Effect of Some Non steroidal Anti-Inflammatory Drugs on Growth, Adherence and Mature Biofilms of *Candida spp.*

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Abstract *Candida spp.* are the most common cause of fungal diseases and the fourth commonest cause of nosocomial invasive infections which are considered in many cases as life threatening. Among *Candida* spp., *C. albicans* is the most common cause of many fungal diseases, but non-albicans spp. infections are in increase. Non-steroidal anti-inflammatory drugs have previously been shown to have antifungal activity. In this study we determine the antifungal activity of the tested NSAIDs using agar well diffusion method, their effect on the dimorphic transition of *C. albicans* by testing their ability to form germ tube in the presence of human serum. Determining the effect of NSAIDs on the adherence to plastic surfaces and on the mature biofilms formed by the tested *Candida* spp.. The results indicated that *Sodium Diclofenac* showed lower MIC against *C. albicans* and *C. glabrata* while Ibuprofen had lower MIC against *C. krusei*. upon examining the effect of Diclofenac sodium, Ibuprofen and ketoprofen on biofilm formed on polyurethane segments by Scanning electron microscope (SEM), a damage to membranes of the tested species was observed. *Sodium Diclofenac* showed the highest inhibitory effect on the adherence (51.1-76.9% at MIC and 56.6-83.3% at 2XMIC) of *C. albicans* and *C. glabrata* but Ibuprofen showed a higher inhibitory effect against the adherence of *C. krusei*. For mature biofilms, the highest disruptive effect on mature biofilms formed by all tested *Candida Spp.* (37.72-59.29% at MIC and 42.68-63.06% at 2XMIC) was observed by Diclofenac sodium. *Sodium Diclofenac* inhibited dimorphic transition of *C. albicans* but a decrease in germ tube formation was shown by others. In conclusion, the tested drugs showed antifungal, anti-adherent and anti-biofilm activity that make them useful in the treatment of fungal infection and the prevention of biofilm formation on the surface of medical devices.

Keywords: *Ketoprofen, Ibuprofen, Diclofenac sodium, biofilm, Candida, Dimorphic transition*

1. Introduction

Most of microbes were found to be able to adhere and colonize biotic or abiotic surfaces. *Candida* cells, like many other microorganisms, are able to adhere to and colonize mucous membranes and surfaces of medical devices, resulting in the formation of a biofilm mass. *Candida* biofilms occurrence on medical device were proven upon the examination and the visualization of medical devices removed from patients or animal models using scanning electron microscopy, confocal laser scanning microscopy [1,2,3]. Fungal biofilm associated infections are considered as a major clinical concern as these structured microbial communities, embedded in an extracellular matrix, are characterized by increased resistance to host immune defense and antifungal therapy [4]. Biofilm resistance may be attributed to the presence of different mechanisms: (i) expression of efflux pumps, (ii) a changing sterol composition in the membrane, (iii) extracellular matrix as a barrier for the diffusion of antifungals and the molecules of immune system (iv) the presence of persisters (dormant cells not mutants) [5,6]. Medical devices can become colonized by one or more microorganisms which form a biofilm consisting of a

mono- or multilayer cells embedded within an exopolysaccharide matrix [7-13]. Detachment of microorganisms from the biofilm may initiate an acute disseminated infection. Implant-associated infections are difficult to be treated increasing the need of implant removal [8,12,14]. Although the majority of implant infections are caused by bacteria, fungal biofilm associated infections tend to be more serious. Fungal infections are most commonly caused by the pathogenic *Candida* species, particularly *C. albicans*, *C. tropicalis*, and *C. parapsilosis* that are regarded as increasingly important nosocomial pathogens [15]. *Candida* bloodstream infections associated with intravascular catheters represented 40% of all nosocomial septicemias occurring in intensive care unit patients with a high mortality rate [11,16,17]. Also, *Candida* biofilms were found associated with urinary catheters, prosthetic heart valves, cardiac pacemakers, silicone voice prostheses, endotracheal tubes, and cerebrospinal fluid shunts [18].

