Antibiofilm Activity of Nystatin, Aspirin and EDTA Against *Candida albicans* Isolated from Iraqi Women with Vulvovaginitis

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Abstract

The vulvovaginitis candidiasis is often associated with biofilm formation by *Candida albicans* and using of antifungal agents against *C. albicans* biofilms is urgently needed. Microtiter plate assay using crystal violet was used for detection the ability of *Candida albicans* to form biofilm and the microtiter broth dilution method was used for determination the minimum inhibitory concentrations (MICs). Out of 42 *Candida albicans* isolated from vulvovaginitis, 37 (88%) can produce biofilm at the varying degrees. Twenty-eight (75.7%) isolates could form a strong biofilm. The results of minimum inhibitory concentrations (MICs) of Nystatin, Aspirin and EDTA (Ethylenediaminetetraacetic acid) against 28 *C. albicans* isolates which formed the strong biofilm, revealed that range of concentrations of Nystatin were (6.25-100 µg/ml), while the MICs of aspirin and EDTA were more than 1000 µg/ml. It was obvious that the Nystatin had the inhibitory activity at the concentrations 6.25 and 12.5 µg/ml. The highest antibiofilm activity by Nystatin were demonstrated at the subinhibitory concentration 50 µg/ml with biofilm eradication percent (75.80%), while the lowest antifungal effect (2.86-10.70%) was at very low concentrations (3.125-6.25 µg/ml). Also, there was an obvious biofilm eradication of Aspirin and EDTA at the concentrations 500 and 1000 µg/ml but the effect of aspirin at the concentration 1000 µg/ml (70.51%) is more than EDTA (60.12%) in contrast with the concentration 500 µg/ml, it was found that the effect of EDTA (51.29%) is more than aspirin (34.25%). In conclusion, the present study highlights the role of Aspirin and EDTA as antibiofilm agents when used with Nystatin which have the ability to inhibit the growth of *C. albicans* in patients with vulvovaginitis.

Keywords: *Nystatin, Aspirin, EDTA, C. albicans, Biofilm, Vulvovaginitis.*

Introduction

Vulvovaginal candidiasis (VVC) is considered as the main infection caused by *Candida albicans*. Numerous virulence determinants and escalating resistance to antifungal therapy have contributed to its pathogenicity(1). Some virulence factors such as dimorphism and the ability to adhere and form biofilm on medical device and/or the host mucosal epithelium, enhance the pathogenicity of *C. albicans*(2). Vulvovaginal candidiasis defined as a disorder characterized by signs and symptoms of vaginal inflammation when the *Candida* species are found and is an ever living problem affecting 70–75% of women of reproductive age at least once during their life(3). The evolution of drug resistance of *Candida* species to conventional antifungal agents has been a major medical challenge worldwide; attempt to use the potential antifungal agents with appropriate therapy efficacy and minimum effects is considerably growing(4). The mechanism underlying development of antifungal resistance of *C. albicans* are complex and involve multiple pathways and genes. Further,

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these mechanisms continue to change and evolve and challenging the medical clinic [5]. The widespread use of antibiotics, frequent use of indwelling medical devices, and a trend towards increased patient immunosuppression has resulted in a creation of opportunity for clinically important *Candida* to form biofilms and there is growing evidence of the importance of *Candida* biofilms in clinical problems [6]. Therefore the aim of this study is investigate the biofilm formation of *C. albicans* isolates as the causative agent of vulvovaginitis in Iraqi women, also using of some compounds such as Aspirin and EDTA as antifungal agents against the high antifungal resistant isolates.

Materials and Method

***Candida albicans* isolates:** In this study, a total of 42 *C. albicans* clinical strains were collected from women patients with vulvovaginitis from three hospitals in Baghdad, Iraq, during the period from September to December 2019. All strains were previously identified by API Candida system (bioMérieux, France) and confirmed using VITEK 2 compact system (bioMérieux, France).

Quantitative biofilm production assay: The Colonies from all isolates of fresh *C. albicans* cultures (48 hours) were grown at 37°C in Sabouraud dextrose broth medium for 24 hours. Biofilm formation was tested by adding 100 µl of this standardized cell suspension to wells of microtiter plates that contained 100 µl of fresh Sabouraud dextrose broth media and incubating them at 37°C for 48 hours. Thereafter, the medium was removed and planktonic cells were removed by washing the biofilms in phosphate buffered saline. After staining plates with 2% crystal violet for 20 m, excess stain was removed using water. The plates were air dried and then the dye was resolubilized with absolute ethanol. The optical density (OD) of each well was measured at 570 nm using Enzyme-Linked Immunosorbent Assay (ELISA) reader (BioTek, Korea). Optical density cut-off value (ODc) was calculated using the equation: average OD of negative control + (3*SD of negative control) [7].

