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Antimicrobial Activity of Green Silver Nanoparticles against Fluconazole-resistant *Candida albican*s in Animal Model

Amira El-adly[#] and Iman Shabana[*]

*Laser Microbiology Laboratory, National Institute of Laser Enhanced Science, Cairo University, Cairo and * Faculty of Veterinary Medicine, Department of Bacteriology, Immunology & Mycology, Suez Canal University, Ismailia, Egypt.*

FUNGAL infections due to fluconazole-resistant *Candida albicans* are a serious clinical problem, requiring new efficient antifungal treatment. The present study evaluated the susceptibility of fluconazole-resistant *C. albicans* to green silver nanoparticles. A total of 40 isolates were examined for the expressions of CDR1, CDR2, and MDR1 gene by quantitative reverse transcription polymerase chain reaction. The antifungal activities of green silver nanoparticles alone and/or in combination with fluconazole (to improve antifungal activity) were assessed by broth microdilution assay and transmission electron microscopy. The effect of fluconazole and/or green silver nanoparticles on the production of *C. albicans* to protease and phospholipase was also evaluated. And finally, animal model was used to prove the safe and effective use of green silver nanoparticles in the treatment of fluconazole-resistant *C. albicans*. For all the tested *C. albicans* strains, the minimum inhibitory concentrations (MICs) of fluconazole and green silver nanoparticles were 64 and 4.84 µg/ml, respectively. Green silver nanoparticles decrease the production of protease and phospholipase enzymes. The expression of both CDR1 and CDR2 were decreased after exposure to green silver nanoparticles, while the expression of CDR 1, CDR 2 and MDR 1 were all decreased when fluconazole and green silver nanoparticles were used. Green silver nanoparticles may be causing suppression of the CDR1, CDR2 and MDR1 expression in fluconazole-resistant *C. albicans*. The results suggest the use of green silver nanoparticles as a safe and effective treatment against fluconazole-resistant *C. albicans*.

Keywords: *Candida albicans*, Fluconazole resistance, Green silver nanoparticles.

Introduction

Oral Candidiasis is one of the common fungal infections, affecting the human oral mucosa. It is caused by *Candida albicans*, that is an opportunistic yeast fungus of the normal microflora in the human digestive tract. A slight change of the human immune system may support the transformation of *C. albicans* into a pathogen causing serious infections that may be lethal. *Candida* commonly showing asymptomatic colonization in the oral cavity, vagina, and rectum (Pfaller & Diekema, 2004). *Candida* species are able to express genes to acclimatize and causing infections, they are the reason for its virulence (Chen et al., 2010). *Candida* colonization and pathogenicity is regarding to several virulence factors, including the expression of adhesions and invasions, morphogenetic transformation of yeast hyphae, biofilms formation, switching phenotypes and hydrolytic enzymes production (Francois et al., 2013). Hydrolytic enzymes are considered to be an important virulence factors. The production of these enzymes after the invasion of *Candida* to the host tissues destroying or disorganizing the cell membranes, causing disruption and/or dysfunction. The important hydrolytic enzymes are proteinase, that hydrolyzed the peptide bonds and phospholipase, that hydrolyzed the phospholipids (Julian et al., 2003).

Control of clinical fungal infections is complicated due to the continuous development of resistance to antifungal therapy especially in immune-compromised persons (Cowen et al., 2002). Antifungal drugs requiring prolonged use, than the prescribed for antibiotics, which is accompanied by an increase in the incidence of side effects. And the challenge is the limited number of available antifungal drugs (Gomez-Lopez et al., 2008). Recently, the resistance of pathogenic fungi to the commercially available

[#]Correponding author e-mail: amiraaladly@yahoo.com
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fungicides is increasing and become a serious problem; therefore, finding new fungicides and alternatives is important to battle the newly emerged resistant fungal pathogens (Kanhed et al., 2014).

Azole are the main antifungal drugs of infections caused by *C. albicans*, while fluconazole-resistant *Candida albicans* has dramatically increased. Azole resistance has direct and indirect mechanisms. The direct mechanism involve the change of lanostero 114α demethylase (Kristan & Rizner, 2012), which encoded by the ERG11 gene. The indirect mechanism is the common form of azole resistance, including over production of ATP-binding cassette transporter proteins (ABC). ABC transporter proteins causing azole efflux and prevent accumulation (Prasad & Goffeau, 2012). Multidrug resistance in *C. albicans* involved, the ABC transporter-encoding gene CDR1 and the MFS protein-encoding locus MDR1 (Fling et al., 1991). Both CDR1 and MDR1 are localized in *C. albicans* plasma membrane (Kapoor et al., 2010). CDR2 is an ABC transporter-encoding gene, involves azole resistance in case of it's over expression or deletion of CDR1 (Sanglard et al., 1998).

Green silver nanoparticles (AgNPs) are an eminent antimicrobial and has a long history in medication with a well-endured tissue reaction and low toxicity. Since nano-silver has a highly toxic effect on most microorganisms, therefore it may be a good alternative for antimicrobial drugs (Herrera et al., 2000). The green synthesis techniques are synthetic methods that use relatively non-toxic solvents such as water, biological extracts (Yu, 2007). Olive plant able to reduce inorganic salts to the elemental forms and the current trials is to use olive for the reduction of silver nitrate to metallic silver. The advantage of olives is the antimicrobial cababilities against several fungi as *Rhizopus*, *Fusarium*, *Rhizoctonia*, *Alternaria*, *Stemphylium* (Sastry et al., 2003 and Al-Mughrabi et al., 2001). Green bio-reduction methods for the silver nanoparticles synthesis were reported by many researchers using plant extracts such as *Macrotylo mauniflorum* (Vidhu et al., 2011), *Anacardium* mushroom extract (Sheny et al., 2011), *Coleus amboinicuslour* (Narayanan & Sakthivel, 2011), *Medicago sativa* (Lukman et al., 2011), *Citrus sinensis* peel (Kaviya et al., 2011), and *Olea europaea* (Awwad et al., 2012) etc.

