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Antifungal Activity of Silver Nanoparticles Synthesized Using Phenylalanine Conjugated Cholic Acid Salts & Tyrosine Conjugated Cholic Acid Salts against Candida Species

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Abstract:
Silver Nanoparticles were synthesized by reducing and stabilizing Silver nitrate solution using two different amino acids, phenylanine conjugated cholic acid salts and tyrosine conjugated cholic acid salts. Stable nano suspensions of the silver were obtained by adding defined volumes of the reducing agent. The shape and size distribution of the stabilized silver nanoparticle were characterized by UV-Vis Spectrophotometer and Transmission Electron Microscopy. The absorption spectrum of the silver nanoparticles showed a Surface Plasmon Absorption band at 487nm and average size of the nanoparticle was calculated as 10-15nm by transmission electron microscopy studies indicating the presence of spherical and nanosized silver. The antifungal activity of silver nanoparticles was tested against 8 strains of Candida species. These organisms were speciated by standard biochemical tests. The minimum inhibitory concentration was determined by microbroth dilution method using Mueller Hinton broth and RPMI. The nanoparticles exhibited inhibitory effect against the tested Candida species at different concentrations and majority of the species were inhibited at 0.43µg/ml. The present study indicates that silver nanoparticles have considerable antifungal activity and deserves further analysis with large number of species and isolates for clinical use.

1. Introduction

With the emergence and increase of microbial organisms to multiple antibiotics, and the continuing emphasis on health care costs, many researchers have tried to develop new, effective antimicrobial reagents free of resistance and cost. Such problems and needs have led to the resurgence in the use of silver based antiseptics with broad spectrum activity and much lower propensity to induce microbial resistance than antibiotics (Sung Jin Park et al., 2006)

Antimicrobial tests were performed against yeast, *E.coli* and *S.aureus* on MHA plates treated with different concentrations of silver nanoparticles (from 0.2 to 33 nm). Comparing with the positive control, Ag nanoparticles showed growth inhibition against yeast and significant growth inhibition was observed from 13.2 nm. These results revealed that the MIC of silver nanoparticles against yeast falls around some minimal concentrations itself. Few studies have shown the biological effects of Nano-Ag, however, its effects against fungal pathogens have not yet been fully studied. In this study, therefore, the antifungal properties of Nano-Ag against human pathogenic fungal strains were investigated.

With this background, the present study is proposed to synthesize silver nanoparticles and to determine and evaluate the antifungal activity against clinically significant fungal pathogens such as Candida species.

2. Materials and Methods

2.1. Synthesis of Silver Nanoparticles

Silver nitrate solution prepared by dissolving silver nitrate in 25ml of deionised double distilled water. The solution was reduced and stabilized with aminoacids conjugated cholic acid (Phenylalanine and Tyrosine). The silver nanoparticles synthesized were characterised by UV spectroscopy and the Nanosize of the particles were determined by HRTEM (JEOL JEM 2100) equipped with a Gatan imaging filter.

2.2. Species of Candida tested

A total of 15 Candida species each two isolates of *C.albicans*, *C. dubliniensis*, *C. tropicalis*, *C.kefyr*, *C.guilliermondii*, and three isolates of *C. fermentati* and a single isolate each of *C.glabrata* and *C. krusei*. ATCC strain of *C.albicans* 90028 were included as quality control study.

2.3. Media used

RPMI is a general purpose medium that is used in the cultivation of a wide variety of fastidious and non fastidisous microorganisms. This medium is not supplemented with calcium or magnesium ions and widely used as medium for susceptibility testing of Candida species by disc diffusion method and has been recommended by CLSI. This media was used to perform MIC assay by Microbroth dilution method.

2.4. MIC determination by microbroth dilution method

Briefly, each isolate was cultured overnight at 35°C on Sabouraud dextrose agar to ensure purity and viability. Five colonies of the isolate were then suspended in 5 mL of sterile saline (0.85%), and turbidity (read at a wavelength of 530 nm) was adjusted to a 0.5 McFarland standard. The suspension (approximately $1x10^6$ to $5x10^6$ CFU/mL) was used for the assay.

The silver nanoparticles (NP) was serially diluted in Mueller Hinton Broth (MHB) to obtain a concentration ranging from 28µg/ml to 0.109 µg/ml in a sterile disposable microtitre plates. Inoculum of concentration of 2.5×10^3µg/ml whose turbidity was matched with 0.5 Mc Farland standard was added and incubated at 37ºC for 24–48hrs. The MIC was recorded spectrophotometrically at 492nm in a microtitre plate reader. The broth with the inoculum and without the nanoparticles served as growth control. Wells containing the media alone served as blank. The percentage of growth at each drug concentration was calculated with the following equation:

% growth= [(OD_{405} of wells containing the drug/OD_{450} of the drug-free well) x 100]

after substraction of background ODs (ODs of microorganism-free wells).

