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EFFECT OF BIOSYNTHESIZED COPPER NANOPARTICLES (CuNPs) ON THE GROWTH AND BIOFILM FORMATION OF FLUCONAZOLE-RESISTANT *CANDIDA ALBICANS*

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ABSTRACT

Biosynthesized copper nanoparticles through extracellular method were employed in this study to evaluate their effect on fluconazole-resistant *C. albicans*. MIC and MBC of CuNPs against *C. albicans* was 2.5 mg/ml and 5 mg/ml respectively, giving a tolerance level of 2. This was followed by absorbance measurements (OD_{600}) to further confirm the growth inhibiting effect of CuNPs in liquid culture. Furthermore, the well-plate method was performed to check the antifungal activity of two different concentrations of CuNPs (5 mg/ml and 10 mg/ml) against *C. albicans* by observing the zones of inhibition and fluconazole (25 µg/ml) was used as a control. Static biofilm assay was performed to check whether the CuNPs were able to inhibit the formation of biofilm by *C. albicans* which was further analyzed through spectrophotometric measurements. Growth curve analysis showed the inhibitory effect of CuNPs on *C. albicans* at different time intervals. The results from the current investigation suggested that the CuNPs were effective in controlling the fluconazole-resistant *C. albicans* and can be utilized as alternative antifungal agents.

Keywords: CuNPs, resistant, growth inhibition, biofilm, growth curve

INTRODUCTION

Antibiotic resistance is a worldwide issue which poses a threat to clinical practice, particularly in hospitals. This complication is further exaggerated by the less availability of new antibiotics (**Boucher *et al.*, 2009**). Research on finding some novel, harmless and effective therapeutic drugs that can replace the less effective antibiotics can be a means of controlling this menace of antibiotic resistance (**Jones *et al.*, 2004; Nagajyoti *et al.*, 2011**). More complications in human clinical practice arise due to the undesirable spread of fungal infections originating as a result of uncommon and unexpected species of *Candida*, which is furthermore intensified by an increase in the number of immunocompromised patients, posing a threat in human clinical practice. The frequency with which fungal infections are spreading has been specified as a result of certain risk factors including the immunological condition of the host, application of broad-spectrum antibiotics, transplants, continued use of intravascular and urethral catheters, use of corticosteroids and parenteral nutrition (**Francois *et al.*, 2013**). *Candida albicans* is generally believed to be the most pathogenic *Candida* species and so far, this species remains the most persistent *Candida* species that is isolated clinically (**Borman *et al.*, 2016**). *Candida albicans* has a major role in causing candidemia. The other species of *Candida* including *C. krusei, C. glabrata, C. tropicalis and C. parapsilosis* also play a role in causing candidemia (**Silva *et al.*, 2012**). The ability of *Candida* species to thrive on human mucous membranes, which is due to different virulence factors including the transition between yeast and hyphae, defensive ability against the host immune system, adhesion, biofilm-forming capability on host tissues or on medical devices and production of extremely harmful enzymes such as hydrolytic proteases, phospholipases and hemolysin, resulting in the development of various diseases ranging from mucocutaneous overgrowth to diffused infections (**Deorukhkar *et al.*, 2014**). The objective of this study was to evaluate the effect of CuNPs on the growth and biofilm of fluconazole-resistant *C. albicans*.

MATERIAL AND METHODS

Fungal strain and copper nanoparticles

The fungal strain (clinical isolate) i.e. *Candida albicans* used in this study was obtained from Tagore Medical College, Chennai, India after proper approval from institutional ethics committee (BSAU: REG-OFF: 2016/02SLS). The CuNPs synthesized in our previous study were used to evaluate the antifungal effects on *Candida albicans*. The synthesis of CuNPs was performed through a biological method via the assistance of a marine actinomycetes isolated from a seaweed (*Gelidium* sp.). CuNPs were synthesized through an extracellular method utilizing the cell-free extract obtained after culturing the actinomycetes in liquid media. 100 mM CuSO4 was mixed with an equal volume of the extract resulting in the synthesis of CuNPs. Freshly synthesized CuNPs were characterized through UV-Vis spectroscopy, FTIR analysis, SEM, EDAX and TEM and were further assayed for their antibacterial potential against 5 different human pathogenic bacteria (**Rasool & Hemalatha, 2017**).