In the present study, broth microdilution method, and transmission electron microscope were used to evaluate the antifungal activity of green silver nanoparticles (AgNPs) alone or combined with fluconazole on the growth of fluconazole-resistant *Candida albicans* strains. In addition, reverse transcription polymerase chain reaction was used to evaluate CDR1, CDR2, and MDR1 expression before and after the treatment. The effect of green silver nanoparticles (AgNPs) on the virulence of the isolates was also studied in animal model.

Materials and Methods

Microbial isolates and culture condition

Our study included a group of 40 clinical *Candida albicans* isolates were obtained from (El-Manyal Hospital, Faculty of Medicine, Cairo University) with symptoms of oral Candidiasis. In this study, isolates from patients being under antibiotic treatment, or having antifungal drugs in the last 30 days. In addition, the patients informed if they had been previously exposed to azoles at least once in the last year as treatment for oral Candidiasis. Swabs were collected from buccal mucosa and tongue by gentle rubbing on the lesion. Swabs were streaked on Sabouraud's Dextrose agar, and the isolates were identified by corn meal agar, formation of germ-tube, fermentation assimilation and the presence of thick-walled spores. The API 20 system were used to confirm the identification (bioMerieux, France) (Monroy-Perez et al., 2012).

Detection of fluconazole resistance genes

Isolation RNA

Candida albicans isolates were added to tubes with Lysing Matrix C silica beads and homogenization was performed by FastPrep®-24 homogenizer (MP Biomedicals, USA). RNeasy® Mini Kit (Qiagen, Germany) was used for RNA isolation.

Complementary DNA synthesis

cDNA was synthesized by reverse transcription of the isolated RNA, using cDNA Reverse Transcription Kit (Applied Biosystems, USA).

CDR1, CDR2 and MDR1 expression

The expression of CDR1, CDR2 and MDR1 was evaluated by Q-PCR. Primers and Taq Man probes sequences were shown in Table 1. The reactions were done in the Applied Biosystems 7300 Real-Time PCR System (Applied

Biosystems, USA) as the following, Ampli Taq DNA polymerase Gold® 95°C, 10 min; 95°C, 15 sec; 60°C, 1 min. for 40 cycles. Fluorescence was measured at a wavelength (lmax=518 nm). Threshold cycles (CT) values were analyzed and compared by Relative Quantitation software (Applied Biosystems, USA).

TABLE 1. Primers and probes used in the Q-PCR method.

Primer sequence	Probe sequence	Gene
CCGTTTTCGGTCAACTTGTAATGG AAACGATCCAGTGGTTTGACTAAGATT	ACACCGACGACAATAT	CDR1
GGCTTATCAATTTTATTCTTCACACAAATGGA CAGTCAAGGCAACATAAACTCCTAAGA	ATGCAACGGTAATTCC	CDR2
AACATTATTATATCGCAAGGCTAAAAGAT TCCTTCACTTGTGATTCTGTCGTT	CCGGTGATGGCTCTCA	MDR1

Green silver nano-particles (AgNPs) preparation

Green silver nano-particles (AgNPs) were purchased from Chemical lab, Cairo University, Egypt, the particles were prepared by method as reported by Gavhane et al. (2012) with some modification. 50 g of fine cut olive leaves in 1 L glass beaker containing 500 ml sterile distilled water. Mixture was boiling for 10 min. the extract was coiled at room temperature and filtered, cooled and stored in order to used, the color of AgNPs solutions change from pale yellow to grey black indicating the reduction of silver positive ions to silver nanoparticles. The particles were spherical in shape, and about 30± 1.0 nm in diameter, and optical absorption peak range at (440 – 458 nm).

Evaluation of AgNPs on the virulence of C. albicans

Protease activity

The Bovine serum albumin medium (BSA) was used for the protease production assay, 10 µl aliquots of the *Candida* suspension (48 h-old *Candida* culture, $5x10^5$ cells, treated with AgNPs 8 µg/ml alone or combined FLC/AgNPs 8/2.17 µg/ml) was inoculated into the wells of the medium and incubated for two days at 37ºC. 20% trichloracetic acid was used for fixation, stained with 1.25% amido black and decolorized with 15% acetic acid. Agar opaqueness around the wells and absence of black color, indicating proteolysis. The diameter of unstained area referred to protease production. The protease activity (Pz) is the ratio of the well diameter to the diameter of the unstained zone (Vidotto et al., 2004). The Pz equal 1, indicating no protease production, while a high production of protease is indicated by low Pz.

Phospholipase activity

Phospholipase production was assayed by using Egg-yolk agar medium (EYA). 10 µl aliquots of the *Candida* suspension (48 h-old *Candida* culture $5x10^5$ cells , treated with AgNPs 8 µg/ml or FLC/AgNPs 8/2.17µg/ml) was inoculated into the wells of the egg-yolk medium and incubated at 37ºC for 48 h. Hydrolysis of lipids resulted in the formation of a calcium complex with fatty acids, producing a precipitate around the colony. Phospholipase activity (Pz value) was the ratio between the diameter of the colony and the diameter of the precipitation area (Vidotto et al., 2004). When the Pz equaled 1, indicating no phospholipase production, while high production of the phospholipase is indicated by low Pz.