The MIC_{80} values was calculated for each species (MIC_{80} is defined as the lowest concentration of the nanoparticle that inhibited 80% of the growth as determined by comparison to the OD values obtained by the growth of isolates in the control wells. The MICs were recorded after 48hrs of incubation at $37\ ^0$C.

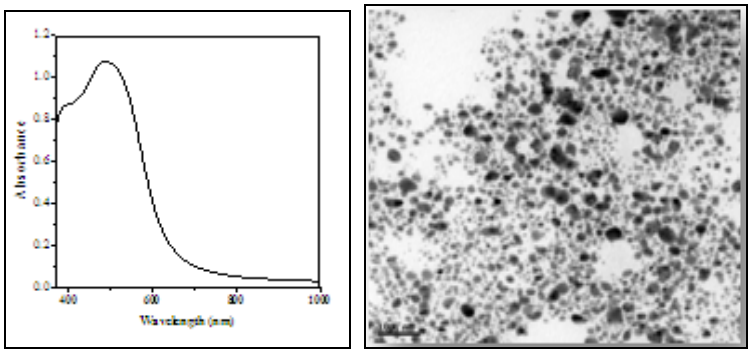
3. Results and Discussion

The phenylalanine and tyrosine residue in the peptides is responsible for the reduction of metal ions to the respective metals, possibly through electron transfer. The UV-visible absorption spectrum of the resultant silver nanosuspensions exhibited a SPR at 415 nm along with a weaker band at 370 nm. In the present study the absoprption spectrum showed a peak at 487nm with the use of phenylalanine conjugates cholic acid salts as both reducing and stabilization agents. To date, there are no reports on the use of such agents in the preparation of silver nano particles.

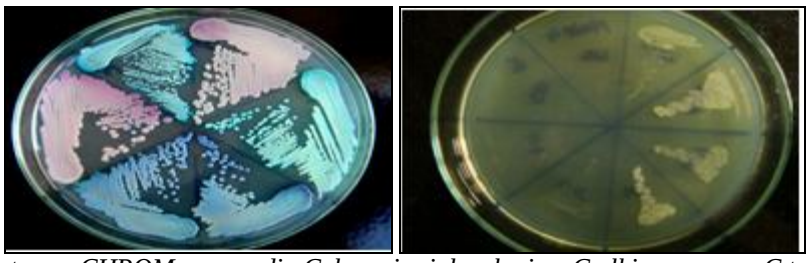
The shape and nanosize of AgNP was characterized by TEM studies and was carried out in HRTEM (JEOL JEM 2100) equipped with a Gatan imaging filter. The transmission electron microscopic (TEM) images (Figure 2) of the aminoacid conjugated cholic acid salts based synthesized silver nanoparticles revealed the formation of spherical particles. The average diameters of the silver nanoparticles were calculated to be ranging between 10-15nm.

3.1. Colony Morphology of the Candida isolates on CHROMagar Candida

The Candida isolates were revived from the stock collection of our department and was subcultured on to SDA plates to obtain pure and isolated colony. The colony colour and morphology was recorded after incubation of the isolates on CHROMagar Candida. *C.albicans* species appeared green and *C.dubliniensis* light green, whereas *C.tropicalis* appeared metallic blue , while isolates of *C.krusei*, *C.kefyr*, *C.guillermondii*, *C.glabrata* and *C.fermentati* showed varying shades of pink to light purple coloured colonies .The Candida isolates grown on CHROMagar showing various coloured colonies are shown in Plate.



Surface Plasmon absorbance with size range from 10-15 nm
&
TEM images of the silver nanoparticles band at 487 nm



Candida isolates on CHROMagar media C. krusei- pink colonies; C.albicans-green C.tropicalis- blue
&
MFC of Candida tested for its susceptibility to silver nanoparticles Anticandidal activity

The synthesised Ag nanoparticles stabilised Phenylalanine & Tyrosine conjugated cholic acid salt was tested for its antifungal activity of Candida species. The silver nanoparticles synthesised with an average diameter of 10-15 nm was evaluated for its antifungal activity with reference to Candida species. A total of 15 Candida species each two isolates of *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. kefyr*, *C. guilliermondii*, and three isolates of *C. fermentati* and a single isolate each of *C. glabrata* and *C. krusei*. ATCC strain of *C.albicans* 90028 was included as quality control throughout the study. The minimum inhibitory concentrations of the nanoparticles were determined by microbroth dilution method as per the CLSI guidelines. RPMI broth was used for the test.

The MIC_{80} of the 15 isolates of candida tested was in the range between 0.21µg/ml to 1.75µg/ml. The two isolates of *C.albicans* were inhibited at 0.43µg/ml, *C.dubliniensis* isolates at 0.87 and 0.21 µg/ml. The isolate of *C.krusei* showed slightly higher value than *C.albicans* with inhibitory concentration at 0.87µg/ml. 3 isolates of *C. fermentati* were inhibited at 87µg/ml while single isolate of *C.guilliermondii* at 0.43 µg/ml and *C. glabrata* 0.87µg/ml. One of the two isolates of *C. kefyr* tested had MIC_{80} value of 1.75µg/ml while the other isolate tested had a MIC_{80} value of 0.87 µg/ml and the single isolate of *C. glabrata* tested had a MIC_{80} of 0.87µg/ml (Table 1).