Media preparation, autoclaving and inoculation

All the media preparations were carried out using analytical grade chemicals. LB broth (supplemented with sucrose for proper growth of *Candida albicans*) and Luria-Bertani agar (LBA) were used as liquid and solid media respectively in order to grow *Candida albicans* and to perform the experiments. The isolate was continuously sub-cultured and maintained as a slant.
Before performing any experiments, all glassware and media were properly autoclaved at 121^{0}C for 15 min to eliminate any sort of contamination.

Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC)

Serial micro-dilution and surface drop method were employed to evaluate the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of CuNPs respectively (**Dash *et al.*, 2012**). MIC was performed in a 96-well microplate where CuNPs were serially diluted with LB broth to prepare various concentrations (5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg/ml). MIC was performed in triplicates. The 10µl fresh culture of *Candida albicans* was added to each well. A positive (*Candida albicans* + LB broth) and a negative control (LB broth alone) were used. Fluconazole (25 µg/ml) was used as an antibiotic control. After performing the MIC experiment, 96-well microplate was incubated at 37^{0}C and was visually observed after a period of 24 h.
Surface drop method was employed to determine the Minimum fungicidal concentration (MFC) of CuNPs. 10µl aliquots from all the MIC dilutions were inoculated as a drop at eight different points on sterile LBA plates. The plates were incubated at 37^{0}C for 24 h. This experiment was performed in triplicates.

The 96-well microplate used to determine the MIC of CuNPs was subjected to spectrophotometric measurements (OD_{600}) to further confirm the antifungal activity of CuNPs.

Antibiotic susceptibility testing and antifungal activity of CuNPs through the well-plate method

To check whether the isolate used in the study is resistant to fluconazole (25 µg), an antifungal disc of antibiotic (fluconazole, 25 µg/ml) was placed on a solid agar plate seeded with an overnight culture (OD adjusted to 0.5 McFarland standard) of *Candida albicans*. The plate was incubated at 37^0C for 24 h and was later visually observed for the zone of inhibition (**Ekwealor *et al.*, 2016**).
The antifungal activity of CuNPs against *Candida albicans* was carried out through the well-plate method (**Kaur *et al.*, 2016**). *Candida albicans* was freshly cultured in a test-tube using Luria-Bertani broth as a medium of inoculation and seeded on LB agar plates. After proper absorption of fungal culture on the plate, proper wells were cut and loaded with 5 mg/ml and 10 mg/ml of CuNPs. $CuSO_4$ solution was used as a negative control. 25 µg/ml disc of fluconazole was used as a positive control. The plates were incubated at 37^0C for 24 h and were observed for the zones of inhibition.

Static biofilm assay

This experiment was performed to check the biofilm inhibition potential of CuNPs by following a method as described by **Reshamwala and Noronha (2011)** with slight modifications. Briefly, an overnight culture of *C. albicans* was diluted in fresh LB broth and adjusted to 0.5 McFarland standard using borosilicate glass tubes (70 × 10 mm). *C. albicans* was treated with the concentration corresponding to MIC of CuNPs. A culture without treatment was used as a positive control, culture treated with fluconazole (25 µg) was used as a negative control and LB broth alone was used as a blank. After performing the experiment, the tubes were incubated at 37^0C for 18-24 h. The detection of a static biofilm was carried out by discarding the LB medium and washing the tubes twice with double distilled water. This was followed by the addition of 0.1% crystal violet. The tubes were allowed to stand for 15 min. After properly washing out the dye with double distilled water, the appearance of the violet ring in the tubes demonstrated the presence of biofilm. This was followed by spectrophotometric measurements after the addition of 30 % glacial acetic acid to all the tubes in order to quantify the biofilm.

