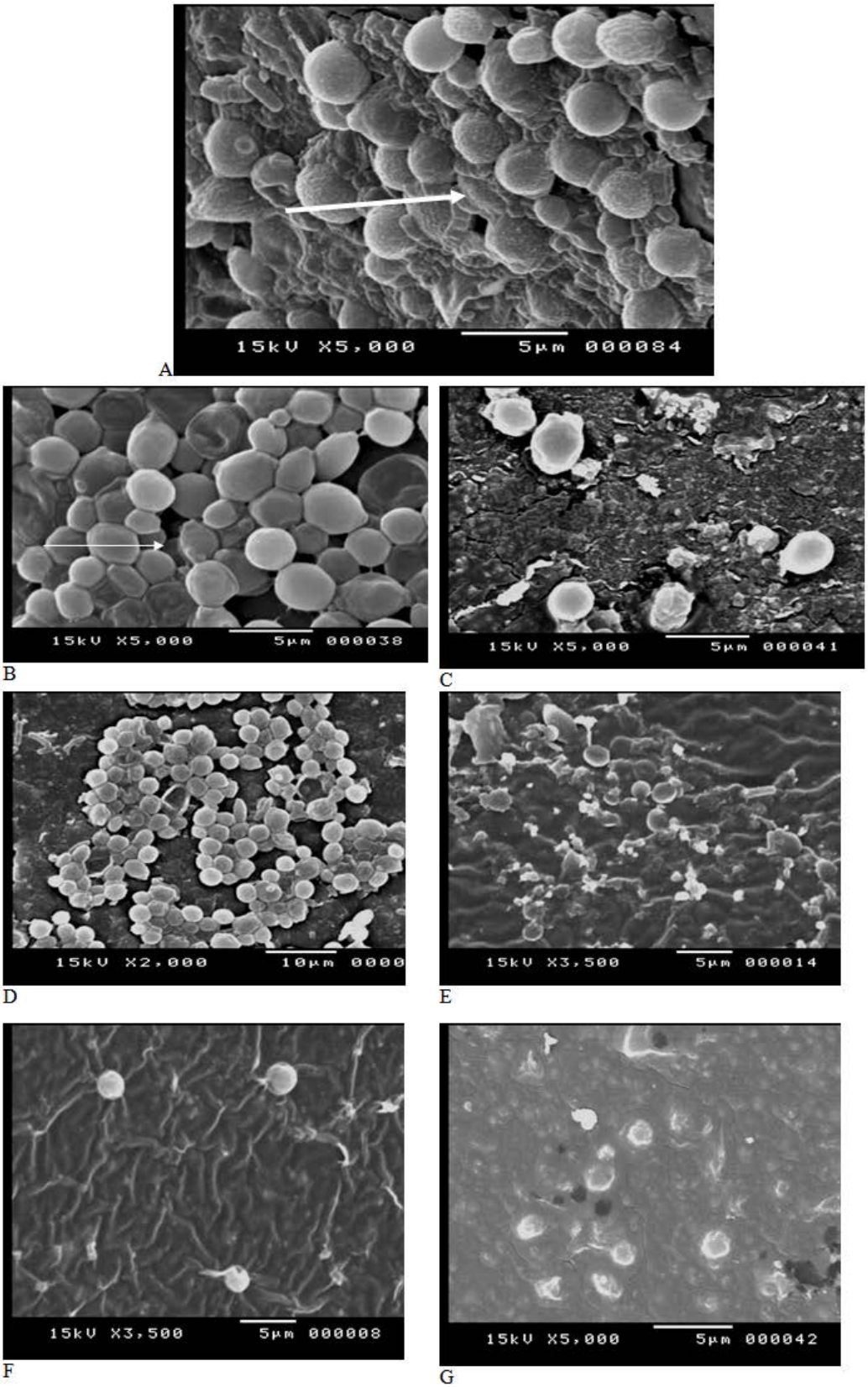


Figure 4. Scanning electron micrographs showing the effect of Ketoconazole, N-acetylcysteine each alone and in combination on a performed *C. albicans* biofilm developed in vitro on a polyurethane segment