Antifungal activity of green silver nanoparticles (AgNps)

Minimum inhibitory concentration (MIC) of fluconazole (FLC), Sliver nanoparticles (Ag NPs) on *C. albicans* cells was determined by broth microdilution assay according to the National Committee for Clinical Laboratory Standards (CLSI, 2012) guidelines. A suspension of *C. albicans* was adjusted to 75 % transmittance using a double-beam UV–visible spectrophotometer. CFU/ml was quantified by plating of a 10 ml suspension on SDA and colonies counting. The suspension concentration

was adjusted to 5×10^5 cells/ml using RPMI 1640 medium (Sigma). 100 µl Aliquot of the suspension, were inoculated into a 96-well microdilution plate containing 100 µl of dilution of (1 – 10 µg/ml) AgNPs or (2 - 128µg/ml) of Fluconazole. The effect of combinations of FLC with nanoparticles on *Candida* cells was assessed. The MIC was recorded as no turbidity showed.

Transmission electron microscopy (TEM)

A suspension of *C. albicans* cells (5×10^5 cells/ml) prepared from a culture grown for 24 h at 37°C in Yeast extract peptone dextrose (YPD). Demineralized sterile water was used for cells suspension, the suspension centrifuged at 3500 rpm for 10 min. 25 ml sterile demineralized water was used for the re-suspended of the pellets. The suspension was distributed in six tubes, each containing 1 ml suspension. Two tubes were treated with AgNPs (8 µg/ml), and the other two tubes with combined FLC/ AgNPs (8/2.17); the last two tubes with the spore suspension in remained without treatment and was used as a control. The cells were harvested and washed in 0.1 M sodium phosphate buffer, then prefixed with glutaraldehyde for 1 h, and left in 1% osmium tetra oxide for 1 h at 4°C. Then was washed in distilled water and dehydrated in a graded acetone series (35 to 100%). The cells were embedded in Epon and the small blocks of samples were cut with an ultra-microtome (Leica Ultracut), then analyzed at 80 KV using (JEOL 1010) TEM (Sangetha et al., 2009) .

Toxicity assay of the prepared AgNPs

Cell line (Hep-2 cells) were grown onto microtiter plates containing 100 µl Dulbecco's modified Eagle's medium (DMEM) for 24 h. Different concentrations (2, 5, 10 µg/ml) of AgNPs, or combined with FLC (serial two fold dilutions of 2-128 µg/ml) were added. 100µl of each concentration was inoculated into the cells and incubated at 37°C for 24 h. The viable cells stained with staining buffers for 1-2 h. 33% citric acid was used for elution, and the absorbance was measured at 595 nm. Determination of the non-toxic concentration of the AgNPs or FLC/ AgNPs combined was done by the microscopic examination (Marcato et al., 2013).

Animal model

Animals and experimental oral candidiasis

This experiment was investigate the possibility of using the combined application of fluconazol and green silver nanoparticles to improve antifungal activity against fluconazole-resistant *Candida alicans* in animal model. Animal housing and treatment conditions were approved by the ethics committee of Institutional Animal Care (IACUC), Egypt .

Forty female healthy Mice (6 weeks old) weighing between 20-25 gm were used in this study, Mice were kept in stainless steel cages under controlled temperature (22 ± 2^0C), humidity was at 55±10% and 12/12 h cycle of light and dark with an access of food and drinking water. The animals were divided into four groups, ten mice each. Group (1): control group which received physiological saline solution, the other animal groups were infected with *C. albicans* and divided into, Group (2): *Candida* group (none treated), Group (3): treated with AgNPs (10 µg/ml), and Group (4): treated with combined FLC/ AgNPs (8/2.17 µg/ml).

Oral candidiasis in mice was induced by *Candida albicans*. It was prepared by harvested one colony of *C. albicans* cells in RPMI 1640 medium (UFC Biotch, KSA) and 5×10^5 viable cells/ml cell suspension was adjusted. The animals were immunosuppressed with subcutaneous injections of prednisolone on days 1, 5, 9 and 13 of the experiment at a dose of 100 mg/kg body weight according to the evaluated period (24 h and 7 days after treatment). Tetracycline (0.83 mg/mL) was added to the drinking water throughout the trial. On day 2, sterile swabs were soaked in *C. albicans* suspension and rubbed on the dorsum of the tongue of sedated animals with chlorpromazine hydrochloride 0.1 ml (2 mg/mL) to induce candidiasis. At day 6, a cotton pad was used to swab the whole oral cavity, then put in a 5ml sterile saline and vortexes thoroughly. A serial 100-fold dilutions of the cell suspension were plated on Sabouraud dextrose agar and incubated for at 37°C 24 h. The growing *Candida* colonies were then counted (Takakura et al., 2003).

Treatments and evaluation

The infection was verified by visual examination on day 7, the animal groups were treated daily from day 7 to 11. The animals were anesthetized by I/M with pentobarbital. The tongues of the animals were gently taken out of the oral cavity, AgNPs diluted in water (10 µg/ml) & combined FLC/AgNPs (8/2.17µg/ml) was

applied by pipette on to the dorsum of the tongue of anesthetized mice using 50µl/mice once a day for 5 days. The negative control group did not infected with *C. albicans* or received treatment. The control group treated with physiological solution. The microbiological evaluation was carried out every 24 h for 7 days after treatments to avoid the decrease of colony forming units (CFU/mL), because swabbing of the oral lesions may reduce yeast load in the local tissue.

The animal's body weight was taken in day 0 and day 11 before dissection; the body weight changes statistically analyzed using One-way ANOVA. Histological examination was done for negative and positive control group (1st and 2nd group) for comparison and evaluation. Tongue was washed by saline then natural buffered formalin. Tissue samples were taken and processed with graded alcohols and xylene, and embedded in paraffin blocks in automatic processor. Serial longitudinal sections of 4.6 µm from tongue were stained with H and E for histological examination. Specimens showed various patterns of cellular activity under light microscopy.

Statistical analysis

The data were analyzed statistically using student's T test. P value of 0.05 or less was considered statistically significant (SPSS 14, 2006).

