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Effect of Histatin 5 Peptide on *ERG11* and *CDR1* Gene Expression in Fluconazole-Resistant *Candida albicans* Isolated from Vaginitis

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Abstract: *Candida albicans* with antifungals resistance and biofilm formation may cause complicated infections among women with Vaginitis. Therefore, the search for new drugs capable of overcoming antifungal resistance is essential. One of the alternative therapeutic agents is Histatin 5 which is an antimicrobial peptide that can be found naturally in human saliva. This study was aimed to evaluate the role of Histatin 5 as antimicrobial and antibiofilm agent against fluconazole-resistant *C. albicans* isolates. Also, study the effect of this peptide on the gene expression of the fluconazole-resistant genes (*CDR1* and *ERG11*). The results of minimum inhibitory concentrations (MICs) using microtiter plate method revealed that the MICs of seven fluconazole-resistant *C. albicans* isolates were (62.5-250 µg/ml). The antibiofilm activity of Histatin 5 demonstrated that this peptide was able to reduce the biofilm formation to 23% at the concentration 62.5 µg/ml, and to 41% at the concentration 31.25, in comparison with the control (100%). Relative quantification (RQ) was used to calculatethe fold change in gene expression using the delta delta Ct value and the gene expression of *C. albicans* isolates was calculated before and after treatment with the subinhibitory concentration of each isolate. Before to Histatin 5 treatment, the fold of gene expression in the *CDR1* gene was slightly higher (1), and the fold of the resistant isolates after Histatin 5 treatment was low (0.07-0.38). Also for ERG11 gene, Before Histatin 5 treatment, the fold of gene expression was slightly higher (1). After Histatin 5 treatment, the fold of gene expression was low (0.11-0.31). In conclusion, the obvious activity of Histatin 5 peptide as antimicrobial and antibiofilm agent against fluconazole-resistant *C. albicans* isolates may be contributed in the management and control the vaginitis infections among Iraqi women.

Keywords: *Candida albicans;* Histatin5; *ERG11*, *CDR1*, Gene expression.

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Introduction

Candidiasis is one of the most important fungal infections in hospital settings. This infectious disease may be caused by at least 20 different yeast species of the genus *Candida* especially *Candida albicans* (1). Different species of *Candida* yeast can act as opportunistic microorganisms in people with imperfect and suppressed immune systems (2). *Candida albicans*, along with other closely related *Candida* species, are the primary causative agents of vulvovaginal candidiasis (VVC); a multifactorial infectious disease of the lower female reproductive tract resulting in pathologic inflammation. Unlike other forms of candidiasis, VVC is a disease of immunocompetent and otherwise healthy women (3,4). Studies revealed that the use of azole antifungal agents

such as fluconazole has increased. This is mostly due to the increasing number of *C. albicans* infections. Most of these cases have higher azole minimum inhibitory concentrations (MICs) and the infections they cause are mostly difficult to cure. High recurrence rates of *C. albicans* vaginitis, cannot be successfully suppressed by fluconazole in long term therapies, and drug resistance is growing (5,6). The resistance to antifungal is one of the biggest health problems in the world. Several factors contribute to the rise of cases of infections caused by multidrug resistant microorganisms: the increase in patients with suppressed immune systems due to diabetes, cancer and AIDS. The higher number of patients who need invasive treatments; and the higher prevalence of treatment with steroids, hyperglycemia, use of broad-spectrum antibiotics and antifungals in sub inhibitory concentrations (7).The antimicrobial peptides isolated from the human body with antimicrobial activity are classified as antimicrobial peptides (AMPs). In general, they are characterized as small basic cationic peptides derived from proteins that exhibit antimicrobial activity. These ones include lactoferrins, defensins, cathelicidins and histatins. A member of the Histatin family, Histatin 5 (Hst5) is found naturally in human saliva; with the greatest antifungal activity in the family, and it can inhibit the growth of yeasts and hyphae of *C. albicans* (8).

Antifungal resistance is generally driven by several factors, including point mutations of the cellular target, overexpression of target molecules, and efflux pump extrusion of antifungals (9). There were many evidences to the important role of biofilm formation in the virulence (10), and in the histopathogenesis of vaginal candidiasis and the recalcitrance of the infection to antifungal treatment, where biofilm growth on the vaginal epithelium also led to high resistance to antifungal treatments and promoted the formation of antifungal-tolerant persister cells (11). The aim of this study is to evaluate the effect of the antimicrobial peptide Histatin5 on the growth of *C. albicans* isolated form Vaginitis and on the gene expression of fluconazole resistant genes (*ERG11* and *CDR1*).

