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Cyclooxygenase Inhibitors Reduce Biofilm Formation and Yeast-Hypha Conversion of Fluconazole Resistant *Candida albicans*

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The incidence of fluconazole-resistant *Candida albicans* has been increasing worldwide. Both biofilm and fungal morphogenesis are main virulence factors of *C. albicans* cells. Extracellular fungal prostaglandins are synthesized during biofilm adhesion and development and through yeast-hypha conversion. Hence, we targeted prostaglandin synthesis with various cyclooxygenase (COX) inhibitors (aspirin, diclofenac, ketoprofen, tenoxicam, and ketorolac) and assessed their effect on fungal adhesion, biofilm formation, and yeast-hypha conversion in clinical isolates of Fluconazole resistant *C. albicans*. Significant reduction in fungal adhesion and detachment of mature biofilm was attained down to 1 mM concentrations of anti-inflammatory agents. Microscopical examination of fungal cells in the presence of the tested drugs showed significant reduction of germ tube formation. Therefore, COX inhibitors have a significant effect on reduction of *Candida* adhesion and biofilm development in correlation with fungal morphogenesis. Moreover, inhibition of *C. albicans* by COX inhibitors gave synergistic activity with fluconazole suggesting that combination therapeutic strategies may be fruitful for management of infection of Fluconazole resistant *C. albicans*.

***Keywords*:** *C. albicans*, fluconazole resistant, cyclooxygenase inhibitors, biofilm, morphology, prostaglandin

Introduction

Candida albicans is a commensal organism of skin and mucosal tissues. It causes serious opportunistic infection in immunosuppressed patients and in persons with distorted normal flora associated with antibiotic therapy, cardiac surgery, and indwelling catheters (Hube, 2006; Pfaller and Diekma, 2007). *Candida* overgrowth results in both superficial and serious systemic disease and it is now the most common hospital-acquired fungal infection (Perlroth *et al*., 2007). Fluconazole is the most commonly used drug to treat candidiasis. Concomitant with this widespread use, there has been an increasing incidence of fluconazole-resistant *C. albicans* (Silva *et al*., 2012). The majority of severe candidiasis manifestations are associated with the formation of biofilms on inert (implanted devices such as catheters) or biological surfaces (Cannon and Chaffin, 1999; Ramage *et al*., 2001; Douglas, 2003). *Candida* biofilms are clinically important because they are very resistant to antifungal agents, including fluconazole and amphotericin B (Bink *et al*., 2011). Also, *Candida* biofilm has the ability to resist host immune defenses and has the potential for causing failure of implanted devices (Hawser and Douglas, 1995; Ramage *et al*., 2005).

Hyphae are essential elements for providing structural integrity to *Candida* biofilms (Baillie and Douglas, 1999). The ability of *C. albicans* to reversibly switch between yeast and filamentous forms are important for its pathogenicity (Ghormade and Deshpande, 2000). At the infection site, the unicellular yeast-like form of *C. albicans* switches into an invasive, multicellular filamentous form (Whiteway and Oberholzer, 2004; Whiteway and Bachewich, 2007). *C. albicans* is able to produce eicosanoids and related compounds (oxylipins) including a prostaglandin E2-like molecule (Noverr *et al*., 2002, 2003). Prostaglandins function as regulators of the dimorphic structure of *C. albicans* could also promote fungal colonization and chronic infection since colonization is often involved in biofilm formation (Douglas, 2003). Candidiasis is also often associated with high levels of PGE2, and it has been shown to enhance germ tube formation (Noverr *et al*., 2001). Culture supernatants of *C. albicans* contained prostaglandins, and when treated with cyclooxygenase (COX) inhibitors, cell viability and the production of prostaglandins is reduced (Noverr *et al*., 2001).

Antifungal drug resistance has become a major problem worldwide (Pappas *et al*., 2009). This is especially true for *C. albicans* and as antifungal drugs are not effective against biofilms of *C. albicans*, the recommended therapy for *Candida* biofilm infection of a medical device includes device removal, which is associated with increased morbidity and health care expenditures (Pappas *et al*., 2009). Alternative therapies for the treatment of resistant *Candida* isolates are currently being sought. Some studies suggested that COX dependent synthesis of fungal prostaglandin might act as a regulator for biofilm development in *C. albicans* (Alem and Douglas, 2004; Ghalehnoo *et al*., 2010; deQuadros *et al*., 2011; Bink *et al*., 2012).

