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 \(2^{-\Delta\Delta CT}\) 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-Munoz 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

Isolates 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 Sabouraud dextrose agar at 37°C (±2°C) 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 55°C. 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 35°C for 24 hours.

Stock nano-silver solution

Stock nano-silver solution taken from biology department / college of science / University of Baghdad (Tamkeen and Al-Bahrain, 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, 20 µl 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERG11</td>
<td>Forward</td>
<td>5’TTGTTGGTGAGTAACATA3'</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’TCTGCTGTTACGTAGGT3'</td>
<td></td>
</tr>
<tr>
<td>ACT</td>
<td>Forward</td>
<td>5’ACCGAACGTCCAATGAAATCCAAAATCC3'</td>
<td>516</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’GTTTTGTCATACCCAGCAGCTTCAA3'</td>
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</tbody>
</table>

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^ΔΔCT 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 permeability. 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.

<table>
<thead>
<tr>
<th>Susceptibility</th>
<th>No. (%)</th>
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</thead>
<tbody>
<tr>
<td><em>S</em></td>
<td>31 (67%)</td>
</tr>
<tr>
<td><em>R</em></td>
<td>9 (20%)</td>
</tr>
<tr>
<td><em>S-DD</em></td>
<td>6 (13%)</td>
</tr>
</tbody>
</table>

biosynthetic 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 drug 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.

**Acknowledgements**

The authors thank the Iraqi Ministry of Higher Education and the University of Baghdad (College of Science and College of Engineering) for the support for this research.

Table 3: AgNPs MICs among resistance Candida albicans isolates.

<table>
<thead>
<tr>
<th>AgNPs con, µg/ml</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>Sub MIC</td>
</tr>
<tr>
<td>12.5</td>
<td>MIC</td>
</tr>
<tr>
<td>25</td>
<td>No growth</td>
</tr>
<tr>
<td>50</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

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

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