Non steroidal anti-inflammatory drugs are widely used, especially as anti-inflammatory and analgesics. NSAIDs inhibit the cyclooxygenase isoenzymes COX-1 and COX-2, which are involved in the biosynthesis of mammalian prostaglandins. Prostaglandins are small lipid molecules showing different functions [19]. They are also found to be produced by *C. albicans* cells but their role in fungal biology is unknown [20]. Erb-Downward and Noverr [21] suggested that prostaglandins can act as regulators for *C. albicans* virulence. As eicosanoids pathway was reported to show a vital role in controlling yeast-hyphal transition and biofilm. So, drugs targeting fungal prostaglandins pathways may show great effects on fungal colonization and infection [21]. In addition, some studies showed that *Sodium Diclofenac* affect *C. albicans* morphogenesis and biofilm formation. [20,22]. Also, Ibuprofen show fungistatic activity at low concentrations and fungicidal activity at high concentrations [23]. In this study we tested the antifungal activity of three NSAIDs on different *Candida* Spp. and their effect on biofilm formation

2. Materials and Methods

2.1. Microbial Strains

Three different species of *Candida*, *C. albicans*, *C. glabrata*, *C. Krusei* obtained from the department of microbiology and immunology, Faculty of medicine, Assuit university 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 [24]. *C. albicans* ATCC 10231 was obtained from MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University, Egypt.

2.2. Drugs

The following drugs were used Sodium Diclofenac (Glaxo, Egypt), Ibuprofen (Kahira/Abbott, Egypt) and Ketoprofen (Amriya, Egypt).Stock solutions were prepared as shown in Table 1. Working solution concentrations were ranged from (20-0.078125 mg/ml).

Table 1. Preparation of NSAIDs stock solution

Drug	Stock solution conc.	Drug (mg)	Solvent (ml)
Sodium Diclofenac	20 mg/ml	2 gm	Water (100 ml)
Ketoprofen	20 mg/ml	2 gm	Water (100 ml)
Ibuprofen	20 mg/ml	2 gm	DMSO (100 ml)

2.3. Detection of Biofilm Formation by Tissue Culture Plate Method (TCP)

The TCP assay is most widely used and was considered as standard test for detection of biofilm formation. All isolates were screened for their ability to form biofilm by the TCP method as described by Christensen *et al.* [25] with a modification in duration of incubation which was extended to 24 hours, according to O'Toole and Kolter [26].

2.4. Evaluation of Antifungal Activity of the tested NSAIDs Using well Diffusion Technique

Candida spp. (0.5 ml) of 1×10^8 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. Each sample was applied as triplicate. The plates were allowed to stand for one hour for prediffusion of the extract to occur then incubated overnight at 37°C. All plates were examined and zones of inhibition were recorded [27,28,29].

2.5. Determination of Minimum inhibitory Concentrations (MIC) of the Tested NSAIDs:

Two fold serial dilutions were performed on the tested NSAIDs. Equal volumes of each dilution were applied separately to each well in three replicates using a micropipette. All plates were incubated at 37°C for 24 hours, then the formed zones of inhibition 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 of the tested NSAIDs 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 [29].

2.6. Germ Tube Formation

C. albicans strains were cultivated on Sabouraud's dextrose agar for one day. Cells were suspended in sterile saline and adjusted to density of 0.5 Mcfarland turbidity. 400 µl of human serum was added to 1 ml of cell

suspensions of *C. albicans*. Drugs were added to the suspensions to a final concentration ranged from 100 to 1000 µg/ml and saline was added to the control tube. The cell suspensions were incubated with gentle shaking at 37°C for 2 hours and were examined for the presence of germ tubes by using a light microscope. Photomicrographs of colonies and invasive growth were taken with a DMRXA microscope (Leica, Germany) [30].

2.7. Effect of Ketoprofen, Diclofenac Sodium, Ibuprofen on the Adherence of the Tested *Candida* spp. on Plastic Surfaces

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 hours. 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 hours, the cells were centrifuged and washed twice with 0.5 ml PBS (phosphate buffered 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.