Growth curve study

C. albicans was allowed to grow in LB broth till it reached to its log phase. Later, a fresh LB broth was prepared and the grown *C. albicans* was added so that an optical density of 0.1 was attained. A solution of CuNPs was prepared (MIC concentration) which was added to the culture medium. The culture was incubated at 37^0C in an incubator shaker at 200 rpm. Growth rate and the concentration of *C. albicans* was determined by measuring OD at 600 nm at 5 different time intervals and the growth curve was analyzed (**Maiti *et al.*, 2014**).

RESULTS AND DISCUSSION

MIC and MFC determination

Minimum inhibitory concentration (MIC) of biosynthesized CuNPs against *Candida albicans* was carried out in a 96-well microplate. MIC value was found to be 2.5 mg/ml. MIC was taken as the concentration of CuNPs where there was no visible growth. Following MIC, minimum fungicidal concentration (MFC) of CuNPs against *Candida albicans* was carried out and it was observed that 5 mg/ml of CuNPs completely inhibited the growth of *Candida albicans*. All other concentrations were least effective with more or less growing *C. albicans*. Here, MFC value was the exact double of MIC value.
After MIC and MFC were calculated, the 96-well microplate was analyzed via spectrophotometric measurements to calculate OD_{600} to get a clear picture of the growth inhibition of *Candida albicans* by CuNPs. Results are shown in figure 1. It was observed that with a decrease in the concentration of CuNPs, the OD values increased parallelly. 5 mg/ml of CuNPs was the most effective followed by other concentrations and 0.3125 mg/ml of CuNPs was the least effective. The antifungal properties of CuNPs against *C. albicans* have been reported in various other studies, even though the MIC values reported in our study differ from those studies (**Amiri *et al.*, 2016; Usman *et al.*, 2013; Soltani *et al.*, 2017**). There are various reasons that lead to the difference in the activities such as the size of the nanoparticles which is an important factor. It is generally known that the size and shape of nanoparticles have an influence on their chemical, optical and thermal properties. So, the size and other characteristics of the nanoparticles can be the reason for variable antimicrobial properties (**Akther *et.al.*, 2018**).The stabilizing agent used in the synthesis of nanoparticles can also have an effect on the properties of nanoparticles (**Vazquez-Munoz *et al.*, 2014**). The effect of nanoparticles is also species dependent (**Yoon *et al.*, 2007**). In our study, CuNPs which were originally synthesized via the assistance of a marine actinomycetes, had a growth inhibiting effect on *C. albicans* where 5 mg/ml of CuNPs proved more effective and 0.3125 mg/ml proved least effective. Still, the least effective concentration of CuNPs (0.3125 mg/ml) was able to inhibit the growth of *C. albicans* more effectively compared to the control (untreated) as shown in figure 1.
A ratio of MFC/MIC was also calculated to get an idea about the fungicidal (MFC/MIC < 4) or fungistatic effect (MFC/MIC ≥ 4) of CuNPs on *C. albicans* (**de Castro *et al.*, 2015**). The ratio of MFC/MIC was calculated as 2 which means that CuNPs had a fungicidal effect on *C. albicans*.

Table 1 MIC and MBC values of CuNPs against *C. albicans*

Strain	MIC (mg/ml)	MBC (mg/ml)	MFC/MIC
Candida albicans	2.5	5	2

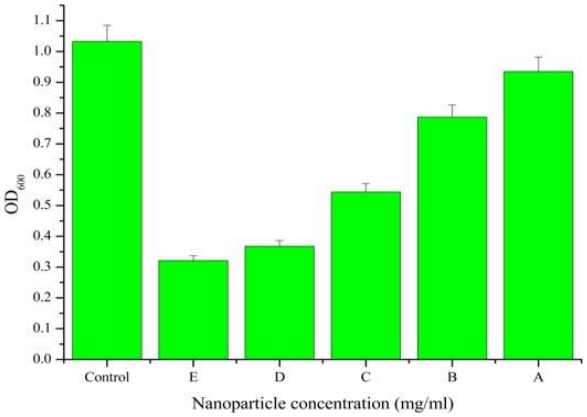

Figure 1 Graphical representation of OD_{600} (From A to E, the concentration of CuNPs increases from 0.3125 mg/ml to 5 mg/ml. The graph represents an increase in the spectroscopic measurements as the concentration of CuNPs decreases)