Materials and methods

Candida albicans isolation and identification

Vaginal samples(250) were inoculated in sterile tubes with 9 ml of Sabouraud Dextrose Broth (SDB) (Himedia, India) and incubated aerobically for 24 h at 37°C. A 10 µL sample was then taken from each sterile tube and inoculated on Sabouraud Dextrose Agar (SDA) (Himedia, India) with chloramphenicol and gentamicin. Petri dishes were incubated at 37°C in an aerobic environment for 24–72 h, and growth from at least 5 colonies was analyzed. These colonies were examined macroscopically, followed by Gram stain, for microscopic examination. *Candida albicans* species and other organisms were identified according to germ tube formation and (some (biochemical tests API). Also, the flucanazole resistant strains were detected by disc diffusion test (12).

Antimicrobial peptide histatin 5

Antimicrobial Peptide. Histatin 5(DSHAKRHHGYKRKFHEKHHSHRGY) was obtained from Genemed Synthesis, Inc. (San Francisco, CA). Analysis of this material by HPLC and mass spectroscopy revealed a purity of 98%. The peptide was dissolved in 10 mM potassium phosphate buffer (PPB; pH 7.0) to a final concentration of 10 mg/ml.

Minimum inhibition concentration (MIC) of histatin 5

Minimum inhibition concentration (MIC) value of *Candida albicans* was determined by broth dilution method with microtiter plate method described in the National Committee for Clinical Laboratory Standards (2020). The Histatin 5 was added into Mueller Hinton Broth (MHB) to achieve concentrations ranging from 0.97 to 500 µg/ml. Then, the *Candida albicans* inoculate were added into 10 mL tube containing 2 mL MHB (containing different concentrations of Histatin 5) as the medium to approximately achieve an initial inoculum of 1×10^7 CFU/mL. 6.25% DMSO was used as negative control.

Antibiofilm activity of histatin 5

This test was performed on three strains that showed strong biofilm formation ability in the biofilm production assay. The effect of different concentrations of Histatin 5 (7.81-250 µg/ml) to inhibit the ability of *C. albicans* cells to form a biofilm was assessed using the TCP method adopted by (13). 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.

PCR primers and conditions

For the purpose of PCR assay, the specific primers of MHB fluconazole resistant genes (*ERG11* and *CDR1*) as shown in Table (1): (14).

Table (1): Oligonucleotide primers sequences of fluconazole resistant genes (*ERG11* and *CDR1*) genes used in this study

Gene Name		Sequence 5'-3'	Annealing temperature (C^o)	Product size (bp)
CaERG11b	**F**	TTTGGTGGTGGTAGACATAGAT	60	128
CaERG11b	**R**	TAATCAGGGTCAGGCACTTT		
CaCDR1	**F**	GATTCTCAAACTGCCTGGTC		158
CaCDR1	**R**	CCAAAATAAGCCGTTCTTCCAC		
18S Rrna	**F**	TCTTTCTTGATTTTGTGGGTGG		150
18S Rrna	**R**	TCGATAGTCCCTCTAAGAAGTG		

Using 25µL of PCR reaction, 2.5 µl DNA template (100 ng/µl) is amplified by using 12.5 µl of Go *Taq*® green master mix 2X (Promega, USA) and 1 µl of each primer (10 pmol/µL) for each specific gene, up to the final volume 25 µl with nucleases free water. The extracted DNA, primers and PCR premix is thawed at 4°C, vortexes and centrifuged briefly to bring the contents to the bottom of the tubes. Optimization of polymerase chain reaction was accomplished after several trials. Negative control contained all material except DNA, that D.W. was added instead of template DNA. PCR programs were set on Thermal-cycler (Applied BioSystem, Singapore). The reaction performs according to (14). Agarose gel electrophoresis was used for detection the PCR products. (25) µl of the PCR products were mixed with (2) µl of 6x loading buffer dye and loaded into the individual wells. The

electrophoresis was performed in Tris Acetate EDTA (TAE) buffer for one hour. At the end, the gel was stained in ethidium bromide solution (1 µg/ml) for 15 minutes. The results were analyzed according to the product length which were visualized on gel documentation system and photographed.

