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EFFECT OF THE SILVER NANOPARTICLES ON *ERG11* EXPRESSION IN FLUCONAZOLE-RESISTANT *CANDIDA ALBICANS* ISOLATES.

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Abstract

Candida is a polymorphic opportunistic human fungal pathogen. *Candida albicans* usually treated with azole such as fluconazole, but treatment failure is common especially in immunocompromised patients. So, it's important to find alternative drag to treat infection. This study aimed to test AgNPs susceptibility on FLC resistance *Candida albicans* isolates from different clinical samples and determination ergosterol biosynthesis gene (ERG11) expression after treated with AgNPs. One hundred samples were collected from different clinical samples of patients suffering from candidiasis, aged between 10-70 years old, during the period from December, 2018 and March, 2019. Identification of samples by VITIC2 results showed that 7 species of *Candida* were collected. Out of 100 sample, 46 identified as *Candida albicans*, where 54 were non-albicans *Candida* species. Fluconazole susceptibility of *C. albicans* isolates were tested using well diffusion method. From 46 isolates, 31 were susceptible, 6 were susceptible dose dependent and 9 were resistant. On the other hand the biosynthesis of AgNPs developed a rapid, eco-friendly and convenient green method for the synthesis of stable silver nanoparticles (AgNPs) with an average diameter of 30 ± 60 nm and like spherical in shape, using the aqueous solution of the olive tree (*Olea europaea*) leaves extract as reducing and capping agent. Antifungal effect of AgNPs was tested on 9 FLC resistant isolates by broth dilution method.result show that FLC resistant isolates is sensitive to AgNPs at 12.5 µg/ml.*ERG11* gene expression was done for seven FLC resistant isolates after treated with AgNPs by qRT-PCR and analysis data by $2^{\wedge-\Delta\Delta CT}$ method (Livak equation). Result shows, silver NPs have considerable anti-fungal properties that could reduce the resistance of *C. albicans* isolates by decreasing the expression of one of drug resistance genes with different mechanisms, compared to the conventional antifungal agents.

Key words: *Candida albicans*, Silver nanoparticles, *ERG11*.

Introduction

Fungal infections are one of causes of infectious diseases, with *Candida* being the most representative model of pathogenic yeasts in humans. *Candida albicans* is a dimorphic normal flora yeast in healthy people, but in conditions of a weakened immune system *Candida* becomes an opportunistic pathogen following a transition from a commercial to a pathogenic phase (Vazquez-Muñoz *et al.*, 2014). A fungal infection caused by any type of genus *Candida* is called Candidiasis, which is the most prominent cause of fungal infections and a very ubiquitous infectious disease (Sullivan *et al.*, 1996; Coutinho, 2009). Candidiasis represents 57% of fungal infections. In Iraq, Candidiasis ranked as the fourth among sexually transmitted diseases (STD) with an incidence of 76.73 per 100,000 population (Alwan, 2004). *Candida albicans* represents more than 50% of the species causing candidiasis, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* (Pfaller and Diekema, 2007, Vincent *et al.*, 2009).

Azol, especially fluconazole is the most widespread antifungal drug prescribed by practitioners for mucosal and disseminated candidiasis. Fluconazole is also a recommended prophylactic drug to prevent fungal invasion in leucopenic patients and to avoid opportunistic an nosocomial candidiasis in AIDS patients. The mode of action of fluconazole involves inhibition of lanosterol 14-α demethylase in ergosterol biosynthesis which encoded by *ERG11* (Navarathna *et al.*, 2005). The overexpression of *ERG11* gene leads to an increase in the target drug. Therefore, a higher concentration of drug is required to react to all of enzyme molecules present in the cells and thereby decrease the susceptibility to azole

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in *Candida* species (Li *et al.*, 2012). The advent of this drug resistance necessitates the development of new antifungal agents to overcome the resistant strains.