Results and Discussion

Candida albicans was identified in 75% (n=30) of the samples. All isolates were sensitive to nystatin, whereas 93.3% (n=28), 86.7% (n=26) were resistant to fluconazole and ketoconazole, respectively. The most resistant fluconazole-*Candida albicans* was taken in the further experiments (data not shown).

The expression of CDR1, CDR2 and MDR1

Table 2 showed the relative expression of *CDR1*, *CDR2*, *MDR1* in *C. albicans* isolates before and after the treatment with AgNPS(8µg/ml) and/or FLC/AgNPs combined (8/2.17µg/ml). Their expression was studied using the Relative Quantitation software (RQ) (Chau et al., 2004 and Chen et al. 2010). *CDR1, CDR2 and MDR1* genes are the major azole resistance determinants (Hu et al., 2015), their products are active transport pumps resulting in the efflux of azole from the Candid's cell (Sanglard, 2002). The data showed decreasing their relative expression after treatment with AgNPS ($P > 0.05$) and combined FLC/AgNPS ($P < 0.05$). A synergistic effect may be exerted between the green silver nanoparticles and fluconazole

Table 2. The relative expression of CDR1, CDR2, MDR1.

Gene	RQ (2-rrCT) mean		
	Untreated	AgNPS	FLC/AgNPS
CRD1	31.2	28.3	25.1
CDR2	0.4	0.3	0.2
MDR1	0.8	0.6	0.5

Enzymes activity

Enzymes are important weapons providing the pathogen the ability to invade host tissue. *Candida albicans* are able to produce proteinase and phospholipase enzymes which increase its virulence. In this study. The data in Table 3 showed variation in the enzyme activity after treatment with AgNPs or combined FLC/AgNPs. Treatment with AgNPs induced variable reduction in proteinase and phospholipase activity (0.88 & 0.85 mm, respectively) in comparing with the control (0.659 & 0.607, respectively). A negative effect of AgNPs on the activities of exoenzymes, including b-glucosidase, acid phosphatase, dehydrogenase, urease and Keratinase was reported by Shin et al. (2012). The inhibitory effect of AgNPs on proteolytic enzymes activity maybe due to their preferential adsorption or attachment on the surface of enzyme (Fischer et al., 2002 and Bayraktar et al., 2006), which induces changes in function, regulation and configuration of the enzyme (Wu et al., 2009), or may possibly limit the access of substrate to the active site of the surface-bound enzyme (Bhinder & Dadra, 2009). Moreover, it was also proposed that positive silver ions released from AgNPs can react with sulfur-

containing proteins, resulting in the inactivation of enzyme functions (Gupta & Silver, 1998 and Matsumura et al., 2003). Further reduction of proteolytic enzyme activity was recorded on using AgNPs in combination with floconazole. It has also been suggested that there can be release of silver ions by the nanoparticles (Feng et al., 2000) and these ions can interact with the essential serine residue at or near the active site of the proteinase and phosphlipase and resulted in the inactivation of the enzyme. In other cases, the charged silver ions may ultimately attach with the sensing surface of the C-terminal residue of amines in enzyme, causing its modification. It has to be mentioned that reduction activity, even for treatment with AgNPs alone, or combined with fluconazole were significantly higher.

TABLE 3. Effect of AgNPs and/or FLC/AgNPs combined on the activity of protease and phospholipase enzymes against fluconazole-resistant *Candida albicans* .

	Protease	Phospholipase
	Pz (mm)	
Control	0.659	0.607
AgNPS	0.88	0.85
FLC/AgNPS	0.92	0.93

Pz = 1 (negative), Pz< 0.9–0.99 (+) , Pz< 0.8-0.89 (++) , Pz< 0.70-0.79 (+++), Pz< 0.70 (++++) .

Antifungal activity

Fluconazole is the drug of choice for the treatment of candidiasis. However, it has several side effects, including nausea, vomiting, and headache, liver damage (Lilly, 2012). Reports on fluconazole-resistant *C. albicans* strains have been increased in the last few years (Pfaller, 2012). Consequently, an urgent need emerges to find alternative therapies for candidiasis. So, the present study tested the green sliver nanoparticles (AgNPS) produced by Olive leaves for their effect against fluconazole-resistant *C. albicans* isolates. The MIC of AgNPs alone or in combination are shown in Table 4. *Candida* isolates were resistant to FLC with MIC value of 64µg/ml. AgNPs showed an antifungal effect with MIC value of 4.84 µg/ml. The effect on *Candida* was selective, as the cytotoxic concentration of AgNPs on Hep-2 cells was higher than 10 µg/ml (data not shown). The combination between AgNPs and FLC had a marked reduction in FLC MIC value (8/2.17 µg/ml). Our results was in accordance with Ishida et al (2014), who reported a fungicidal effect of green AgNPs on *C. albicans*, with MIC and MFC values of 1.68 and 3.40 µg/ml, respectively. No cytotoxic effect was shown up to a concentration of 10 µg/ml of AgNPs for Hep-2 cells (Marcato *et al*., 2013 and Lima et al., 2013). Our results emphasize the decrease of fluconazole MIC of *C. albicans* when combined to AgNPs. The resistance to fluconazole in *C. albicans* is strongly associated with over expression of efflux pumps encoding genes (Odds et al., 2003). The mechanisms of Ag-NPs against *C. albicans* are not fully understood, While AgNP-treated *C. albicans* shows disruption of the cell wall and cytoplasmic membrane (Kim et al., 2009). Moreover, AgNPs increasing in the production of reactive oxygen species and hydroxyl radical, which causing the damage of the cell membrane (Hwang et al., 2012). Since AgNPs alters the cell membrane permeability, we may assume that they may facilitate the entry of FLC, which interferes with the biosynthesis of ergosterol (Xia et al., 2016), as the growth of the pathogenic fungus *T. asahii* was inhibited by silver. Ag penetrated the cells, damage the organelles, including mitochondria and ribosome and caused condensation and margination of chromatin, a marker of apoptotic cell death. Moreover, due to their small size, the silver nanoparticles may attach to the cell surface and get into the cells directly without damaging the cell wall and then cause the death of the cell (Xia et al., 2016)

TABLE 4. MIC µg/ml of fluconazole and/or green silver nanoparticles or fluconazole combined with green nanoparticles against fluconazole-resistant *Candida albicans*.