Inhibition of fungal biofilm formation and remediation of mature *Candida* biofilms are of our interest. This could assist in controlling fluconazole-resistant *Candida* infection and contribute in development of combination therapy between antifungal drugs and anti-inflammatory agents for treatment of the resistant fungal infections. In the present study, we have determined the effects of a variety of COX

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inhibitors on biofilm and yeast-hypha conversion of resistant *C. albicans* strains. In addition, we investigated the effect of combined anti-inflammatory agents with fluconazole and their potential for treating resistant clinical isolates of *C. albicans*.

Materials and Methods

Organisms

Three clinical isolates of *C. albicans* were used in this study. *C. albicans* strains were isolated from infected patients at Mansoura University hospital, Dakhalia, Egypt. *C. albicans* strain Sp1 was obtained from sputum, *C. albicans* Sp2 was obtained from ear and *C. albicans* Sp3 was obtained from blood. The three isolates of *C. albicans* were obtained from the Clinical Microbiology Laboratory at Mansoura University College of Medicine where the strains were identified. Briefly, positive fungal growths on sabouraud dextrose agar were routinely Gram-stained and found to be yeast-like colonies under microscope. They were also tested for germ tube formation and chlamydoconidial arrangement on rice agar. Both a carbohydrate assimilation test and culture in Tween 80 agar were performed (Elmer *et al*., 1992; Slifkin, 2000; Marinho *et al*., 2010). In addition, species identification was confirmed by its growth on cycloheximide, inability to hydrolyse urea and its ability to grow in hypertonic sabouraud broth (Alves *et al*., 2002). All strains were stored on glycerol stocks at -80°C and subcultured on Sabouraud dextrose agar slopes (Difco).

Medium and culture conditions

Organisms were grown in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% dextrose). Batches of medium were inoculated from fresh culture slopes and incubated at 28°C in an orbital shaker at 200 rpm. Under these conditions, C. *albicans* grew entirely as budding yeast. Cells were harvested after 24 h and washed twice in phosphate-buffered saline (PBS; pH 7.4) [10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM sodium chloride] (Sigma Aldrich Co., USA). Before use in biofilm experiments, washed cells were resuspended in filament-inducing medium (YPD medium supplemented with 5% serum) and were standardized to an optical density of 0.4 at 600 nm.

Susceptibility to fluconazole

Susceptibility of the tested strains to fluconazole was analyzed using the NCCLS M27A2 broth microdilution method (Pfaller *et al*., 2002). Briefly, we used the spectrophotometric method of inoculum preparation corresponding to a concentration of 0.5×10^3 to 2.5×10^3 cells/ml for each of the tested isolates. Cells were inoculated in two-fold serial dilutions of each tested drug in a 96-well plate. Drug-free inoculations were the positive controls. The microtiter plates were then incubated at 37°C, the endpoints were read visually after 48 h and the minimal inhibitory concentrations (MICs) were determined.

COX inhibitors and their growth inhibitory concentrations

Solutions of aspirin, diclofenac, ketoprofen, tenoxicam, and ketorolac were used at different concentrations: 10 mM, 5 mM, and 1 mM. Susceptibility of the tested strains to anti-inflammatory drugs was analyzed using the NCCLSM-27A2 broth microdilution method as mentioned previously.

Biofilm formation and drug treatment

Biofilms were formed on commercially available sterilized, polystyrene, flat-bottom, 96-well microtiter plates (Nett *et al*., 2008). Biofilms were formed by pipetting 100 µl of standardized cell suspensions into selected wells of the microtitre plate and incubated for 3 h at 37°C (adhesion period). Non-adherent organisms were removed by washing twice with PBS and the plates were then incubated for a further 48 h at 37°C while they were submerged in 100 µl of growth medium (biofilm formation). In order to study the effect of anti-inflammatory agents on biofilm formation, 100 µl of each drug concentration (10, 5, and 1 mM) was added at the beginning of the adhesion period and again after removing the non-adherent organism at time zero of the subsequent 48 h incubation. Controls included wells with no cells, wells with cells and solvent but no drug, and wells with drugs but no cells.