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, MIC, above MIC and under the MIC concentrations of each of the following solutions: Ketoprofen, ibuprofen, and Sodium Diclofenac was added to each well. The plates were covered to prevent evaporation and incubated at 25°C for 24 hours. 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. Experiment was performed in triplicate.

2.8. Effect of Ketoprofen, Diclofenac Sodium, Ibuprofen on the Preformed Biofilms of the Tested *Candida* spp. on Plastic Surfaces

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 hours. 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 sub MIC, MIC, above MIC of each of the following solutions: Ketoprofen, ibuprofen, and *Sodium Diclofenac* was added to wells each alone. Normal saline without any agents was added to the control wells. The plates were incubated at 25°C for 24 hours. 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 [31]. Experiment was performed in triplicate.

2.9. Scanning Electron Microscopy (SEM)

The untreated *Candida* spp (control) and *Candida* spp. treated with the tested drugs were fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco PBS (PH 7.2) for 1.5 hours, 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) [32].

2.10. 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

3.1. Biofilm Production

Four *Candida* spp. were tested for their ability to form biofilm and according to TCP method strains can be classified into weak adherent at OD <0.120, moderate 0.120-0.240 and strong >0.240 (Table 2)

Table 2. Biofilm production by *Candida spp*.

Microorganisms	Ability to adhere to TCP surface
C. albicans ATCC 10231	Moderate
C. albicans	Strong
C. glabrata	Moderate
C. Krusei	Strong

3.2. Minimum Inhibitory Concentration of NSAIDs

Sodium Diclofenac showed a higher activity and lower MIC against *C. albicans* and *C. glabrata* while Ibuprofen showed the higher activity against *C. krusei*. (Table 3).

Table 3. Minimum inhibitory concentrations of Ketoprofen, Ibuprofen and *Sodium Diclofenac* against the tested *Candida* spp.

	MIC (µg/ml)		
	Ketoprofen	Ibuprofen	Diclofenac sod
C. albicans ATCC 10231	1577.5	500	52.6
C. albicans	1642.5	500	166.5
C. glaberta	1045.7	250	147.6
C. Krusi	1579.9	250	1000.5

3.3 Effect of the Tested NSAIDs on *Candida* spp. Membranes

Figure 1 showed the effect of *Sodium Diclofenac* on *Candida spp*. membranes. At low concentration, Pores were formed on *Candida* membranes (Figure 1a). At higher concentration, complete lysis and a release to the contents of cells was observed (Figure 1b).

3.4. Effect of NSAIDs on the Dimorphic Transition of *C. albicans*

By testing the effect of Diclofenac sodium, Ibuprofen and ketoprofen on yeast to hyphae transition of *C.*

albicans, it was found that Diclofenac at 500 µg/ml inhibited germ tube formation while Ibuprofen and ketoprofen inhibited their formation to lesser extent in all tested concentrations (Figure 2).

3.5. Effect of Diclofenac Sodium, Ibuprofen and Ketoprofen on the Adherence And Mature Biofilms

The effect of the tested NSAIDs on adherence and mature biofilms was found to be concentration dependent (P<0.05). At MIC, *Sodium Diclofenac* showed the highest inhibitory activity against the adherence of *C. albicans* and *C. glabrata* but Ibuprofen showed the highest inhibitory effect on the adherence of *C. krusei*. On mature biofilms, *Sodium Diclofenac* showed the highest activity against the tested *Candida spp* (Figure 3, Figure 4). At 2X MIC the same results were obtained (Table 4, Table 5).
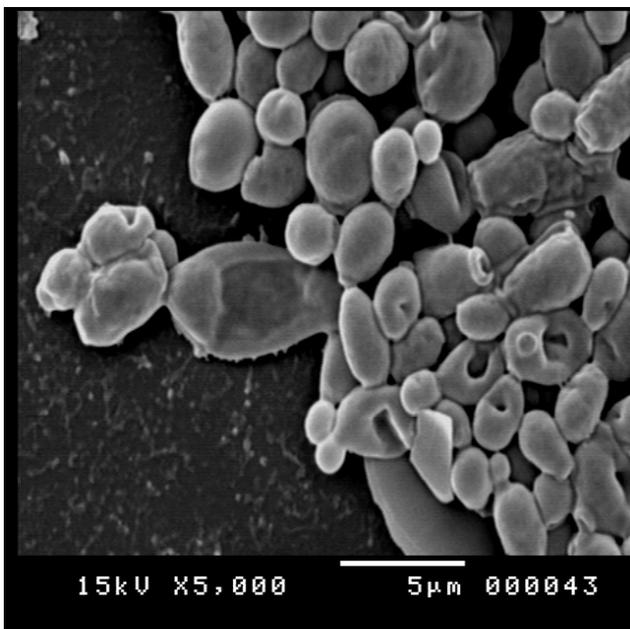