	MIC±SD
Fluconazole	640.50 ±
AgNPS	4.84±0.60
FLC/AgNPS	8/2.17±0.30

Transmistion electron microscope (TEM)

the ultrastructure alterations or organelles changes in *C. albicans* after treatment with AgNPs and/or combined FLC/AgNPs was detected by Transmission electron microscopy. Photo (1-a) of untreated *Candida* cells (control group) appeared with a uniform central density, structured nucleus, and cytoplasm with several endomembrane elements and had a regular intact cell wall and plasma membrane. On the other hand, treated *Candida* cells with AgNPs and/or combined FLC/AgNPs, showed cell lysis, with absence of the cell wall and ruptured cytoplasmic membrane, causing loss of intracellular material and cell wall abnormalities. Some cells showed invaginations of the cytoplasmic membrane with marked disorganization of the cytoplasm and reduction of the intracellular volume (Photo1- b-c).

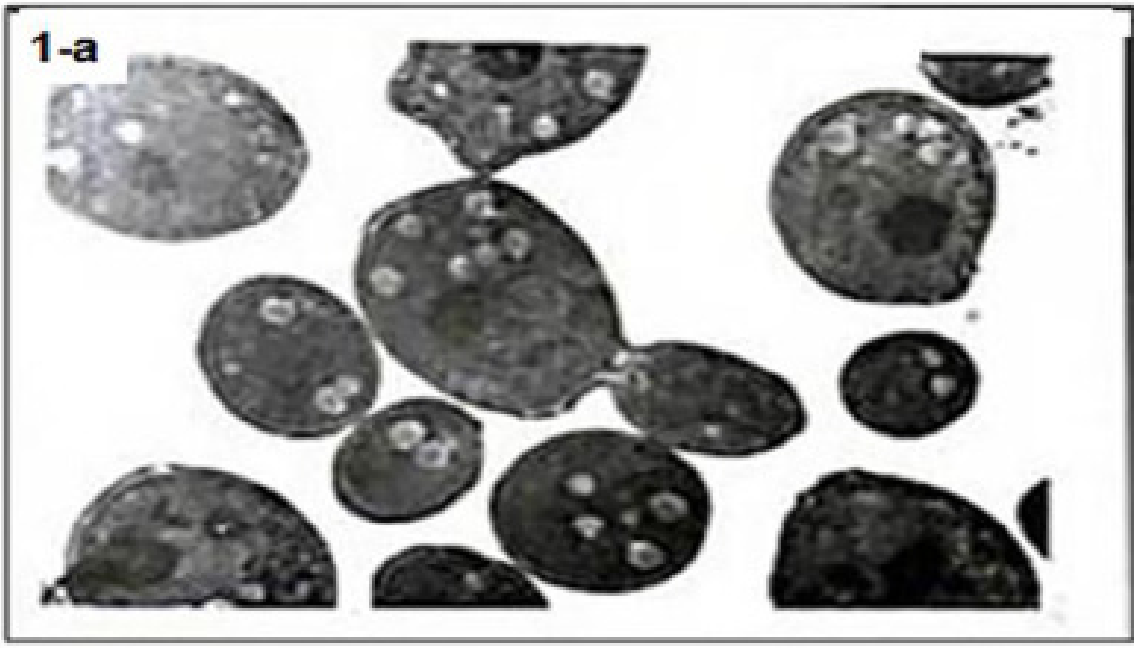

Photo 1-a. TEM micrographs of the untreated *Candida albicans* cells (control group).

Photo 1-b. TEM micrographs of AgNPs effect on *Candida albicans* cells.