Gene expression using RT-PCR technique

The experiment was designed using (3) isolates of flucanazole resistant *C. albicans* that had the two genes (*ERG11* and *CDR1*). The gene expression of the two genes in the resistant isolates was measured before treatment with the peptide and after the treatment. The concentrations of Histatin 5 used in the treatment were below the MIC value to allow the bacterial growth with induction of resistance. To examine the effect of sub-inhibitory concentrations of the peptide on the gene expression.

RNA isolation by TRIzol™

The amount of 500 µl from *Candida* culture was added into a 1.5 ml tube containing 700µl Trizol for each patient and healthy individual and the tube was inverted many times for mixing. Five minutes of incubation to permit complete dissociation of the nucleoproteins complex. The chloroform was added as 0.15 mL of TRIzol™ Reagent used for lysis. The Incubation for 2–3 minutes. The sample was centrifuged for 15 minutes at 12,000 × g. The mixture was separated into a lower red phenol-chloroform, interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA is transferred to a new tube. The RNA was precipitated by adding 0.45 mL of isopropanol to the aqueous phase. The mixture was incubated for 10 minutes. Then Centrifuged for 10 minutes at 12,000 × g. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.

The supernatant was discardedby the micropipette. The pellet gets resuspended by 0.75 mL of 75% ethanol. Then the vortex was used to dissolve the pellet and centrifuge for 5 minutes at7500 × g. The supernatant was discarded by the micropipette. To dry the RNA, pellet the tube opened for 15 minutes. Then the pellet was resuspended by 20 µl of RNase- Free water and incubated at 60 °C for 15 minutes by using a thermomixer. The RNA concentration was measured by Qubit 4.0.

RT-qPCR protocol

Synthesis of cDNA from RNA through a specific primer for (*ERG11* and *CDR1*)and *18S rRNA* transcripts and protoscript cDNA synthesis kit was used. Five microliters from each extracted total RNA sample added into the new PCR tube. Protoscript reaction mix that contain dNTPs, buffer and other essential components added as 10 µl for each sample. MuLV Enzyme added into reaction as 2 µl per sample.

Two microliter oligoT, and the volume completed up to 20 µl by adding 1 µl. The mixture was incubated for 1 hour at 42^{O}C by using thermocycler and this followed by 80 ºC for inactivation of enzyme. The second section of this protocol it's done by choosing the cDNA sample from the patient and control at the same run, for each sample, there are three PCR tubes, one tube for each gene, (*ERG11* and *CDR1*)and *18S rRNA* which is considered as a housekeeping gene in this study. The detection of quantity is based on the fluorescent power of SyberGreen(Table 2). PCR tubes were spine to remove the bubbles and collect the liquid (1 minute at 2000g), and then the program for Real-Time PCR was set up with indicated thermocycling protocol as shown in Table (3). The result was collected and analyzed by Livak formula.

Table (2): RT-qPCR protocol and volumes

Component	20 µl Reaction
Luna Universal qPCR Master mix	10
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Template DNA	5
Nuclease-free Water	3

Table (3): Thermocycling protocol and condition

Cycle Step	Temperature °C	Time	Cycles
Initial Denaturation	95	60 seconds	1
DenaturationExtension	95 60	15 seconds 30 seconds (+ plate read)	40-45
Melt Curve	60-95	40 minutes	1

Delta delta Ct (ΔΔCt) method

The cycle number (Ct) at which signals crossed a threshold set within the logarithmic phase was recorded. The differences in cycle threshold (ΔCt) and fold changes evaluated between the treated groups and calibrators of each gene. These values were normalized to House Keeping gene (*rpo*B) expression as showed below: Relative quantification:

- Delta delta Ct (ΔΔCt) method
- ΔCT =CT gene - CT House Keeping gene (HKG)
- ΔΔCT =ΔCT Treated - ΔCT Control
- Folding $=2^{-\Delta\Delta CT}$

Firstly, the ΔCt between the target gene and the HKG gene is calculated for each sample (for the unknown samples and also for the calibrator sample).

- ΔCt = Ct target – Ct reference gene

Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value:

- ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) calibrator

The normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$ and this value can be used to compare expression levels in samples (15). The samples were analyzed in duplicates and standardized against gene expression. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method, ($2^{-\Delta\Delta Ct}$) between the peptide-exposed and peptide non-exposed *C. albicans.*

Statistical analysis

The Statistical Analysis System-SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Results and discussion

The antifungal activity of Histatin 5 against *C. albicans* isolated from Vaginitis by measured the minimum Inhibitory Concentrations (MICs) of Histatine 5 peptide against *C. albicans* isolates by microtiter plate assay with blue(resazurin dye) color change from blue to pink ; the results revealed that the MICs range of (7) isolates was 62.5 and 250 µg/ml, while one of the isolates was exhibited high resistance where the MIC was above the 500 µg/ml (Figure 1 and Table 4).