A. *Candida albicans* with a high biofilm mass (Cotton like mass) on the surface of a polyurethane stent incubated with *Candida albicans* suspension for 48 h (control).
B: The effect of N-acetylcysteine (4 mg/ml) on *C. albicans* biofilm. Cotton like mass was highly decreased and cells appeared swollen with disrupted membranes.
C. The effect of N-acetylcysteine (8 mg/ml) on *C. albicans* biofilm. Cells appeared scattered with disrupted membranes and no biofilm mass.
D. *C. albicans* biofilm exposed to Ketoconazole at MIC concentration. Cells with irregular membranes. A decrease in the amount of biofilm mass.
E. Effect of Ketoconazole at 2X MIC. Cells appeared swollen, scattered. No biofilm mass observed.
F. Effect of Ketoconazole-N-acetylcysteine combination (MIC/4 mg/ml). No biofilm mass
G. Effect of Ketoconazole/NAC combination (2 X MIC/8mg/ml) on *C. albicans* biofilm. No biofilm mass or cells observed.

SEM showed the disappearance of the hyphael forms which are essential elements for the integrity and the formation of highly structured mature/fully developed biofilms and the presence of the yeast form (Figure 4). Also, Figure 4 showed the effect of the tested agents on cells morphology and the texture of biofilm mass. SEM images showed that the decrease in biofilm mass was observed at concentrations of 4 and 8 mg/ml (lower than MICs of NAC) that didn't affect *C. albicans* growth.

Table 4. Effects of ketoconazole and N-acetylcysteine combinations on preformed biofilm by the tested *Candida albicans*

Candida albicans	Ketoconazole/N-acetyle systiene		
	Conc.	Mean±S.E	% of reduction
1	CTR	0.128±0.000	
	1\4[a]	0.013±0.001**	89.80%
	2\8[b]	0.008±0.000**	93.70%
2	CTR	0.150±0.000	
	1\4 [a]	0.012±0.000**	92%
	2\8 [b]	0.000±0.000**	100%
3	CTR	0.247±0.000	
	1\4 [a]	0.069±0.05**	72.06%
	2\8 [b]	0.037±0.06**	92.70%
4	CTR	0.134±0.001	
	1\4 [a]	0.046±0.000**	65.60%
	2\8 [b]	0.043±0.000**	67.90%
5	CTR	0.152±0.000	
	1\4 [a]	0.042±0.001**	72.30%
	2\8 [b]	0.040±0.001	73.60%
6	CTR	0.137±0.000	
	1\4 [a]	0.012±0.057	91.20%
	2\8 [b]	0.007±0.000**	94.80%
7	CTR	0.155±0.001	
	1\4 [a]	0.042±0.000**	72.90%
	2\8 [b]	0.038±0.000**	75.40%
8	CTR	0.124±0.000	
	1\4 [a]	0.031±0.001**	75.00%
	2\8 [b]	0.025±0.000**	79.80%
9	CTR	0.125±0.000	
	1\4 [a]	0.018±0.000**	85.60%
	2\8 [b]	0.009±0.004	92.80%
10	CTR	0.135±0.000	
	1\4 [a]	0.062±0.006**	54.07%
	2\8 [b]	0.055±0.001**	59.20%

CTR: without drug (control).
a: MIC/2 mg/ml; b: 2X MIC/4 mg/ml; * P<0.05: Significant value, compared to controls.
** P<0.01: Significant value, compared to controls, Ketoconazole group and NAC group.. Mean±S.E.M= *Mean values ± Standard error of means of 3 experiment.*

4. Discussion

Surface-associated *Candida* can grow incorporated in extracellular matrix which consists of carbohydrates, proteins and some unknown components, known as a biofilm. Biofilms can be formed readily on biotic (mucous membranes) and abiotic surfaces (Foley catheters and intrauterine devices (IUDs)), that render the embedded *Candida* isolates resistant to antifungal agents, especially azoles and promote persistence of fungal infections [30]. The ability of *C. albicans* to adhere to host mucosal surfaces is a prerequisite for subsequent biofilm formation and colonization of the host mucosal surfaces such as buccal and vaginal mucosa [31].

Many studies have shown that Candida biofilm development is associated with the generation of an extracellular matrix and that mature biofilms show a highly heterogeneous structure and grow variably depending on the topography of the substrate [25,32]. Scanning electron microscope examination of biofilms revealed the presence of both adherent yeast form and invasive hyphal form cells forming the basal and upper layers, respectively. Both yeast and the hyphal forms were enclosed in an extracellular polymer matrix consisting of polysaccharides, proteins and forming a three-dimensional structures with water channels [33].