Minimum inhibitory concentration of Nystatin, Aspirin and EDTA: Nearly, 100 µl (0.5 McFarland) of the *C. albicans* culture was inoculated into each well of a 96-well microtiter plate containing 100 µl Nystatin, Aspirin and EDTA at different concentrations (0.39–200 µg/ml). Wells without Nystatin, Aspirin and EDTA were used as a positive control while those without *Candida* were considered as negative controls. After 24 hours incubation at 37°C, the wells were visually inspected for the growth. The MIC was considered as the lowest concentration of Nystatin, Aspirin and EDTA that inhibits the Yeast growth[8].

Antibiofilm activity of Nystatin, Aspirin and EDTA: This test was performed on four strains that showed strong biofilm formation ability in the biofilm production assay. The effect of different concentrations of Nystatin (3.125-100 µg/ml), while Aspirin and EDTA (31.25-1000 µg/ml) to inhibit the ability of *C. albicans* cells to form a biofilm was assessed using the TCP method adopted by Khodavandi *et al*. (2011) [9]. Nearly, 100 µl of 0.5 McFarland yeast cultures was dispensed into each well of 96-well polystyrene microtiter plates in the presence of 100 µl of the antibiofilm agent at different concentrations, and plates were incubated at 37°C for 48 hours. Antimicrobial agent free wells served as positive controls for the biofilm growth. After incubation, the medium and non-adherent cells were removed and wells were washed three times with sterile PBS. The plates were air dried and then the dye was resolubilized with absolute ethanol. The OD of each well was measured at 570 nm using ELISA reader (BioTek, Korea). Each assay was performed in triplicates.

Statistical Analysis: The Statistical Analysis System- SAS (2012) [10] program was used to detect the effect of difference factors in study percentage. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Least significant difference LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

Results and Discussion

Biofilm formation: Out of 42 *Candida albicans* isolates, 37 (88%) can produce biofilm at the varying degrees. Twenty-eight (75.7%) isolates could form a strong biofilm, while 6 isolates were the moderate producer, and only 3 isolates were weak biofilm formers. Also it was found that five isolates don't have the ability to formation the biofilm (table 1). Microtiter plate assay using crystal violet was used for detection the ability of *Candida albicans* to form biofilm (figure 1).

Table 1. Distribution of biofilm formation ability among *Candida albicans* isolates.

Candida albicans	Biofilm formation			
	Weak	Moderate	Strong	Negative
Total no of Isolate = 42	3	6	28	5
%	7.14%	14.29%	66.67%	11.90%
Chi-Square (χ^2)	12.073 **			

** (P≤0.01).

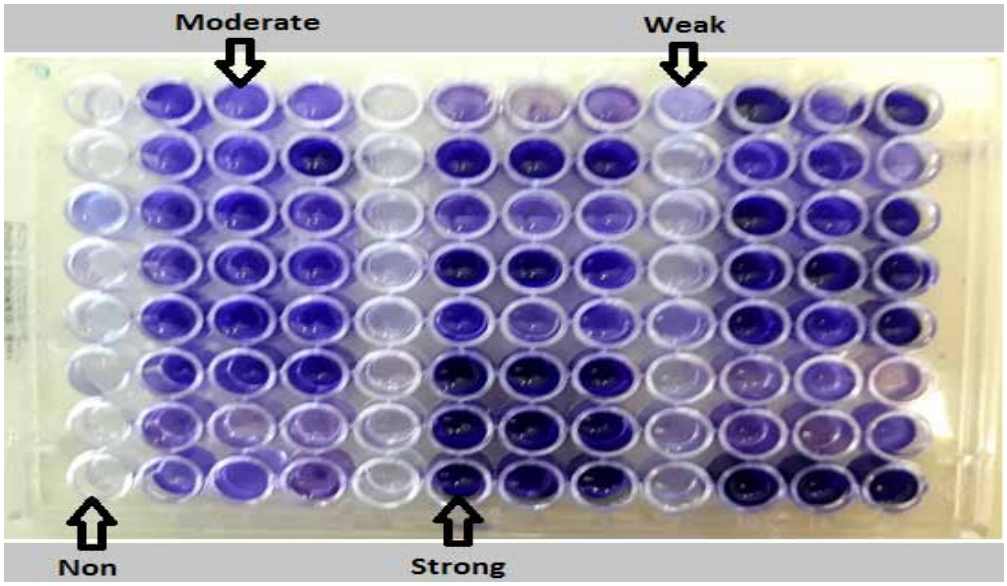

Figure 1. Biofilm formation detection of *Candida albicans* isolates by microtiter plate assay.