PC=Positive Control, NC=Negative Control

Figure (1): The minimum Inhibitory Concentrations (MICs) of Histatine 5 peptide against *Candida albicans* isolates by microtiter plate assay with resazurin dye

Table (4): The minimum Inhibitory Concentrations (MICs) of Histatine 5 peptide against eight of flucanazole resistant *Candida albicans* isolates

Antimicrobial Peptide	*Candida albicans* Isolates	Antimicrobial peptide Concentrations (µg\ml)									
		500	250	125	62.5	31.25	15.625	7.812	3.90	1.95	0.97
Histatine5 peptide	Isolate1	+	+	+	+	+	+	+	+	+	+
	Isolate2	-	-	-	+	+	+	+	+	+	+
	Isolate3	-	-	-	-	+	+	+	+	+	+
	Isolate4	-	-	+	+	+	+	+	+	+	+
	Isolate5	-	-	-	+	+	+	+	+	+	+
	Isolate6	-	-	+	+	+	+	+	+	+	+
	Isolate7	-	-	-	+	+	+	+	+	+	+
	Isolate 8	-	-	-	+	+	+	+	+	+	+

New molecules have been the target of several types of research in recent years because it is necessary to search for potential antifungal treatments that do not present resistance. These can be obtained from different sources such as natural products, synthetic agents or polymeric materials. Marine organisms, entophytic fungi, saponins, alkaloids, peptides and proteins were also investigated (16).

Peptides are molecules of great interest since they can be found naturally in the human body and some already have a function as antifungal agents. Antifungal peptides (AMPs) display a large range of activities, being one of the first lines of defense in the human body as they are able to rapidly inhibit a broad spectrum of pathogenic microorganisms (17). Histatins are an important family of endogenous AMPs rich in histatin, that have antifungal activity against *C. albicans*, as wells as immunomodulatory, and pro-wound healing effects (18). Histatin 5 displays fungicidal activity against a variety of fungi including *Candida albicans, Candida glabrata, Candida krusei, Cryptococcus neoformans*, and unicellular yeast-like *Saccharomyces cerevisiae* (19).

The results of antibiofilm activity of Histatin 5 against *C. albicans* isolated from vaginitis using subinhibitory concentrations of Histatin 5, indicated to the obvious effect of this peptide by reduction the percentage of biofilm formation at the most of peptide concentration used. The current study revealed that the concentration 62.5 µg/ml inhibited the biofilm formation to 23% in contrast with the control (100%).

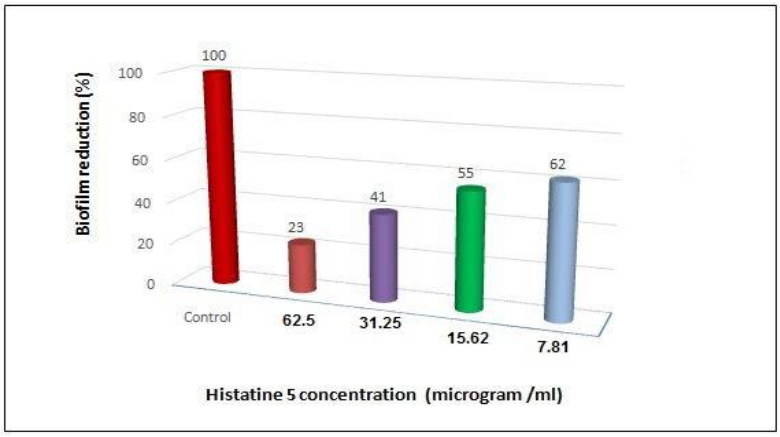

Figure (2): The percentages of biofilm reduction of *Candida albicans* at different subinhibitory concentrations of Histatine 5 peptide (O.D. at 630nm). The result represents the average of 3 isolates

Among pathogenic fungi that affect humans, *C. albicans* is the species most frequently associated with biofilm development. It was found that the effect of Histatin 5 on *C. albicans* biofilm formation revealed that histatin 5 peptide decreased the metabolic activity of the biofilm of inhibition at the highest concentration used (62.5 and 31.25µg/ml). Studying the effect of histatin 5 on *C. albicans* biofilm, Konopka (20) showed that histatin 5 was able to decrease biofilm metabolic activity by concentrations ranging from 62.5µg/mL to 31.25 µg/ ml. The difference between these results and the data obtained in our study may reflect the genetic variability among *C. albicans* clinical isolates. Histatine5 showed a fungal effect that is likely to be useful for the treatment of clinical infections. Histatine5 may be important for the future development of antibiotics against *C. albicans*, especially in multidrug-resistant isolates.