Quantitative measurement of biofilm formation

At the end of the 48 h incubation at 37°C, the contents of each well was aspirated using a Pasteur pipette and each well was rinsed three times with 200 µl of PBS (pH 7.4). The plates were vigorously shaken in order to remove all non-adherent cells. The remaining attached fungi were fixed with 150 µl of absolute ethanol for 15 min, plates were emptied and left to dry, then stained by adding 150 µl/well of crystal violet (1% w/v) for 20 min. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 150 µl/well of 33% (v/v) glacial acetic acid. The optical density (OD) of each well was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader

Table 1. Minimal inhibitory concentrations of the COX-inhibitors against *C. albicans* clinical isolates Sp1, Sp2, and Sp3

	MIC of *Candida* (Sp1) mg/ml (mM)	MIC of *Candida* (Sp2) mg/ml (mM)	MIC of *Candida* (Sp3) mg/ml (mM)
Ketorolac	1.88 (7.30)	7.50 (29.40)	1.875 (7.3)
Declofenac	3.13 (9.83)	6.25 (19.65)	3.13 (9.83)
Ketoprofen	12.50 (49.10)	12.50 (49.10)	12.50 (49.10)
Tenoxicam	10 (29.60)	10 (29.60)	10 (29.60)
Aspirin	18 (99.90)	9 (49.90)	4.50 (24.9)
Fluconazole	18.75 (61.22)	18.75 (61.22)	18.75 (61.22)

(Stepanovic *et al*., 2000; Abdi-Ali *et al*., 2005).

Effect of anti-inflammatory drugs on mature biofilms

Mature biofilms were formed as described above, where biofilms of tested strains of *C. albicans* were grown in the absence of drugs for 48 h. The free, unbound cells were washed out by PBS. Subsequently, fresh growth medium containing different concentrations of each drug (10, 5, and 1 mM) were added to the 24 h mature biofilms and incubated for a further 48 h at 37°C (Alem and Douglas, 2004; Plerce *et al*., 2009). Quantitative measuring of the biofilms formed with and without drug treatment was performed as mentioned before.

Germ tube formation

Cultures of *C. albicans* grown overnight in YPD medium were harvested, and the cells were washed twice with PBS (pH 7.4). The cells were then resuspended in the same medium containing 5% serum and standardized to an optical density of 0.4 at 600 nm. At zero time, the tested drugs were added to a final concentration of 1 mM, 5 mM or 10 mM. Cell suspensions were incubated at 37°C for 3 h. After the 3 h incubation, treated suspensions were examined by light microscopy to determine the percentage of germ tubes formation, as compared to control untreated cells in the absence of COX inhibitors using light microscope (Alem and Douglas, 2004).

Measuring the combined effect of fluconazole and COX inhibitors

Combinations of ketorolac/fluconazole, ketoprofen/fluconazole, and declofenac/fluconazole were tested using checkerboard microdilution method (Pfaller *et al*., 1989; Lewis *et al*., 2002). Two fold serial dilutions of declofenac, ketoprofen, ketorolac, and fluconazole were prepared. The concentrations of each tested drug ranged from 4-fold to 1/16 the MIC. One hundred microliters of each concentration of the COX inhibitor drugs was combined with one hundred microliters of each concentration of fluconazole in 96-well flat bottom microtitration plates. The initial inoculum of isolate Sp1 was prepared as described for broth-microdilution susceptibility testing. After inoculation, plates were incubated for 24 h and 48 h at 37°C. To evaluate the interaction, the fractional inhibitory concentration (FIC) was calculated for each combination as follows: FIC of drug A (FICA) = MIC of drug A in combination/MIC of drug A alone, FIC of drug B (FICB) = MIC of drug B in combination/MIC of drug B alone. FIC index (FICI) was calculated as the sum of each FIC. Synergy was defined as an FICI<0.5, antagonism was defined as an FICI>4.0 and indifference was defined as an FICI>1. Additivity was defined as an FICI>0.5 (Eliopoulos and Moellering, 1996).

Statistical analysis

For statistical analyses, the Excel data analysis package was used to calculate the mean, standard deviation of the mean and standard error. Data was analyzed using the GraphPad Instate software package (version 3.05) according to the Tukey-Kramer multiple-comparison test at a P value <0.05 or P<0.01. All results are calculated from the mean of four separate experiments.