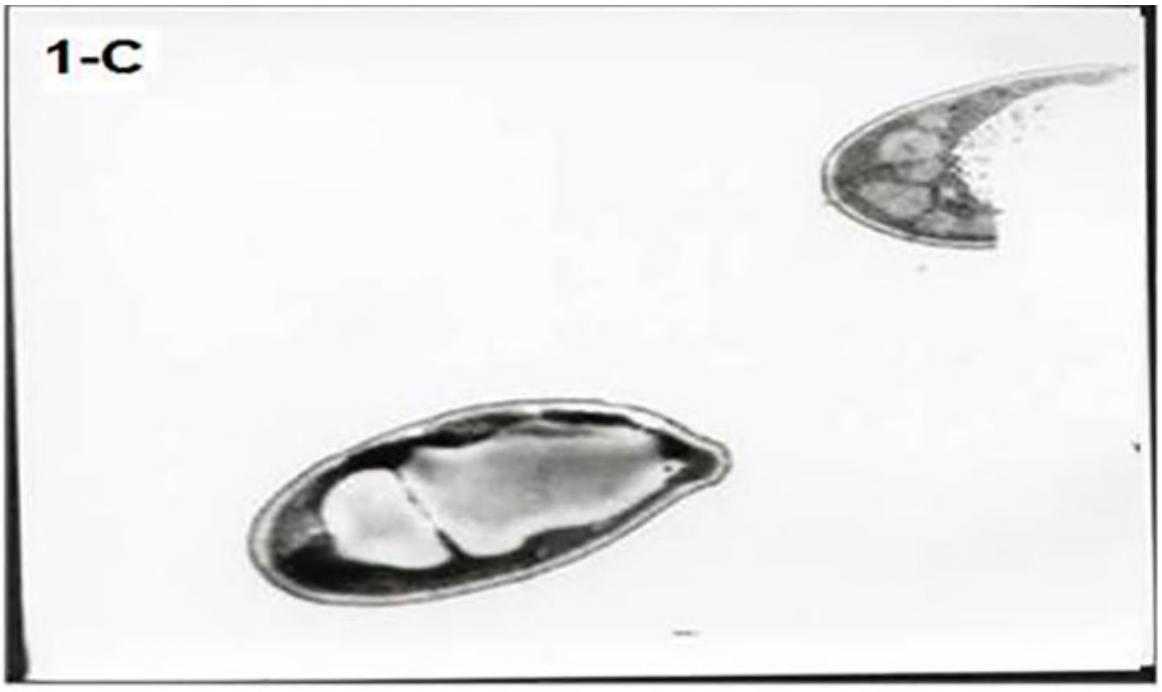

Photo 1-c. TEM micrographs of FLC/AgNPs combined effect on *Candida albicans* cells.

Vivo experiment

The present investigation, used the induced oral candidiasis in immunocompromised mice as a proposed model. All animals showed white patches or pseudomembrane on the dorsum of the tongue with inflammatory infiltrate with numerous hyphae. At day 6, there was a statistically significant difference in animal's body weights, the body weight were decreased after immunosuppression with two subcutaneous injections of prednisolone. Mean and standard deviation of the body weight in infected and treated groups were calculated and showed decrease in values compared with control group. Combined FLC/AgNPs treated group showed less changes in body weight than other groups (Table 5). The body weight loss in all groups was highly significant when the result was compared with animal body weight of control group ($P<0.001$). This result was similar to the results of Solis & Filler (2012) .

TABLE 5. The body weight of the animal groups.

Animal group	Mean of body weight		
	Zero day	5th day	Change in weight (g)
Control	20.70	22.38	1.68
Candida	20.00	17.50	2.5-
AgNPS	22.00	20.30	1.70-
FLC/AgNPs	22.00	21.21	0.79-

$P < 0.001$

In this study, the oral candidiasis were treated after inoculation with either AgNPs or AgNps combined with fluconazole. The number of *C. albicans* cells in oral cavity before and after treatment was counted by plating *Candida* suspension from a cotton pad that swapped from oral cavity of animals after infection and after treatment. The control group showed no *Candida* cells growth while, *Candida*-infected group had 6.58 $\log_{10}$ CFU/ml *Candida* cells (Table 6). The treated groups (Green silver nanoparticles, and combined FLC/AgNPs) showed high reduction in the number of *Candida* cells after treatment (2.70 $\log_{10}$ CFU/ml). The treated groups showed statistically significant reduction in *Candida* cells count ($P<0.001$) as compared to *Candida*-infected group. Our results are in accordance with the finding by Maneewattanapinyo et al (2011) of safe administration of colloidal AgNPs to oral, eye and skin of the laboratory animals for short periods of time.

TABLE 6. Microbiological evaluation of *Candida albicans* cells number in oral cavity before and after treatment with AgNPS and/or FLC or FLC/AgNPs.

Treated animal group	Log 10 CFU/ml of candida cells±SD	
	Before	After
Control	0.0	0.0
Candida	6.58±0.08	6.50±1.03
AgNPS	6.54±0.06	2.70±0.67
FLC/AgNPS	6.57±0.07	2.70±0.86

The histological examinations supported the macroscopic changes of candidiasis on the tongue dorsum after treatment. *Candida* penetrates vertically from the keratinized layer to the upper part of the prickle layer in the untreated group. While in the combined AgNPs /FLC-treated group, the fungal structures exposed on the tongue surface without reaching the inner layers of keratin. The elongated filiform papillae were similar to the control group. The other treated groups showed different degrees of recovered with semiformal epithelial of tongue dorsum (Photo 2- a-d).

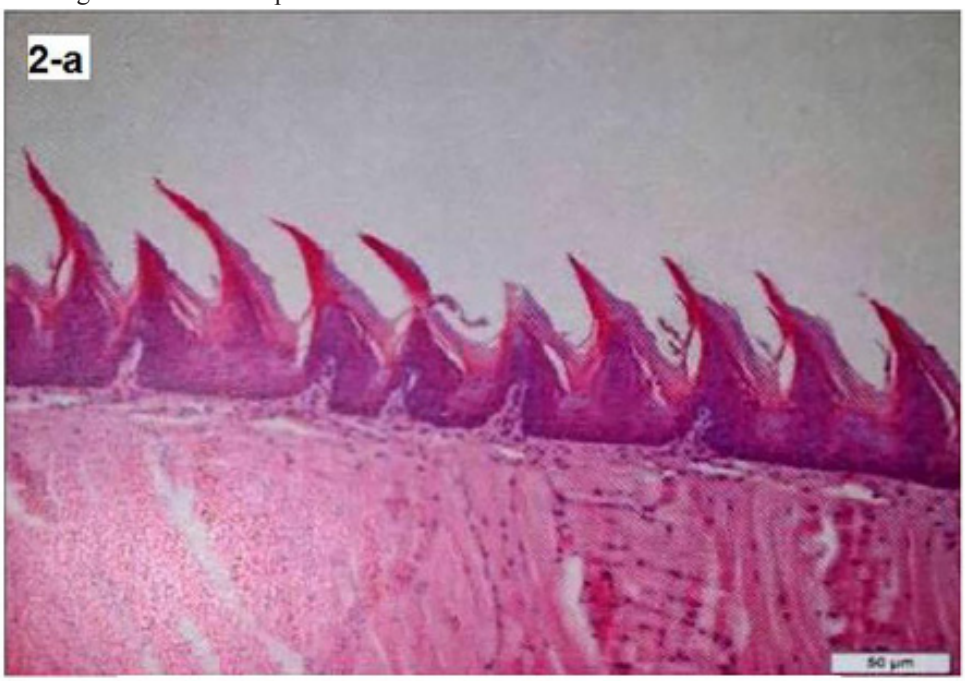

Photo 2-a. Sagittal section of the tongue dorsum of mice (control group), stained with H&E at magnification of 400 x, showing normal keratinized filiform papillae.