This study revealed that the most of *Candida albicans* isolated from Iraqi women patients with vulvovaginitis formed a strong biofilm. Many studies demonstrated the ability of *Candida albicans* clinical isolates to form heterogeneous biofilms. The presence of these communities in vulvovaginitis may explain why *C. albicans* infections remain unresponsive to therapy, and there was a relationship between biofilm formation and antifungal resistance among *Candida* isolates [11, 12]. The study of Shreif *et al.* (2019) [13] in Egypt which included one hundred *Candida albicans* isolates from patients with nosocomial infections revealed that the biofilm capacity was identified by the microplate method in 58% of *C. albicans* and the optical density was intense in 20 isolates, moderate in 21 isolates and mild in 17 isolates. The local study of Mohammed *et al.* (2017) [14] demonstrated that *C. albicans* was the most predominant species among vaginal specimens in percentage (45%) and all *C. albicans* isolates were biofilm producers with variable strength, Among vaginal isolates; 10/22 (45.5%) were weak biofilm formers whereas moderate or strong biofilm formers were 12/22 (54.5%). In a previous study, it was found the *Candida* biofilms have important clinical implications since the biofilm associated with *Candida* or *Gardnerella* genital infections may act as a chlamydial reservoir contributing to the transmission of *Chlamydia trachomatis* in the population, alongside its dissemination in the female upper genital tract [15]. The biofilm formation is very important virulence factor in *C. albicans*, where this species expresses hyphal-specific adhesins and regulators required for adhesion. Also, the morphological dimorphism in *Candida albicans* supports noticeable phagocyte escape mechanism [16].

The present study investigated the role of some compounds as antibiofilm agents against the biofilm formation in *C. albicans* isolates, these compounds are Nystatin, Aspirin and EDTA. At first, the minimum inhibitory concentrations (MICs) of these compounds were measured by microdilution method in 96 well microtiter plates with resazurin dye and then detection the antibiofilm concentration by exposing the yeast

to subinhibitory concentration in the same plate with crystal violet staining.

The results of minimum inhibitory concentrations (MICs) of Nystatin, Aspirin and EDTA against 28 *C. albicans* isolates which formed the strong biofilm, revealed that range of concentrations of Nystatin were (6.25-100 µg/ml), while the MICs of aspirin and EDTA were more than 1000 µg/ml. The table 2 and figure 2 demonstrated the MICs of 2 isolates by using the double concentrations (from 0.39 to 200 µg/ml) and it was obvious that there is no effect of Aspirin and EDTA on the growth of *C. albicans* at the used range while the Nystatin had the inhibitory activity at the concentrations 6.25 and 12.5 µg/ml against the isolates 1 and 2 respectively.

Table 2. The minimum Inhibitory Concentrations (MICs) of antibiofilm agents against *Candida albicans* isolates.

Antibiofilm agent	Candida albicans	Minimum Inhibitory Concentration (MIC) (µg/ml)									
		0.39	0.78	1.56	3.12	6.25	12.5	25	50	100	200
Nystatin	Isolate 1						+				
	Isolate 2					+					
Asprin	Isolate 1	-	-	-	-	-	-	-	-	-	-
	Isolate 2	-	-	-	-	-	-	-	-	-	-
EDTA	Isolate 1	-	-	-	-	-	-	-	-	-	-
	Isolate 2	-	-	-	-	-	-	-	-	-	-