Research and development of new antifungal agents is complicated by the paucity of selective targets, since fungi are eukaryotic cells and high levels of toxicity of some the current antifungal. Additionally, drug resistance of *C. albicans* against antifungal such as azoles and echinocandins represents an increasing problem. Therefore, novel antifungal drugs against these uncontrollable infections are urgently needed. With the advances on Nanotechnology, silver nanoparticles (AgNPs) have confirmed to be broad-spectrum bactericidal, virucidal and fungicidal. Silver has powerful antimicrobial agent contrast with other metals, also Silver display great effect on microbial cell in the time to show lower harmless to eukaryotic cells. For this reasons it chosen in nanotechnology to prepare AgNPs. (Lara *et al.*, 2015). Silver has antimicrobial effect even at low concentrations. Due to the importance of silver in health care. Recent studies suggest that AgNPs may be safer to use than ionic or colloidal silver. In modern research, Potential positive of AgNPs have proven their effects against *C. albicans* by prevention of biofilm formation, disrupting the membrane and forming pores causing ion leakage and other materials, inducing apoptosis and causing ultra structural changes. Moreover, it has also been reported that AgNPs have synergistic interactions with different antimicrobial drugs (Vazquez-Muñoz *et al.*, 2014).

Material and Methods

Isolation and identification samples

Isolates collection was performed between December, 2018 and February, 2019. 100 samples were collected from patients suffering from fungal infection diagnosed by consultant physicians in Baghdad; (Medical city) teaching hospital, isolates taken as subculture from samples taken as: sputum from patients with respiratory tract infections, swabs from vulvo-vaginal infected women and patients with oral thrush and as urine. All samples were cultured on SDA. The isolates identified as *Candida* depending on morphological features on culture medium, chromogenic agar (Ghelardi *et al.*, 2008) and VITEK 2 system (Pincus, 2006; Hata *et al.*, 2007).

Fluconazol susceptibility test

Antifungal susceptibility test performed by Well diffusion method. (Magaldi *et al.*, 2004). *Candida albicans* subcultured on Sabrouraude dextrose agar at 37ºC (±2ºC) for 24 h, inoculum prepared by picking five distinct colonies from 24 hrs old culture, colonies then suspended in 5 mL of sterile 0.145 mol/L saline (8.5 g/L NaCl; 0.85% saline), the suspension then vortexed for 15 seconds and it's turbidity adjusted visually by either adding more saline or more colonies to adjust the transmittance to that produced by McFarland standard for obtaining yeast stock solution of 1×106 to 5×106 cells per mL to produce semi-confluent growth of *C. albicans*.

The well diffusion test was performed using Mueller Hinton agar. 20 ml of media were melted, cooled to 55ºC. The agar was poured into the assay plate (9 cm in diameter) and allowed to cool down on a leveled surface. Once the medium had solidified, 20 µl of the organism suspension was applied and stringed then, well with 4mm in diameter, were cut out of the agar and 20 µl of the antifungal agent were placed in it, then incubated at 35ºC for 24 hours.

Stock nano-silver solution

Stock nano-silver solution taken from biology department / college of science / University of Baghdad (Tamkeen and Al-Bahrani, 2019). The biosynthesis of AgNPs was accomplished according to Green synthesis of silver nanoparticles (AgNPs) method. Which is rapid, eco-friendly and convenient method for the synthesis of stable silver nanoparticles (AgNPs) using the aqueous solution of Olive tree (*Olea europaea*) leaves extract as reducing and capping agent. AgNPs were characterized based upon specific surface Plasmon resonance peak, shape, size and interaction between protein and NPs. by using Ultraviolet-visible spectroscopy:, Atomic force microscopy (AFM) and Scanning electron microscopy (SEM) (Prasad et *al.*, 2011; Oliveira *et al.*, 2005).

AgNPs susceptibility test

MIC was determined using the microdilution method in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) (M27-A2). The suspension of AgNPs was first diluted in de ionized water in a geometric progression. The final concentrations of AgNPs in the dispersion ranged from 100 µg/ml to 1.65 µg/ml (100, 50, 25, 12.5, 6.5, 3.3, 1.65 µg/ml). Then, 100 µg/ml of each silver concentration obtained previously was added to 1 ml Mueller Hinton medium in each microtiter plate well. Inoculation from 24 h yeast cultures on SDA were adjusted to a turbidity equivalent to a 0.5 McFarland standard in saline solution (0.85% NaCl). The yeast suspension was diluted (1:5) in saline solution. Each yeast inoculum (100 µg/ml) was added to the respective well of microtiter plates containing 100 µg/ml of each specific concentration of SN suspension. Controls devoid of SN were also included. The microtiter plates were incubated at 37°C, after 24 h, 20µl of each concentration was cultured on SDA agar plate,incubate for 24h at 37°C,

Table 1: Oligonucleotide primers used for Real time polymerase chain reaction.