Results

Antifungal susceptibility of fluconazole and anti-inflammatory agents

The MIC of *Candida* isolates (Sp1, Sp2, and Sp3) against fluconazole were found to be 18.75 mg/ml under the specified growth conditions. In addition, tested strains were susceptible to the anti-inflammatory agents at concentrations more than 5 mM (Table 1).

Different concentrations of anti-inflammatory agents inhibit biofilm formation of tested strains

Five anti-inflammatory agents (aspirin, diclofenac, ketoprofen, tenoxicam, and ketorolac) were assessed for inhibition of biofilm formation of the three clinical isolates of *C.*

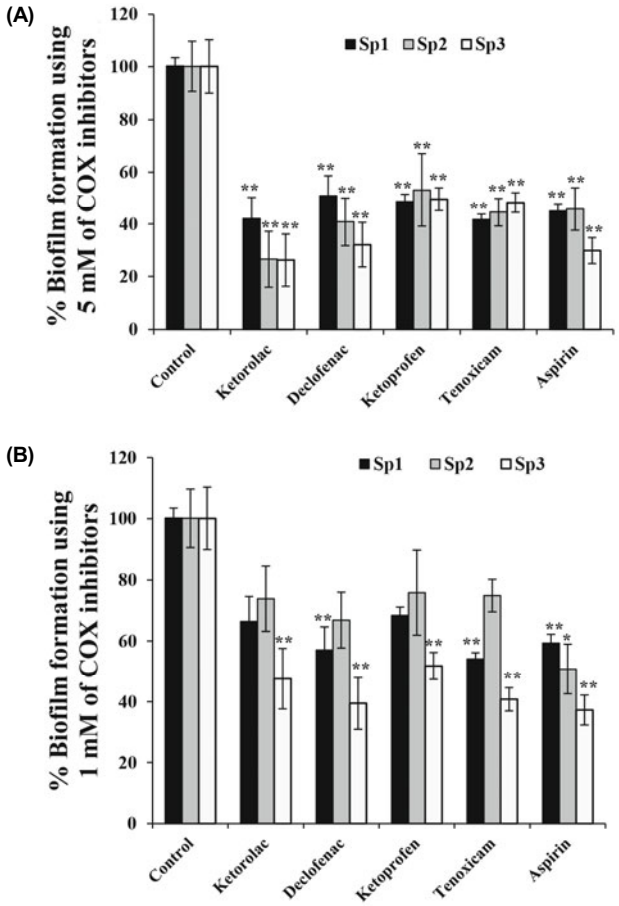

Fig. 1. Biofilm formation in presence of anti-inflammatory agents. (A) Effect of sub-inhibitory concentration (5 mM) of anti-inflammatory agents on biofilm formation of the tested isolates Sp1, Sp2, and Sp3. (B) Effect of lower concentrations (1 mM) of tested agents on adhesion of the cells of Sp1 and Sp3. (**) highly significant (P<0.01) and (*) significant (P<0.05).