Biofilms associated drug resistance is a result of many factors: (i) The exopolymer matrix of biofilms decrease or inhibit the penetration of immune system components and antimicrobials [34]. (ii) In *Candida*, the matrix consists of carbohydrates, proteins, hexosamine, phosphorus and uronic acid [35]. These components have the ability to bind antifungals, preventing their access to the antifungal targets in the cell and results in resistance [36]. Also, extracellular polymeric material may act as an adsorbent to the antimicrobials. (iii) The growth rate differentials. (iv) Production of antimicrobial-degrading enzymes [37].

Many studies attributed the anti-biofilm activity of NAC to a number of factors which are: (i) its ability to bind surfaces, increasing their wettability that results in decreasing of microorganisms adhesion. (ii) NAC can detach the adherent microbial cells to steel surfaces. (iii) its ability to reduce the amount of exopolysaccharide (EPS) by direct effect in which a possible reaction of its sulfydryl group occurs with the disulfide bonds of the enzymes involved in EPS production or excretion, which renders these molecules less active. Also, its competitive inhibition of cysteine utilization and indirect effect by affecting cell metabolism and EPS production due to its antioxidant activity [19,20]. So that it is expected that an antibiofilm/antimicrobial agent combination would be synergistic which lead us to use it in our study on biofilm formation by *Candida albicans*.

Harriott *et al*. [38] were the first who reported that *C. albicans* can form biofilms on vaginal mucosa. As they found that vaginal *C. albicans* have biofilm architecture typical of *in-vitro* grown *C. albicans* biofilms, consisting of yeast and hyphae forming a complex network surrounded by extracellular matrix. Our results showed that by examining *Candida albicans* biofilm developed in an *in-vitro* model, it was found that biofilm formed showing the yeast form and the hyphal form.

The use of ketoconazole at MIC and 2 MIC reduced biofilm formation or adherence by 25-87.4% (P<0.05) and disrupted the mature biofilm causing a reduction in optical density of 22-80.7% but Baillie and Douglas [39] found that 20 times the MIC of commonly used antifungals such as amphotericin B, fluconazole, or flucytosine is required to cause a significant reduction in cell numbers. Chandra *et al*. [25] reported that *C. albicans* required low MICs of polyenes and fluconazole during the early biofilm development phase. However, during biofilm maturation, they became highly resistant to these drugs. The potency of ketoconazole as anti-biofilm was also reported by Chebotar and Parshikov [40], who showed that ketoconazole was the most potent anti-biofilm as it resulted in 100%

cell death after 48h exposure of *Candida* biofilms in comparison to the effect of nystatin and fluconazole.

N-acetylcysteine was previously reported to have a high antibiofilm activity, a high disruptive effect on mature biofilms and increase the therapeutic activity of some antimicrobials as ciprofloxacin and tigecycline against biofilm producing bacteria [41]. In a study by Olofsson *et al.* [19], it was found that the initial adhesion of bacteria to stainless steel surfaces is dependent on the wettability of the substratum. Aslam and Darouiche [42] reported that NAC is fungistatic and has a significant effect on biofilm formation by *Candida albicans* which agree with our results. Also our results agree with those obtained by Venkatesh *et al.* [43] who showed that NAC showed synergistic action in combination with amphotericin B and fluconazole against C. albicans biofilms.

This study showed that by using NAC and ketoconazole (each alone or in combination), *Candida albicans* became unable to form the hyphal form which lead to the formation of thin biofilm (easily removed). Similar results were obtained by Ramage *et al*. [44] and Lewis *et al.* [45] who reported that strains of *Candida albicans* that unable to filament form poor biofilms lacking in three-dimensional structure and composed mainly of sparse monolayers of elongated cells.

5. Conclusion

Our results showed that NAC has a great anti-biofilm and can disrupt the preformed mature biofilms and antifungal properties. In addition, both NAC and ketoconazole can inhibit the dimorphism of *Candida albicans* which plays an important role in the maturation of biofilm. So, it will be valuable to use NAC in the treatment of candidal infections as oral candidiasis or to prevent biofilm formation on different medical devices surfaces as voice prosthesis or ureteral stents.

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