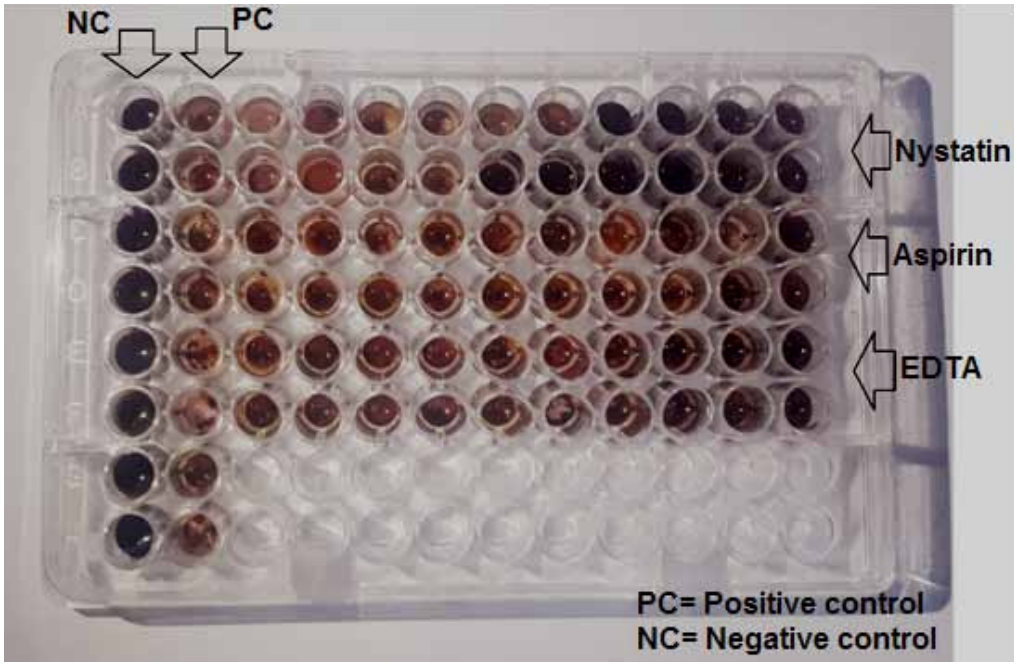

Figure 2. The minimum Inhibitory Concentrations (MICs) of antibiofilm agents against *Candida albicans* isolates by microtiter plate assay with resazurin dye.

The previous study included 14 yeast strains 7 control strains were investigated regarding their *in vitro* susceptibility to the polyene antifungal agent nystatin, the Minimum inhibitory concentrations (MICs) for nystatin were measured by both visual examination, and spectrophotometric measuring after 24 and 48 hours incubation time at 36°C. The visual read-out of growth inhibition revealed MICs for nystatin in a range from 0.625 to 1.25 µg/ml for all *Candida* species tested, where the *Candida albicans* strains, and both

strains of *C. glabrata* and *C. tropicalis,* showed low MIC values 0.625 µg/ml [17]. Many previous studies found that nystatin MICs for *C. albicans* isolated from vulvovaginitis ranged from 1 to 16 µg/ml, with a MIC inhibiting 90% of isolates (MIC_{90}) of 4 to 16 µg/ml [18,19].

The current study consistent with Al-Bakri *et al*. (2009) [20] study which indicated to need of high concentrations of Aspirin and EDTA to achieve MIC against *C. albicans*, where Aspirin MIC values (mg/ml) of 2.03, 1.2 and 2.65 were achieved against *P. aeruginosa, E. coli* and *C. albicans*, respectively. An EDTA concentration as high as 60 mg/ml failed to attain the MBC value, while aspirin MBC values (mg/ml) of 4.8, 4.9 and 5.28 were reported against *P. aeruginosa, E. coli* and *C. albicans*, respectively. Also, the results of Cederlund and Mardh (1993) [21] demonstrated that Aspirin possesses a relatively weak broad-spectrum antimicrobial activity where relatively high concentrations of aspirin are needed to effect biostatic activities and even higher concentrations are needed for biocidal activities. Antipyretics such as Aspirin primarily act by inhibiting prostaglandin synthesis. Fungi produce prostaglandins, and although their exact function is uncertain, it is thought that they influence virulence, in particular controlling the yeast-to-hypha transition and biofilm production. Also, changing the surface hydrophobicity of microbes and modifying the susceptibility of microbes to antimicrobial therapy[22;23].

It is also known that anticoagulant and calcium and magnesium chelator EDTA (Ethylenediaminetetraacetic Acid) may have antimicrobial activity against several Gram-positive bacteria and *Candida* spp. EDTA forms chelation with divalent metals such as Mg(2+) and Ca(2+), which are required by various essential enzymes[24,25]. EDTA demonstrated the highest antifungal activity in comparison with routine antifungal drugs by prevent the binding of *C albicans* to the proteins in a dose-dependent manner and reduces the growth of *C albicans* by removing calcium from the cell walls and causing collapses in the cell wall, and by inhibiting enzyme reaction[26]. EDTA acts on the cell surface, resulting in the rapid release of approximately half of the lipopolysaccharide with a negligible loss of other cell components. The disruption of the lipopolysaccharide structure in the outer membrane of Gram-negative bacteria occurs because EDTA chelates divalent cations. The release of lipopolysaccharides increases the membrane permeability to other agents, hence the potentiating action[27].