Antibiotic susceptibility and antifungal activity through the well-plate method

Susceptibility testing revealed that *C. albicans* used in this particular study was resistant to 25 µg/ml of fluconazole which is a standard concentration of this antibiotic prescribed for any fungal infection caused by *C. albicans* (**CLSI, 2017**). There was no zone formation with the antibiotic disc used showing the higher resistance of *C. albicans* towards the antibiotic. Antifungal activity of CuNPs through well-plate method displayed smaller zones of inhibition where 10 mg/ml of CuNPs showed little higher zones (12.5 mm) compared to 5 mg/ml (11.5 mm). The reason behind the less activity of CuNPs in solid media couldn't be known which needs to be investigated more. No zones were observed with $CuSO_4$ solution (negative control) as well as the blank. Results of susceptibility and antifungal activity through well-plate method are shown in figure 2.

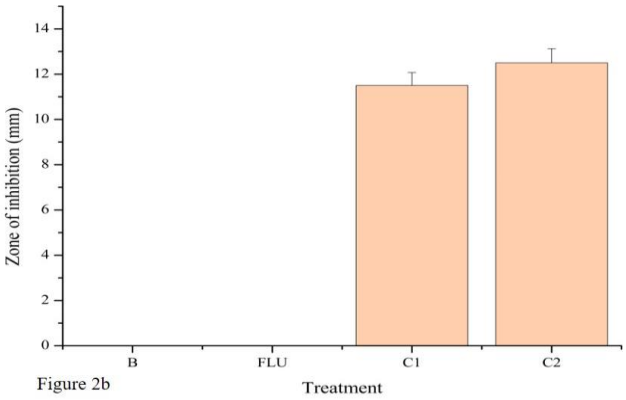

Figure 2 Graphical representation of zones of inhibition of CuNPs (well-plate method), against *Candida albicans* (C1=5 mg/ml and C2=10 mg/ml of CuNPs, FLU=Fluconazole, B= Blank $CuSO_4$)

Biofilm inhibition

The tube method employed for controlling the biofilm formation in *C. albicans* was effective. The diagrammatic representation of the biofilm inhibition by CuNPs and graphical representation with spectrophotometric measurements that were carried out after the addition of 30 % glacial acetic acid is presented in

figure 3. It is evident from both the figures that CuNPs were more effective in controlling the formation of biofilm when compared to fluconazole. Formation of biofilms by *C. albicans* is not a simple gathering of cells, but rather highly structured microbial communities, which is being assumed to carry out different functions such as to ease the process of the influx of nutrients and waste disposal. Most of the researchers agree to the point that the biofilm formation in *C. albicans* comprises of different phases which include initial adherence, colonization, proliferation, maturation and ultimately dispersion so that the process of biofilm formation gets repeated all over again. Biofilm formation by mature *C. albicans* consists of a network of yeasts, hyphae and pseudohyphae which possess a complex three-dimensional structure. (**Uppuluri *et al.*, 2009**). There are some studies carried out on the inhibition of *C. albicans* biofilms. A study by **Bruzual *et al.*, (2007)** reported the inhibition of biofilm formation by fluconazole-resistant *C. albicans* by fluconazole itself. They also stated the effect as biofilm-specific rather than the effect of fluconazole. A study by **Rahimi *et al.*, (2016)** reported the inhibition of *C. albicans* biofilm by copper oxide nanoparticles. They conducted their study on a standard strain of *C. albicans*.
In this study, we found that the biosynthesized CuNPs attenuated the *C. albicans* biofilm formation more effectively compared to the antibiotic fluconazole. A study by **Yu *et al.*, (2016)** reported that the activity of gold nanoparticles in inhibiting the *C. albicans* biofilm to be associated with the electrostatic interaction between nanoparticles and fungal cells. The biosynthesized CuNPs not only can inhibit the pathogenic bacteria, as demonstrated in our previous study, but can also impair the biofilm formation in *C. albicans*. Inhibition of biofilm formation in pathogenic microbes has received a great interest during recent times (**Akther *et al.***),As the biofilm formation depends on cell-to-cell signalling facilitated by quorum sensing (QS) molecules, maximum approaches towards biofilm inhibition are targeted towards the signalling system. A single possible mechanism that can be involved here is the strong electrostatic attractions between CuNPs and cell wall surface of *C. albicans* leading to an interruption in the adhesin-mediated interaction between the cells of *C. albicans* and the substrate surfaces.