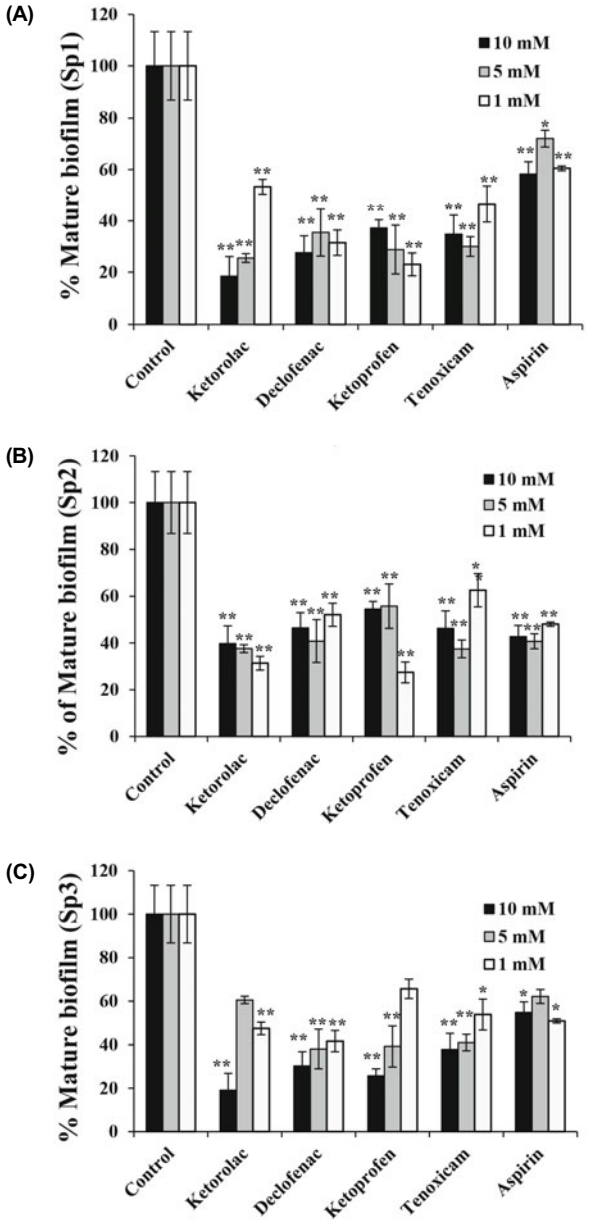

Fig. 2. Reduction in mature biofilms formed by *Candida* isolates grown in the presence of anti-inflammatory agents calculated as percentage of control untreated cells. Different concentrations of anti-inflammatory agents were tested against three isolates Sp1 (A), Sp2 (B), and Sp3 (C). (**) highly significant ($P<0.01$) and (*) significant ($P<0.05$).

albicans. Three concentrations of these agents were used; 10, 5, and 1 mM.

Quantitation of adherent filaments within the first hours indicated that all agents resulted in significant inhibition in percentage of biofilms formation compared to control untreated wells (Fig. 1A). Also, Sp1, and Sp3 demonstrated a significant decline ($P<0.01$) in adherent biofilm when treated with the lower concentration (1 mM) of the anti-inflammatory agents (Fig. 1B).

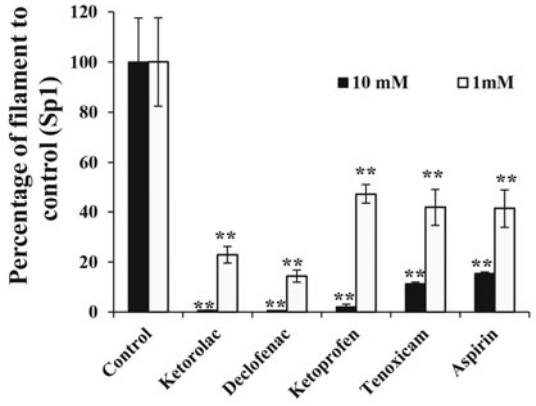

Fig. 3. Effects of different concentrations of anti-inflammatory agents on germ tube formation of clinical isolate Sp1. Germ tube formation is expressed as a percentage of that for control cells incubated in the absence of inhibitors (**$P<0.01$).

Reduction in mature biofilm by different concentrations of anti-inflammatory agents

To investigate the effect of anti-inflammatory agents on the mature 48 h biofilms, COX inhibitors at different concentrations were incubated with mature biofilms for a further 48 h. Inhibitory concentrations of tested drugs (10 mM) and sub-inhibitory concentrations (5 and 1 mM) significantly inhibited biofilm activity after 48 h (Fig. 2). Ketorolac (5 mM) produced the greatest effect on biofilm formation; however, the other tested drugs also eliminate biofilm formation to a lesser, but still significant, extent. After 48 h of incubation with the 10 mM of drugs, biofilm activity was reduced by 18 to 58% (Fig. 2A). Moreover, utilizing lower concentrations (5 mM and 1 mM) still significantly diminishes biofilm viability for all tested strains (Figs. 2B and 2C). The OD of the control strain did not increase for the further 48 h of incubation.

Candida morphogenesis

The yeast to hypha transition is often associated with pro-

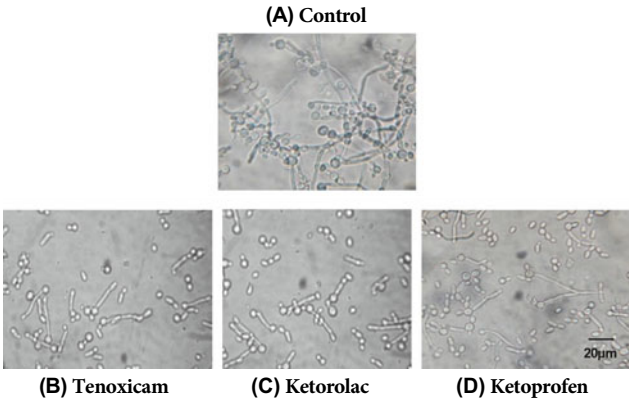

Fig. 4. Microscopical examination of yeast-filament transition of clinical isolate Sp1; lower concentrations (1 mM) of (B) Tenoxicam, (C) Ketorolac, and (D) Ketoprofen showed yeast cells with few hyphal cells, compared to (A) control untreated cells.