In *CaCDR1* gene when the subMIC value was high (62.5 µg/ml) in resistant was slightly higher and the fold of the resistant isolates after treating them with Histatine5 was low (0.38-0.07). Table(5).

Table (5): Folding of gene expression of *CaCDR1* gene in *C. albicans* isolates before and after treatment with Histatin 5

The isolate	*18SrRNA*	*CaCDR1*	DCT	DDCT	Fold change
Control	17.90	21.68	3.78	0.00	**1.00 ±0.00 a**
A3	20.25	26.92	6.67	1.39	**0.38 ±0.11 b**
A4	15.06	21.01	5.94	3.33	**0.10 ±0.04 b**
A8	17.49	24.83	7.34	3.88	**0.07 ±0.02 b**
LSD	--	--	--	--	0.327 **
P-value	--	--	--	--	0.0062
**** (P≤0.01).**					

Number studies on the resistance mechanisms in *C. albicans* have been carried out, but there are few researchers who are focusing on *C. tropicalis* isolates. There are different mechanisms of resistance; those of the most importance are alterations in the target enzyme or upregulation of the *CDR1* and *MDR1* genes encoding active efflux pump transporters (21). The expression levels of *CDR1*, *CDR2*, and *MDR1* genes increased in most clinical isolates with fluconazole MICs of ≥ 64 µg/mL, while the disruption of these genes resulted in hypersensitivity to azoles (22). Furthermore, the increased

expression of *CDR1*, *CDR2*, and *MDR1* genes was a major contributor to azoles resistance in clinical isolates (23) in this study; our results suggested that the *CDR1* gene was upregulated in fluconazole-resistant isolates. For *CaERG11* gene when the subMIC value was high (32.25 µg/ml) in resistant isolates before treatment withHistatine5, the fold of gene expression was slightly higher and the fold of the isolates after treating them with Histatine5was low (0.31 –0.27).

Table (6): Folding of gene expression of *ERG11* gene in *C. albicans* isolates before and after treatment with Histatin 5

The isolate	*18SrRNA*	*CaERG11*	DCT	DDCT	Fold change
Control	17.90	28.45	10.50	0.00	**1.00 ±0.00 a**
A3	20.25	32.69	12.44	1.68	**0.31 ±0.07 b**
A4	15.06	25.81	10.75	3.18	**0.11 ±0.04 b**
A8	17.49	32.72	15.23	1.88	**0.27 ±0.08 b**
LSD	--	--	--	--	0.224 **
P-value	--	--	--	--	0.0085
**** (P≤0.01).**					

It was concluded that the overexpression of efflux pump transporter genes and a specific point mutation in *ERG11* confers fluconazole resistance in *C. albicans* isolates. Synergism with other inhibitors can also be evaluated to aid new therapeutic options (24). Overxpression of the CDR genes is a common mechanism of resistance, and it appears that the CDR Genes render a cell resistant to many different azoles while over expression of MDR1 appears to be specific for fluconazole (25). Histatine5 peptide showed a fungal effect that is likely to be useful for the treatment of clinical infections. The present reults concluded that the expression gene in *ERG11* and *CDR1* confers fluconazole resistance in *C. albicans* isolates. Synergism with other inhibitors can also be evaluated to aid new therapeutic options., this study provides useful information for the treatment of candidiasis and indicates that clinicians should be cautious of cross resistance within this class of antifungal drugs, especially for the treatment of patients with prior azoles prophylaxis or patients at high risk of *C. albicans* infections (26).

Conclusion

Histatin 5, a cationic histidine-rich salivary peptide of human and higher primates has showed a promising potential to control *C. albicans* infections among women patients. This peptide demonstrated an obvious effect on the growth, biofilm formation and flucanazole resistance genes expression among *C. albicans* isolates.

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