The MFC was found to be slightly higher than the MIC values of the nanoparticles against the Candida species. Mueller Hinton agar plate showing MFC of an isolate of Candida tested for its susceptibility to silver nanoparticles is presented in Plate.

In comparison to the number of antibacterial agents, only a limited number of antifungals are available for use in antifungal therapy. The azole antifungals such as fluconazole, itraconazole are frequently used in the treatment of candidiasis. Prolonged usage of these azoles as well as emergence of *Candida* species that have decreased susceptibility or intrinsic resistance to these agents have resulted in treatment failures.

Many authors have reported on the antimicrobial activity of silver nanoparticles on bacterial species while the antifungal efficacies of the silver nanoparticles documented in the literature are very few. Silver Nanoparticles of average size of 13.5nm has been tested on *E.coli* and inhibition of the bacterial species with a particle size with 5nm against *E.coli* was found to be 10µg/ml. The antifungal effect of silver nanoparticles has received only marginal attention and only few studies are available. In the present study, the MIC of the silver nanoparticles on 15 isolates of Candida species was determined.

In the present study, the silver nanoparticles with an average size of 10-15nm showed more activity and had a lesser MIC Value to all the species of Candida tested when compared to their reports. This may be due to the stabilization of the nanoparticles with agents of biological origin and may be a possible reason for more antimicrobial activity.

In the present study, the silver nanoparticles were synthesized using Phenlyalanine and Tyrosine conjugated cholic acid bile salts as reducing as well as stabilizing agent. The Phenlyalanine and Tyrosine residue in the peptides is responsible for the reduction of metal ions to the respective metals, possibly through electron transfer.

Sl. No	Candida species	No of isolates tested	MIC_{80} µg/ml Silver nanoparticle reduced and stabilized by Phenylalanine conjugated Cholic acid salts	MIC_{80} µg/ml Silver nanoparticle reduced and stabilized by Tyrosine conjugated Cholic acid salts	MIC_{80} µg/ml Itraconazole (azole antifungals)
1.	ATCC C. albicans 90028	Standard Strain	0.87	1.75	0.062
2.	IFM C. tropicalis	Standard Strain	0.43	0.87	0.5
3.	IFM C.krusei	Standard Strain	3.5	3.5	0.25
5.	C.albicans	Clinical strain 1 Clinical Strain2	0.87 0.21	0.43 1.75	0.062 0.062
6.	C. tropicalis	Clinical strain 1 Clinical Strain2	0.43 1.75	0.87 0.87	0.03 0.062
7.	C. krusei	Clinical strain 1 Clinical Strain2	1.75 0.87	0.87 1.75	0.5 0.25
8.	C. guillermondii	Clinical strain 1 Clinical Strain2	0.21 0.43	0.21 0.87	0.062 0.062
9.	C. fermentati	Clinical strain 1 Clinical Strain2 Clinical strain3	0.43 0.43 0.87	0.43 0.43 0.87	0.25 0.5 0.062
10.	C. kefyr	1	0.21	0.21	0.062

Table 1: Anticandidal activity of Silver Nanoparticles by Minimum inhibitory concentration studies (Micro broth dilution method)

In the present study , an attempt was made to synthesise silver nanoparticles using Phenylalanine conjugated cholic acid salts and nanoparticle of average size ranging from 10-15nm was synthesized , characterized and their antifungal activity against Candida species were performed. This study included more species of Candida such as C. *glabrata* and *C.fermentati* than the earlier reports and MIC values were comparatively less than that of the MIC values obtained for the azole antifungals tested. The use of phenylalanine and cholic acid may render the nanoparticles with negligible toxicity and may be used as a alternative and as safer antifungal agent.The study also reports on the susceptibility testing of Candida species to silver nanoparticles such as *C.guillermondii* and *C.glabrata* which has not been previously reported.

4. Conclusion

The increase in fungal diseases, the diversed fungal pathogens, **a**vailability of fewer antifungal agents, emergence of resistance to available antifungals ,the increasing reports of treatment failure in patients and relapse of the disease emphasize the need for alternative agents for antifungal therapy. Nanoparticles are gaining more attention as potential antimicrobial agent. The results of the present study suggests that amino acids may be used as a stabilizing agent in the synthesis of silver nanoparticles which may be used as potential alternative treatment for resistant strains of Candida and the nanoparticles may be considered more safer as the synthesis involves biological molecules as reducing and stabilizing agents. The present study also suggest to explore more aminoacids or biomolecules be tested for their efficiency in producing nanosized particles. The MIC values were comparatively less and suggest that Ag nanoparticle is a potent antifungal agent, with high degree of antimicrobial indentions. The size of the nanoparticles also plays a pivotal in its inhibitory action.

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