Figure 1b. complete lysis to cells were observed at high concentrations

Figure 2a.untreated *C. albicans* (control)

Figure 2b. *C. albicans* with Diclofenac sodium.

Figure 2c. *C. albicans* with Ibuprofen

Figure 2d. *C. albicans* with ketoprofen.
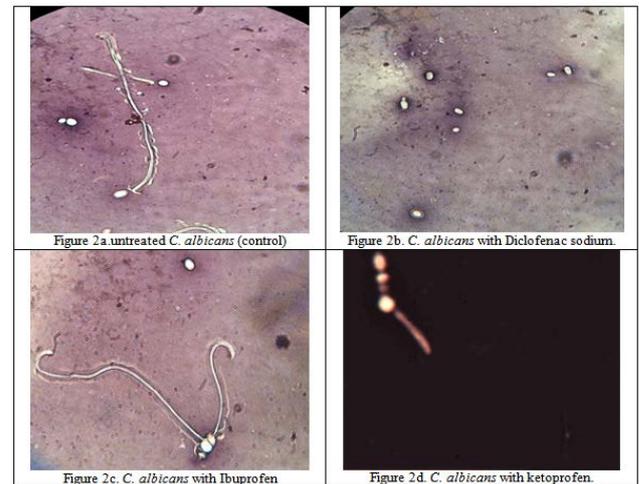
Figure 2. Effect of the tested NSAIDs on germ tube formation by *C. albicans*.

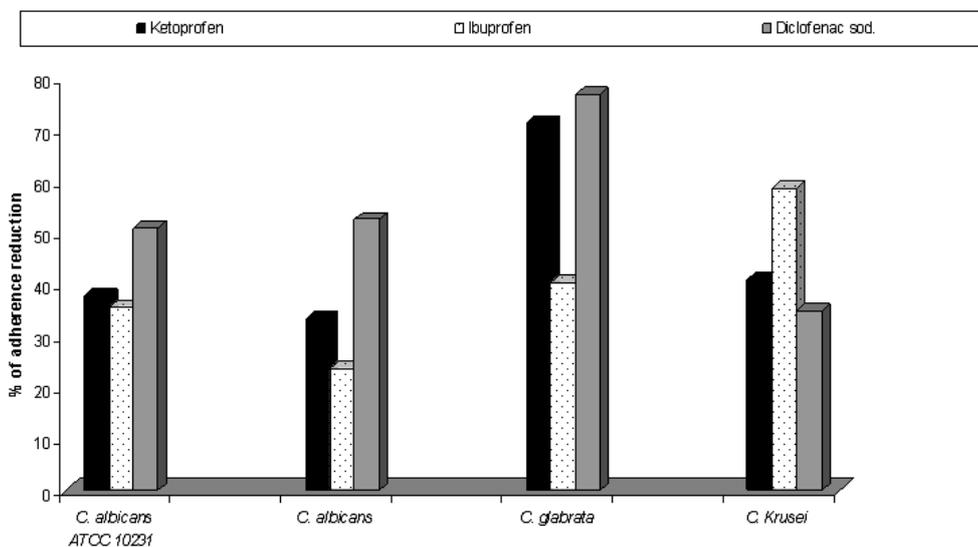**Figure 1a.** At low concentration, pores were formed on the membranes of *Candida* cells