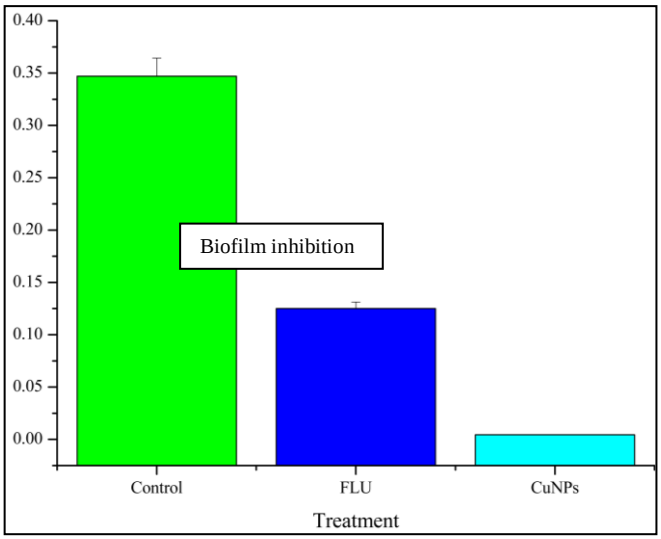

Figure 3 Biofilm inhibition by CuNPs. The above graph displays the inhibition of *C. albicans* biofilm by CuNPs. Compared to fluconazole, CuNPs were more effective in inhibiting the biofilm

Growth curve analysis

After observing and analyzing the effect of CuNPs on *C. albicans* with time and plotting a graph from the observed data, the slope of the *C. albicans* growth curve decreased with the passing time till a straight line was observed. The initial growth curve of *C. albicans* starting at 0.1 nm reduced to 0.013 nm after treatment with CuNPs. Untreated *C. albicans* showed a luxuriant growth with increasing time intervals. Hence, it can be concluded that CuNPs had a time-dependent effect on the growth of *C. albicans*.

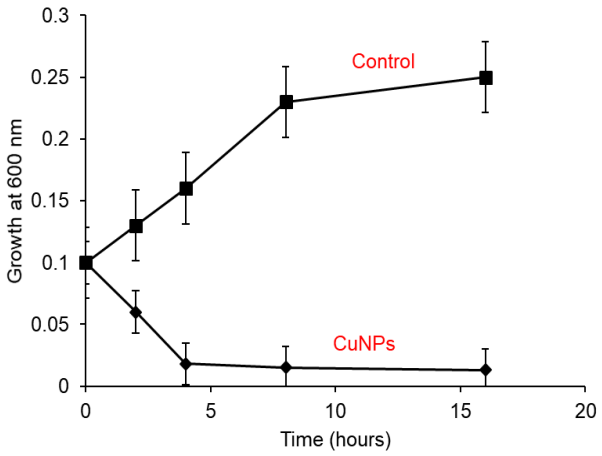

Figure 4 Growth curve with initial OD 0.1. The above graph shows the time-dependent effect of CuNPs on the growth of *C. albicans*

CONCLUSION

This study was carried out to widen the area of biological activities that CuNPs could possess. In this study, CuNPs were effective in controlling the growth in fluconazole-resistant *C. albicans*. After observing and calculating the MIC and MBC values followed by growth assay, it was concluded that CuNPs were effective in controlling the growth of *C. albicans* in liquid media. However, CuNPs were not much effective in controlling the growth of *C. albicans* in solid media which needs some further investigations. CuNPs were also effective in controlling the formation of biofilm in *C. albicans*. It can thus be concluded that CuNPs used in this study can be used as disinfectants or can be applied on liquid settings which are infected with *C. albicans*.

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