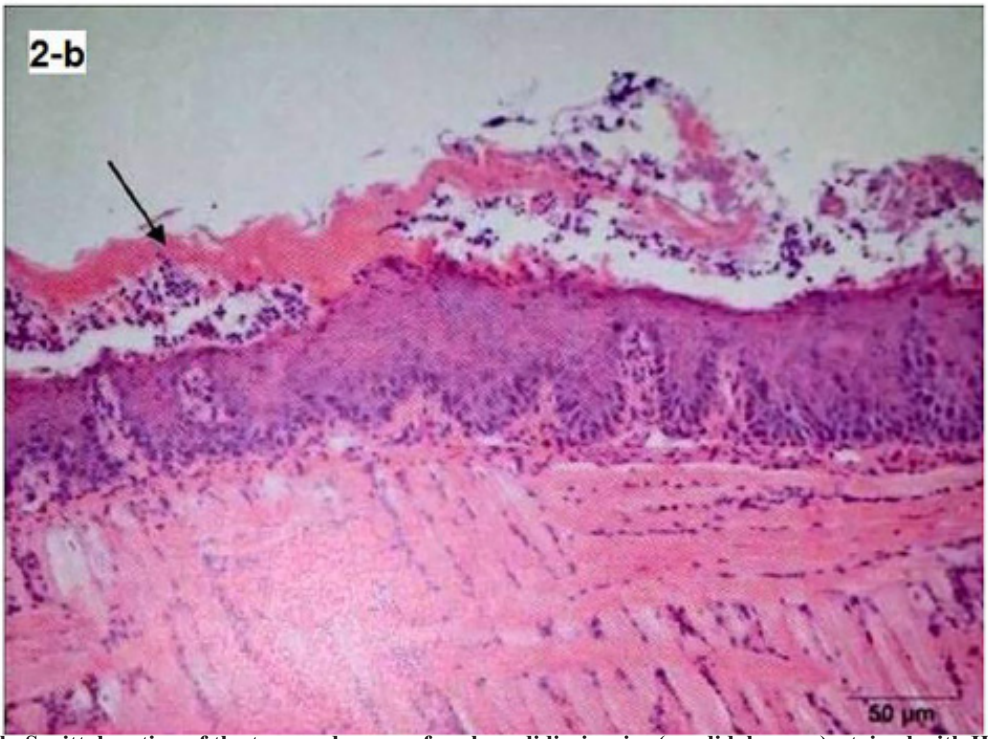

Photo 2-b. Sagittal section of the tongue dorsum of oral candidiasis mice (candidal group), stained with H&E at magnification of 400 x, showing loss of filiform papillae with keratin layer regression.

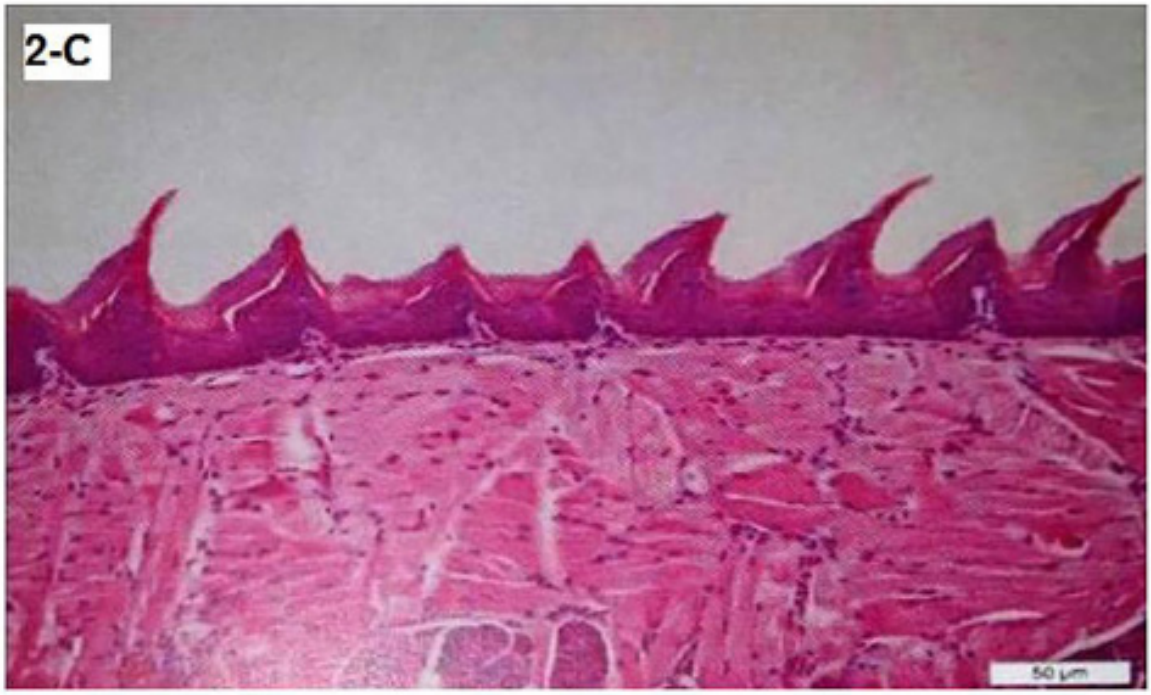

Photo 2-c. Sagittal section of the tongue dorsum of oral candidiasis mice (AgNPs group), stained with H&E at magnification of 400 x, showing healthy filiform papillae covered by undamaged keratinized layer.