Figure 3. Effect of minimuminhibitory concentration of ketoprofen, lbupofen and diclofenac sod on the adherence of *Candida spp. to the tissue culture plate*
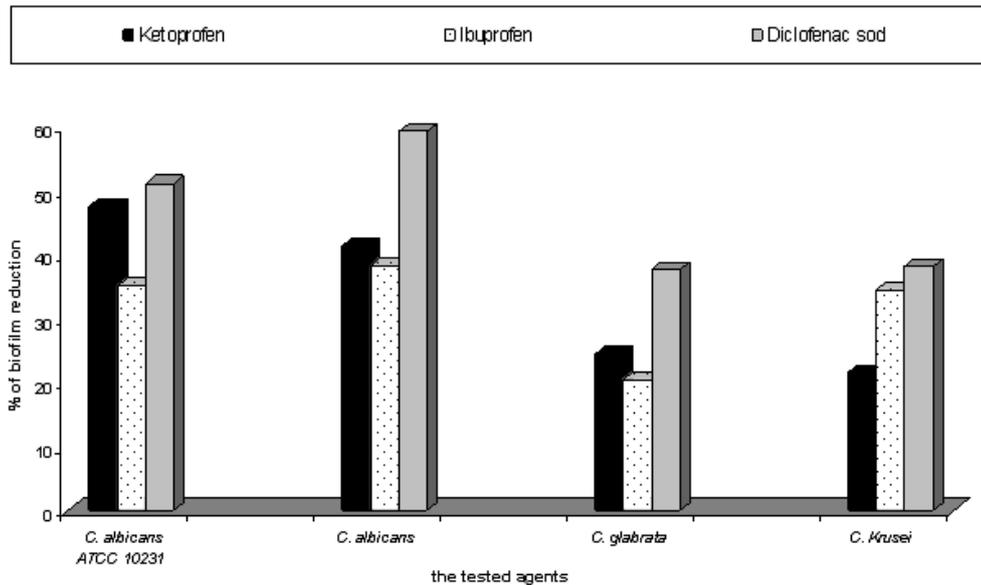
Figure 4. Effect of minimuminhibitory concentration of ketoprofen, lbupofen and diclofenac sod on mature biofilms formed by the tested *Candida spp.*

Table 4. Effect of MIC, Sub-MIC and above MIC concentrations on the adherence of the tested *Candida* spp.

Drugs / Microorganism	Conc	Optical Density± SD (% of adherence reduction)		
		Ketoprofen	Ibuprofen	Diclofenac Sod
C. albicans ATCC 10231	**CTR**	**0.356**	-	
	2XMIC	0.183±0.0001 (48.5)[*]	0.178±0.0001 (50)[*]	0.147±0.0001 (58.5)[*]
	MIC	0.221±0.0003 (37.7)[*]	0.229±0.0001 (35.6)[*]	0.174±0.0001 (51.1)[*]
	0.5X MIC	0.301±0.000 (15.2)[*]	0.31±0.0001 (12.9)[*]	0.353±0.0002 (0.79)[NS]
C. albicans	**CTR**	**0.378**		-
	2XMIC	0.207±0.0002 (54)[*]	0.198±0.0001 (47.5)[*]	0.163±0.0003 (56.6)[*]
	MIC	0.252±0.0003 (33.3)[*]	0.288±0.0001 (23.6)[*]	0.179±0.0001 (52.64)[*]
	0.5X MIC	0.342±0.000 (9.5)[*]	0.325±0.0003 (13.9)[*]	0.302±0.0002 (19.87)[*]
C. glabrata	**CTR**	**0.423**	-	
	2XMIC	0.08±0.0001 (80.9)[*]	0.193±0.0002 (54.21)[*]	0.07±0.0003 (83.3)[*]
	MIC	0.12±0.0002 (71.4)[*]	0.252±0.0001 (40.37)[*]	0.97±0.000 (76.9)[*]
	0.5X MIC	0.21±0.000 (48.3)[*]	0.346±0.0001 (18.13)[*]	0.163±0.0002 (61.4)[*]
C. Krusei	**CTR**	**0.420**	-	
	2XMIC	0.2±0.0001 (51)[*]	0.121±0.0002 (71.04)[*]	0.184±0.0002 (56)[*]
	MIC	0.247±0.0002 (41)[*]	0.174±0.0001 (58.57)[*]	0.27±0.0002 (34.9)[*]
	0.5X MIC	0.286±0.000 (31.7)[*]	0.209±0.000 (50.1)[*]	0.387±0.0001 (7.8)[*]