Primer	Orientation	Sequence	Length (bp)
ERG11	Forward	5'TTGGTGGTGGTAGACATA3'	163
	Reverse	5'TCTGCTGGTTCAGTAGGT3'	
ACT	Forward	5'ACCGAAGCTCCAATGAATCCAAAATCC3'	516
	Reverse	5'GTTTGGTCAATACCAGCAGCTTCCAAA3'	

then determine MIC. The minimal inhibitory concentration (MIC) was defined as the lowest concentration that inhibited 80% of the fungal growth and was determined by a comparison with the fungal growth in the control well. (Monteiro *et al.*, 2011; L. Pereira1, 2014).

RNA extraction and complementary DNA synthesis

RNA were extract from isolates that treated with AgNPs at sub MIC (6.25 µg/mL) and also from the positive control sample. Afterwards, 6×108 cells of the fungal suspension were centrifuged for 2 min/12000 g and the supernatant was removed. RNA was extracted using the RNA extraction kit (ZR Fungal RNA MiniPrep™ Catalog No. R2014) kit. (Sambrook *et al.*, 1989). After centrifugation, the sediment was stored at -20°C until use.

cDNA synthesis was done according to the method of AccuPower® RocketScriptTM RT PreMix Kit provided by Bioneer-Korea**.** In addition to the solutions of the kit, other solutions were used. Oligo primer Oligo (dT)15 in lyophilized form was used to convert RNA to cDNA after dissolving it in sterilized deionized distilled water to make final concentration (10pmol/µl) as recommended by the company.

Real-time polymerase chain reaction

Quantitative PCR was done according to the method of DualStar qPCR PreMix PreMix Kit provided by Bioneer-Korea. *C. albicans ERG11* gene was amplified from the synthesized cDNA with primers as described in table 1. Moreover, act in (*ACT*) was established as a house-keeping gene to normalize the dissimilar RNA concentrations during RNA extraction. The RT reactions were performed in triplicate at 95°C for 4 min, followed by 26 cycles of three-step cycling, denaturation at 95°C for 30 s, annealing, extension and Detection at 58°C for 45 s. The data of relative gene expression were analyzed according to the $2^{\wedge-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Results and Discussions

Identification of *Candida* species

Candida spp. have been isolated most frequently from vaginal swab 42 (42%), followed by sputum 30 (30%), oral swab 17 (17%) and urine 11 (11%). *Candida* isolates identified by VITIC2, *C. albicans* represented the most isolated species 46 (46%) and non-albicans *Candida* contributed to 54% of the samples. The most predominantly isolated species was from female than male.

***In-vitro* susceptibility testing**

The fluconazole susceptibility results for *Candida albicans* isolates are presented in table 2. According to the interpretive criteria for fluconazole susceptibility, Out of 46 isolates of *Candida albicans*, 9 (20%) isolates were FLC resistant, 6 (13%) were S-DD and 31 (67%) were susceptible to FLC. The present results agree with (Zouhair and Rehab, 2011) who revealed the high susceptibility of *Candida albicans* against fluconazole with (78.37%) of isolates being sensitive and (21.2 %) resistant.

Antifungal activity of AgNPs

The results showed that the AgNPs were fungicidal against all of the FLC resistance isolates at low concentrations. These results were confirmed by plating the content of each well on SDA. The MICs of AgNPs at a silver concentration of 12.5µg/mL (Table 3). The control solution without silver did not reveal any effect on the tested yeasts.

When compare MICs of AgNPs with MIC of FLC, relieve that the AgNPs have more lethal characteristics than drugs. These results is similar results were investigated by (Juneyoung Lee and Lee, 2010, Nasrollahi *et al.*, 2011), they indicated that Ag-NPs have remarkable potential as an antifungal agent in treating fungal infectious diseases in contrast of fluconazol.

Effect of AgNPs on *ERG11* expression

Lipids are essential components of membranes and perform a variety of functions that modulate fluidity and perme-ability. Among various classes of fungal lipids, membrane ergosterol (encoded by *ERG11*) is one of the most important constituents of fungal cell membrane. Ergosterol plays a vital role in imparting stability to the cell and loss of sterol leads to destabiliza-tion of membrane resulting in enhanced permeability and thus increase in sensitivity (Mukhopadhyay *et al.*, 2004). Ergosterol acquires importance because of the fact that the ergosterol

Table 2: Fluconazole susceptibility among *Candida albicans* isolates.