The effect of some antifungal and antibiofilm agents (Nystatin, Aspirin and EDTA) on the biofilm formation of *C. albicans* isolates were achieved using microtiter plate assay with crystal violet staining (figure 3).

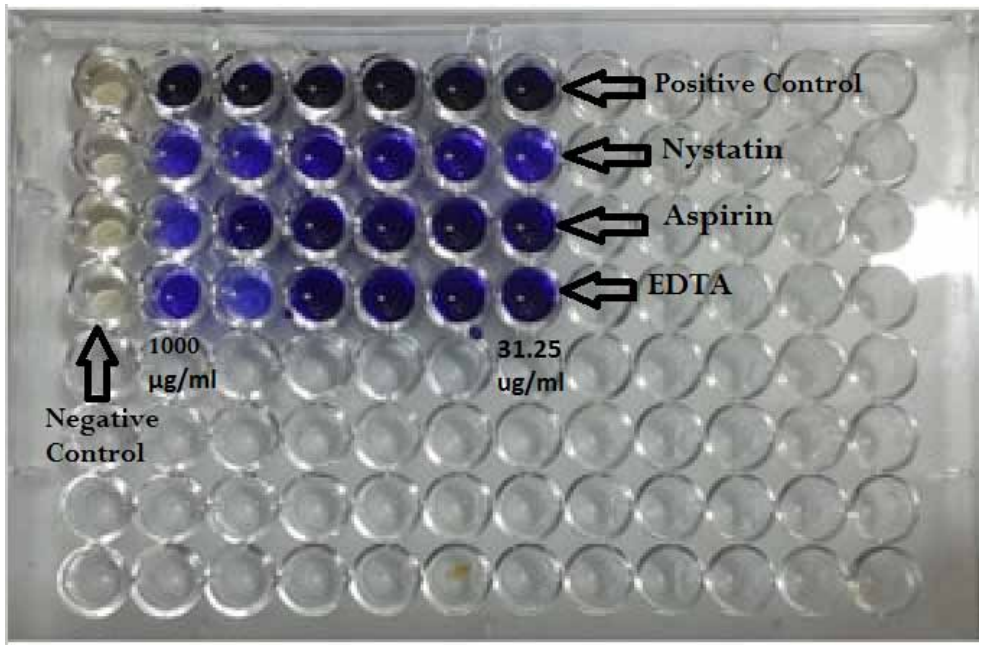

Figure 3. Biofilm quantification by microtiter plate assay of *Candida albicans* (isolate 5) at different subinhibitory concentrations of Nystatin, Aspirin and EDTA.

The results of effect Nystatin, Aspirin, and EDTA at subinhibitory concentrations against the biofilm formation of 28 *C. albicans* isolates which formed the strong biofilm, revealed that the Inhibition activity of biofilm formation by nystatin was stronger relative to Aspirin and EDTA, and this antifungal agent had antibiofilm effect even though at very low concentrations (table 3).

Table 3. The percentages of biofilm reduction by Aspirin, EDTA and Nystatin against *Candida albicans* at different subinhibitory concentrations.

	Biofilm reduction (%)						**Chi-Square (χ^2)**
Antibiofim agent (µg/ml)	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	
Aspirin	61.90–70.51%	29.75–34.25%	8.95–10.71%	1.54–5.18%	12.01–14.33%	2.35–8.58%	12.63**
EDTA	54.43–60.12%	48.27–51.29%	1.83–3.27%	0.74–2.08%	4.00–5.89%	26.19–28.65%	11.08**
	Biofilm reduction (%)						---
Antibiofim agent (µg/ml)	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**	**---**
Nystatin	76.66–83.30%	69.51–75.80%	44.86–57.15%	29.49–34.35%	9.12–10.70%	2.86–4.22%	13.46**

** (P≤0.01).

EDTA (Ethylenediaminetetraacetic Acid): The effect of three antibiofilm agents (Nystatin, Aspirin and EDTA) on the nystatin resistant *C. albicans* isolate 5 (MIC=100 µg/ml) was summarized at the figure 3 and table 4.

Table 4. The absorbance of biofilm formation of *Candida albicans* (isolate 5) at different subinhibitory concentrations of Aspirin and EDTA.