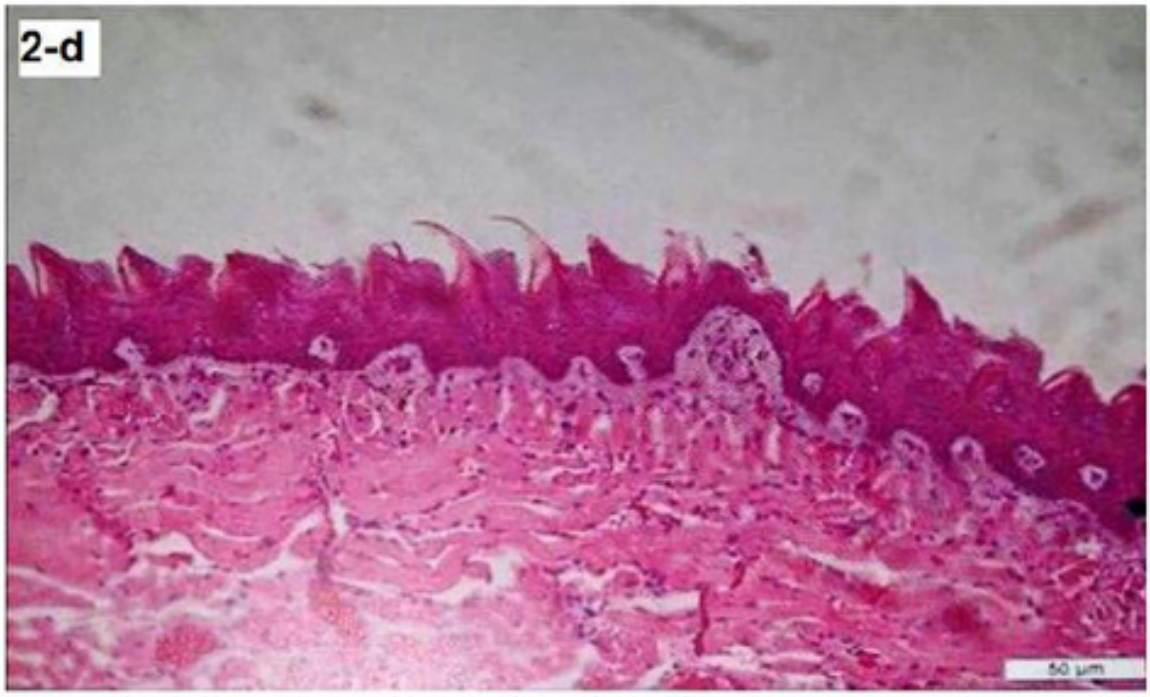

Photo 2-d. Sagittal section of the tongue dorsum of oral candidiasis mice (FLC/AgNPs treated group), stained with H&E at magnification of 400 x, showing recession keratinized layer and increased interpapillary surface with partial distortion of filiform papillae.

Conclusion

Our investigation suggests that AgNPs alone or combined with fluconazole is safe to treat oral candidiasis, since it has antifungal activity without harming the host tissue. We recommend the use green silver nanoparticles against fluconazole-resistant *Candida albicans* in clinical trials.

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التاثير الضد ميكروبى لجزيئات الفضة النانونية الخضراء تجاه فطر الكانديدا المقاومة للفلوكونازول

أميرة عبده محمد العدلى و ايمان إبراهيم ثابت شبانة*
معهد الليزر – جامعة القاهرة – القاهرة و *كلية الطب البيطرى – جامعة قناة السويس – الإسماعيليه – مصر.

الأمراض الفطرية الناتجة عن سلالات الكانديدا المقاومة للفلوكونازول مشكلة سريرية خطيرة و تحتاج إلى مضادات فطرية جديدة وفعالة. في هذه الدراسة قمنا بتقييم تأثير الجسيمات النانوية الفضية الخضراء على الكانديدا المقاومة للفلوكونازول. و قد تم استخدام عدد ٤٠ عزلة من الكانديدا للدراسة الحالية. تم الكشف عن الجينات MDR1، CDR2، CDR1 عن طريق تفاعل البوليميراز المتسلسل العكسي. و تم تقييم النشاط المضاد لكل من الفلوكونازول و/أو الجسيمات النانوية الفضية الخضراء عن طريق التخفيفات متناهية الصغر و الميكروسكوب الإلكتروني القاطع. كما تم تقييم تأثير الفلوكونازول و/أو الجسيمات النانوية الفضية الخضراء على إنتاج الكانديدا لانزيم البروتينيز والفوسفوليبز. وأخيراً، استخدم نموذج الحيوان لإثبات الاستخدام الآمن والفعال للجسيمات النانوية الفضية الخضراء في علاج الكانديدا المقاومة للفلوكونازول. أظهرت النتائج أن التركيزات الأدنى المثبطة لكل من الفلوكونازول وجسيمات النانو الفضية الخضراء هى 64 و 4.84 ميكروغرام/مل على التوالي . و عملت الجسيمات النانوية الفضية الخضراء على انخفاض إنتاج إنزيمات البروتيز والفوسفولیباز و التعبير عن CDR1 و CDR2 المسؤولة عن المقاومة. اظهرت النتائج أن الجسيمات النانوية الفضية الخضراء قد تسبب فى تقليل التعبير عن جينات المقاومة CDR1 و CDR2 و MDR1 في الكانديدا المقاومة للفلوكونازول. وتشير النتائج إلى استخدام الجسيمات النانوية الفضية الخضراء كعلاج أمن وفعال ضد الكانديدا المقاومة للفلوكونازول.