Table 2. Combined effects of COX inhibitors and fluconazole by chessboards method

Drug A	FIC of drug A	FIC of drug B "Fluconazole"	FIC (A+B)	Combined effect
Ketorolac	0.468/ 1.875=0.25	4.6/ 18.75=0.245	0.25+0.24=0.495	Additive
Declofenac	0.780/ 3.125=0.25	2.3/ 18.75=0.123	0.25+0.123=0.37	Synergism
Ketoprofen	1.560/12.50=0.124	2.3/ 18.75=0.123	0.124+0.123=0.247	Synergism

gression of infection by *C. albicans*. Hence, the synthesis of germ tubes in the presence of anti-inflammatory agents was examined microscopically. The treated isolates showed a significant reduction in percentage of filament formation compared to the control with all used drugs concentrations (Fig. 3). In the presence of anti-inflammatory agents at 1 mM, the filamentation was reduced to 14–47% (Fig. 3). Using higher concentrations (10 mM), *Candida* grows almost entirely as yeast cells (Figs. 3 and 4).

Antifungal synergy

In the chessboard assay method for combined activity of fluconazole with ketoprofen and declofenac showed an increase in the susceptibility of the Sp1 isolate towards fluconazole up to ⅛th MIC of fluconazole alone. Combination of declofenac with fluconazole showed no growth of *Candida* strain Sp1 at ⅛th of declofenac MIC when used alone. Also, the combination of ketoprofen with fluconazole showed no growth of *Candida* strain Sp1 up to ¼th MIC of ketoprofen. However, the MIC of ketorolac was not significantly reduced when it was used in combination with fluconazole against strain Sp1. Fractional inhibitory concentration was calculated (Table 2). The FICs of fluconazole/ketoprofen and fluconazole/declofenac were lower than 0.5, indicating synergistic activity. However, the FIC of fluconazole/ketorolac was around 0.5, which representing additive effect.

Discussion

Candida albicans is an opportunistic pathogen associated with implanted medical devices. The severity of this fungal infection is a consequence of antifungal drug resistance and virulence factors (Blankenship and Mitchell, 2006). These factors include adhesion, biofilm formation, and phenotypic switching (Calderone and Fonzi, 2001). *Candida albicans* biofilm is an increasing problem in clinical settings as they adhere to implanted devices and these biofilms are notoriously resistance to common antifungal agents (Douglas, 2003; Kojic and Darouiche, 2004). It has been reported that prostaglandin production is a significant virulence factor in biofilm-associated infections of *C. albicans* (Alem and Douglas, 2005). *C. albicans* and *Cryptococcus neoformans* produced prostaglandins in their culture supernatant and treatment of their infections with the COX inhibitor indomethacin significantly reduced cell viability (Noverr *et al*., 2001).

In this study, we investigated the effect of nonsteroidal anti-inflammatory drugs on biofilm synthesis of fluconazole resistant clinical isolates of *C. albicans* (Sp1, Sp2, and Sp3). The results indicated that five COX inhibitors, which decrease prostaglandin synthesis, repressed biofilm formation even at 1 mM. Higher concentrations (10 mM) within the range of therapeutic doses inhibited fungal growth and biofilm formation after initial contact of cells at adhesion period 3 h and for the next 24 h (Fig. 1). Furthermore, ketorolac, and aspirin (5 mM) particularly, reduced or eliminated biofilm formation of the tested isolates. As well, lower aspirin concentrations (1 mM) significantly reduced biofilm synthesis. These results are parallel with other reports on decreased prostaglandin levels and decreased biofilm formation following exposure to COX inhibitors (Alem and Douglas, 2004, 2005), thus supporting the perception that COX-dependent synthesis of prostaglandins may participate in regulating biofilm formation.