	O.D. (630nm)						**LSD value**
Antibiofim agent (µg/ml)	**1000**	**500**	**250**	**125**	**62.5**	**31.25**	
Aspirin	0.581 b	1.138 a	1.211 a	1.589 a	1.384 a	1.050 a	0.456 *
EDTA	0.695 d	0.808 cd	1.505 ab	1.601 a	1.509 ab	1.195 bc	0.398 *
	O.D. (630nm)						
Antibiofim agent (µg/ml)	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**	**---**
Nystatin	0.262 d	0.411 cd	0.724 bc	1.030 b	1.401 a	1.580 a	0.377 *

Means having with the different letters in same row differed significantly.
* (P≤0.05).
O.D. of positive control = 0.063
O.D. of negative control = 1.569

EDTA (Ethylenediaminetetraacetic Acid): The highest antibiofilm activity by Nystatin were demonstrated at the subinhibitory concentration 50 µg/ml with biofilm eradication percent (75.80%), while the lowest antifungal effect (2.86-10.70%) was at very low concentrations (3.125-6.25 µg/ml). Also, there was an obvious biofilm eradication of Aspirin and EDTA at the concentrations 500 and 1000 µg/ml but the effect of aspirin at the concentration 1000 µg/ml (70.51%) is more than EDTA (60.12%) in contrast with the concentration 500 µg/ml, it was found that the effect of EDTA (51.29%) is more than aspirin (34.25%). The effect of other concentrations (31.25-250 µg/ml) on the biofilm formation was not significant in comparison with the higher concentrations. The results revealed that nystatin at low concentration showed a significant effect as antifungal and antibiofilm agent in comparison with the high concentrations of aspirin and EDTA.

Concerning inhibition of biofilm production in the presence of subinhibitory concentrations of the Nystatin, the present study reflected more promising significant effects for treatment with this antifungal agent (75.80% reduction) which are more than studies reported by other investigators, as in El-Houssaini *et al*. (2019) [28] (30.86% reduction), and the study of Redding *et al*. (2009)[29] (70%

reduction). Many studies showed that the treatment of clinical *C. albicans* isolates with subinhibitory nystatin, concentrations significantly decreased production of extracellular hydrolases, Also, the greatest inhibitory effect on phospholipase and aspartyl protease production and a noticeable significant impact on inhibiting biofilm formation of *C. albicans* clinical isolates [28]. De Prijck and coworkers investigated the effect of nystatin released from modified polydimethyl siloxane disk as a model for incorporating antifungals in medical devices against biofilm formation by *Candida* spp. Nystatin exhibited a concentration-dependent inhibitory effect on *Candida* biofilm formation in a microtiter plate [30].

It was demonstrated that EDTA alone (at 25 and 2.5 mM) significantly reduced fungal metabolic activity in preformed biofilms. Also, EDTA combined with fluconazole significantly reduced the growth of biofilm when compared to biofilm treated with fluconazole alone [31]. EDTA resulted in partial reduction of catheter colonization by *C. albicans*, As previously reported, the combination of EDTA with low minocycline concentration (0.1 mg/ml) resulted in a significant decrease in catheter colonization but combined with higher concentrations of minocycline resulted in complete eradication of *C. albicans* biofilms [32].

According to the results of effect of aspirin on the biofilm formation in *C. albicans*, it was found that cyclooxygenase-dependent synthesis of fungal prostaglandins is important for both biofilm development and morphogenesis in *C. albicans* and may act as a regulator in these physiological processes and that aspirin possesses potent antibiofilm activity in vitro and could be useful in combined therapy with conventional antifungal agents in the management of some biofilm-associated *Candida* infections [33]. Studies have shown that cyclooxygenase (COX) inhibitors, such as aspirin, ibuprofen, and indomethacin, combined with fluconazole can significantly reduce *Candida* adhesion and biofilm development and increase fluconazole susceptibility; the MIC of fluconazole can be decrease from 64 to 2 µg/ml when used in combination with ibuprofen. In addition, *in vivo* studies have also confirmed the antifungal activities of these inhibitors [34]. Aspirin alone or in combination with conventional antifungal drugs is also beneficial for the treatment of vulvovaginal candidiasis by inhibition of cyclooxygenases in host cells and by inhibition of 3-hydroxyoxylipins in *C. albicans* [35].

Conclusion

The present findings indicated to the role of some drugs or compounds such as Aspirin and EDTA when used with effective antifungal agents in control the infections of *Candida albicans* especially in the women with vulvovaginitis by inhibition the biofilm formation which considered as the main virulence factor in Candida species.

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Ethical Clearance: Yes

Conflict of Interest: Nil

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