CTR: without drug (control).* P<0.05: Significant value, compared to controls. NS: Not significant. MIC: Minimum inhibitory concentration.

Table 5. Effect of MIC, Sub-MIC and above MIC concentrations on mature biofilms formed by the tested *Candida* spp.

Drugs / Microorganism	Conc	Optical Density± SD (% of biofilm reduction)		
		ketoprofen	ibuprofen	Diclofenac Sod
C. albicans ATCC 10231	**CTR**	0.384		
	2XMIC	0.174±0.000 (54.55)[*]	0.228± (40.54)[*]	0.159± (58.5)[*]
	MIC	0.2±0.000 (47.59)[*]	0.336± (35.13)[*]	0.187± (51.18)[*]
	0.5X MIC	0.24 ±0.0001 (36.63)[*]	0.336± (12.31)[*]	0.353± (7.9)[*]
C. albicans	**CTR**	0.39		
	2XMIC	0.193±0.0001 (50.44)[*]	0.194±0.0002 (50.12)[*]	0.144±0.0001 (63.06)[*]
	MIC	0.228±0.000 (41.45)[*]	0.24±0.000 (38.30)[*]	0.158±0.0002 (59.29)[*]
	0.5X MIC	0.342±0.0002 (12.1)[*]	0.3±0.0001 (23)[*]	0.238±0.0003 (38.82)[*]
C. glabrata	**CTR**	0.59		
	2XMIC	0.337±0.000 (42.73)[*]	0.362±0.0001 (38.64)[*]	0.338±0.0001 (42.68)[*]
	MIC	0.445±0.0002 (24.5)[*]	0.469±0.0002 (20.4)[*]	0.367±0.000 (37.72)[*]
	0.5X MIC	0.533±0.0003 (9.5)[*]	0.522±0.000 (10.3)[*]	0.51±0.000 (12.51)[*]
C. Krusei	**CTR**	0.878		
	2XMIC	0.589±0.0001 (32.89)[*]	0.436±0.0002 (50.23)[*]	0.426±0.0001 (51.38)[*]
	MIC	0.688±0.0001 (21.6)[*]	0.575±0.0001 (34.47)[*]	0.542±0.0002 (38.20)[*]
	0.5X MIC	0.749±0.000 (14.6)[*]	0.68±0.0001 (22.55)[*]	0.621±0.000 (29.18)[*]

CTR: without drug (control).* P<0.05: Significant value, compared to controls. MIC: Minimum inhibitory concentration.

4. Discussion

Candida spp. can cause opportunistic diseases specially in patients who are immune compromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy or undergoing invasive surgical procedures and organ transplantation. Among *Candida* spp., *C. albicans* is the most common cause of many fungal infections, but non-albicans spp. infections are in increase [33,34].

All *Candida* species can cause urinary tract infections (UTIs) but according to many epidemiological studies C. *albicans* is the most common isolated species [35,36]. Non *Candida albicans* also can cause fungal UTIs, and in many parts worldwide, non *albicans* predominate. The

increase of antifungal resistance increase the need for new antifungals. Many studies showed that NSAID showed activity against *Candida* [37,38,39] that led us to undergo this study on the activity of three non-steroidal anti-inflammatory drugs on *C. albicans* and 2 non-albicans strains

In this study we determined the ability of the tested organisms to form biofilm, antifungal activity of the tested drugs and their effects on the morphological switch of the tested *C. albicans* strains. The tested strains were moderate to strong biofilm producers. Also, the effects of the tested agents were found to be concentration dependent. *Sodium Diclofenac*showed lower MIC against *C. albicans* and *C. glabrata* while ibuprofen showed lower MIC against *C. krusei* in comparison to other NSAIDs. By testing their effect on germ tube formation, it was found that *Sodium Diclofenac*at 500 µg/ml inhibit germ tube formation while ketoprofen and ibuprofen inhibit it to lesser extent that agree with many studies [40,41,42].