Susceptibility	No. (%)
*S	31 (67%)
*R	9 (20%)
*S-DD	6 (13%)

*S: susceptible, *R: resistant,*S-DD: susceptible dose dependent.

Table 3: AgNPs MICs among resistance *Candida albicans* isolates..

AgNPs con.µg/ml	Result
6.25	Sub MIC
12.5	MIC
25	No growth
50	
100	

biosyn-thetic pathway is the cellular target for most of the common antifungal (azoles, polyenes and allylamines) also, it consider as one of goal of AgNPs (Lavie and Liscovitch, 2000; Marambio-Jones and Hoek, 2010; Reidy *et al.*, 2013).

In the present study qRT-PCR was used in order to determine the level of *ERG11* expression in resistant *C. albicans* after treatment with AgNPs, being an antifungal at sub MIC (6.25 µg/mL). Moreover, this potential effect was studied for 7 resistant *C. albicans* isolates chosen from clinical source. The immediately isolated purified RNA from each isolates was used to determine the expression of the goal gene (*ERG11*) involved in drag resistance in *C. albicans* isolates. The expression level of *ERG11* gene was calibrated and normalized with housekeeping gene of purified internal transcription spacer sequence (*ACT*). The gene expression values of housekeeping gene, tested genes and Delta Ct values were illustrated in (Table 4).

When comparing the folding gene change between after and before treatment isolates with AgNPs, a down regulation (0.4 to 0.8) was noticed in *ERG11*expression after treatment with AgNPs in 5 isolates. Nevertheless, one isolate (number 4) demonstrated a slight up regulation (1.1) of *ERG11* gene. While in isolate number 5 show high depression (0.08). This mean, there is Down-regulated expression of the *ERG11* gene was found in *C. albicans* isolates treated with AgNPs at the MIC concentrations. According to the results of this study, Ag NPs could reduce the resistance of *C. albicans* to antifungal drug by decreasing the expression of one of drug-related genes *(ERG11)*.

(Radhakrishnan *et al.*, 2018) in their research found that AgNPs were affect cell membrane integrity and the physical state of the membrane of *Candida* cells. They checked ergosterol contents of the AgNp-treated cells in order to examine the effect of AgNPs on the lipid composition, which might be responsible for the altered physical state of the membrane. They found a reduction in ergosterol content by only 1.3% in the presence of 5 µg/mL AgNPs and there was a marked reduction by 22.8% in the fungal cells treated with 40 µg/mL AgNPs. also, they measured the percentage of fatty acid in *Candida* membrane which they have critical important in membrane fluidity. The results showed a significant increase in the percentage of saturated fatty acids and a simultaneous decrease in unsaturated fatty acids in AgNp-treated *Candida* cells in about 3.2% when cells were grown in the presence of 5 µg/mL AgNPs and a marked reduction about 20% in the presence of 40 µg/mL AgNPs. Therefore, it appears that the increase in saturated fatty acids and simultaneous decrease in unsaturated fatty acids compensated for the reduced ergosterol levels resulting in a net decrease in membrane fluidity after AgNp treatment.

Conclusions

Silver NPs have considerable antifungal properties that could reduce the resistance of *C. albicans* isolates by decreasing the expression of one of the drug resistance genes (*ERG11*) with different mechanisms, compared to the conventional antifungal agents.

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Table 4: Fold change of *ERG11* of *C. albicans* isolates after treatment with silver nanoparticles.

No. of Isolate	Ct of *ACT* before treatment Isolate with AgNPs	Ct of *ACT* after treatment Isolate with AgNPs	Ct of *ERG11* before treatment Isolate with AgNPs	Ct of *ERG11* after treatment Isolate with AgNPs	ΔCT before treatment Isolate with AgNPs	ΔCT after treatment Isolate with AgNPs	ΔΔCT	Fold Change
C1	36.19	36.89	19.19	19.32	-17	-17.57	0.57	0.673616
C2	28.47	23.36	24.75	20.74	-3.72	-2.62	1.1	0.466516
C3	22.56	22.23	19.84	19.89	-2.72	-2.34	0.38	0.768437
C4	22.29	22.65	18.04	18.21	-4.25	-4.44	-0.19	1.140763
C5	28	22.51	20.96	19.05	-7.04	-3.46	3.58	0.083620
C6	32.81	32.12	17.3	17.15	-15.51	-14.97	0.54	0.687770
C7	24.76	24.53	17.83	17.78	-6.93	-6.75	0.18	0.882702

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