The role of prostaglandin E2 (PGE_2) and the fungal-produced prostaglandin EX (PGE_x) in *C. albicans* biofilm development and fungal pathogenesis has been demonstrated. Fungal PGE2 and PGE_x increased adhesion, germination, biofilm mass, matrix and shed cell population (Erb-Downward and Noverr, 2007; Harriott, 2009). Moreover it has been investigated by Alem and Douglas (2005) that prostaglandin synthesis by both planktonic and biofilm cells was sensitive to the cyclooxygenase inhibitors aspirin, diclofenac, and etodolac. In this study, five COX inhibitors significantly decreased biofilm formation by fluconazole resistant *C. albicans* clinical isolates, with some strain variation. Ketoprofen, declofenac, tenoxicam, and ketorolac at all used concentrations, providing the highest influence on Sp1 and Sp2 (Figs. 2A and 2B). Aspirin, one of the oldest and most widely used anti-inflammatory drugs, also has a significant inhibitory effect on mature biofilm of Sp2 (Fig. 2B). Moreover, the tested drugs, particularly declofenac, affected biofilm activity of *Candida* strain Sp3 to a significant but a lesser extent (Fig. 2C).

The ability of *C. albicans* to undergo a morphogenic shift from yeast-to-hypha is an important virulence factor of *C. albicans* and a crucial factor for *C. albicans* biofilm development. Therefore, it was essential to investigate the effect of COX-inhibitors on fungal morphogenesis and yeast filament conversion. Cultures treated with the tested drugs exhibited a dose-dependent inhibition of germ tube formation following a 3 h incubation at 37°C (Fig. 3). Significant reduction in germ tube formation has been achieved here under treatment with 1 and 10 mM anti-inflammatory drugs for 3 h ($P<0.01$) especially with ketorolac and declofenac (Fig. 3). It has been reported that lower concentrations (75–100 μg/ml) produced only about 30% inhibition on germ tube formation (Alem and Douglas, 2004). Declofenac sodium regulated the hyphal transformation of *C. albicans* cells, as it represses the upregulation of some genes involved in hyphal formation of *C. albicans* (Ghalehnoo *et al*., 2010). This result together with those showed by Alem and Douglas (2004) may explain the inhibitory effect of diclofenac sodium on biofilm development and *C. albicans* morphogenesis. Moreover, microscopical examination of fungal development of treated or untreated cells (Fig. 4) showed a decrease

in germination of *Candida* cells treated with 1 mM concentration of drugs. As production of eicosanoids stimulates germ tube formation, morphogenesis and inflammation during infection (Alem and Douglas, 2004). Also, prostaglandin like molecules, (3*R*)-hydroxyoxylipins, which are derived from arachidonic acid, have also been found in *C. albicans* (Douglas, 2003). Other lipid molecules may be involved in the regulation of biofilm formation. The synthesis of these compounds appears to occur in filaments, not yeast cells, and was found to be suppressed by aspirin (Deva *et al*., 2000, 2001).

Resistance of *C. albicans* to antifungal treatment is the main problem in management of fungal infection. Hence, it would be of interest to investigate the combined effect of antifungal agents and COX inhibitors in *Candida* infections. It has been reported that combination of fluconazole with either sodium salicylate or ibuprofen results in synergistic activity against *C. albicans* (Scott *et al*., 1995; Pina-Vaz *et al*., 2000; Mukherjee *et al*., 2003; Zhou *et al*., 2012). In our study, interactions between ketorolac, declofenac or ketoprofen with fluconazole against fungal cell strain Sp1 has been evaluated by the chessboard method. Synergistic effects are found for the combination of declofenac and ketoprofen with fluconazole against *C. albicans* cells and an additive effect was observed in the combination of fluconazole with ketorolac. As Fungal-produced prostaglandin EX (PGE_x) affects susceptibility of antifungal agents, so non-steroidal anti-inflammatory drugs (NSAIDs) provide a synergistic action with fluconazole for inhibiting in vitro *C. albicans* biofilms (Harriott, 2009).

In conclusion, through the following study; we figured out that COX inhibitors have significant effect on reduction of *Candida* adhesion, biofilm development in correlation with fungal morphogenesis. We also investigated synergistic effect of an antifungal drug (fluconazole) in combination with COX inhibitors and the development of novel therapeutic strategies for treating opportunistic *C. albicans* infections. These results open a new view regarding the use of these compounds in the treatment of fluconazole resistant *Candida* infections.

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