In addition, The data demonstrated in this study reveal successful reduction in the *in-vitro* adherence and a significant destructive effect on mature biofilms of *Candida spp.*. *Sodium Diclofenac*showed the highest inhibitory effect on the adherence of *C. albicans* and *C. glabrata* (51.1-76.9% at MIC and 56.6-83.3% at 2XMIC) and the highest disruptive effect (37.72-59.29% at MIC and 42.68-63.06% at 2XMIC) on mature biofilms formed by all the tested spp. in comparison to ibuprofen and ketoprofen. Also, by testing its effect on biofilm formed on polyurethane segments, it was found that no biofilm mass formed and the formation of pores in the membranes of *Candida* spp. followed by complete lysis with higher concentration of diclofenac sod.

Ghalehnoo *et al.,* [41] and Bink *et al.,* [42] have documented the effect of diclofenac on *C. albicans* biofilms or on cells grown on solid media. The second study [42] showed inhibition of the yeast-to-hyphae transition by diclofenac on solid YPD media. The first study demonstrated a significant reduction of biofilm formation in YPD on small polyvinyl chloride disks by diclofenac when added during the period of adhesion and biofilm formation, quantified by XTT measurements.

Alem and Douglas [43] attributed the antifungal and antibiofilm activity to the diclofenac-induced increase of the membrane permeability of the *C. albicans* biofilm cells which may be a result of the effect of diclofenac on the biosynthesis of fungal prostaglandins. Also, they demonstrated that biofilms of various *C. albicans* isolates and strains, grown in the presence of diclofenac, showed increased susceptibility to caspofungin. Even the activity of caspofungin against biofilms of a caspofungin-resistant isolates could be increased by diclofenac pretreatment.

Our study showed a marked activity of Ibuprofen against the tested *Candida spp.* especially *C. krusei* that agree with several studies [38,44]. The mechanism proposed for fungicidal activity of ibuprofen is in conformity with its lipophilic character [45]. Fungicidal activity of Ibuprofen is concentration dependent as it showed fungicidal activity with high doses but fungistatic activity with low doses. High doses leads to the direct damage of membranes similar to that shown by SEM images in our study. Pina-Vaz *et al.,* [43] showed that ibuprofen at low concentration inhibit growth in a manner resembles that shown by lipophilic azoles by interfering with the metabolic pathways more than by direct damage to membranes. Also, The concentration of ibuprofen in urine of patients under the conventional standard systemic regimens reaches values above those shown to possess an antifungal activity, pointing to the potential usefulness of ibuprofen for urinary tract candidosis.

In addition, our results showed that *C. krusei* produced extensive biofilm mass on surfaces. This may be due to that C. *krusei* strains are more hydrophobic than C. *albicans,* C. *tropicalis, C. kefyr, C. parapsilosis, C. glabrata* or *C. lipolytica* isolates. C. *krusei* is endowed with a greater ability to colonize inert surfaces such as implants and catheters by virtue of its cell surface hydrophobicity [46].

Ketoprofen antifungal activity, their ability to decrease adherence and to detach mature biofilms were also proven by Chowdhury and Mukhopadhyay [45] and Abdelmegeed and Shaaban [47].

5. Conclusion

Diclofenac sodium, Ibuprofen and Ketoprofen showed antifungal activity, antiadherent activity, and can detach mature biofilms. Also, *Sodium Diclofenac*can inhibit morphological switch of *C. albicans* at a concentration of 500 µg/ml. So, these drugs are suggested to show a useful role in the treatment or the prevention of biofilm associated diseases and prevent adherence or disrupt mature biofilms of *Candida Spp.* to medical device surfaces.

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