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BIOSYNTHESIS OF SILVER NANOPARTICLES USING BIOMASS OF *CLADOSPORIUM CLADOSPORIOIDES* AND ANTIFUNGAL ACTIVITY AGAINST PATHOGENIC FUNGI CAUSING ONYCHOMYCOSIS

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Abstract

Onychomycosis is a fungal infection of the toenails or fingernails that can involve any component of the nail unit, including the matrix, Bed and plate. Recently, Fungal isolates showed increasing resistance to antifungal agents and this had contributed to high morbidity among the patients. For that the study aimed isolation and identification of Dermatophytes fungi, Non-dermatophytes fungi and Yeast caused of Onychomycosis and biosynthesis of silver nanoparticals (AgNPs) using biomass *Cladosporium cladosporioides* and evolution of antifungal against pathogenic fungi causing onychomycosis. The isolates were distributed as Dermatophyte fungi, non-dermatophyte mold and yeast. Bio synthesis of AgNPs using biomass of *C. cladosporioides*. Several techniques where used to characterize AgNPs: X-ray Diffraction Analysis (XRD), UV-Visible Spectroscopy, Scan Electron microscope (SEM) and Fourier Transform Infrared Spectroscopy (FTIR). The effect of AgNPs at concentrations (25, 50 and 100) µg/ml against (2) isolates of *Trichophyton* spp. and (5) isolates of mold study by Food poising method. Also study the effect of AgNPs against (4) isolates of *Candida* spp and (1) isolates of *Rhodotorula* spp. by agar plate well diffusion assay. The results showed that all *Trichophyton* spp, mold isolates and some species of yeast were susceptible to AgNPs and the inhibition rate increases with the increase of concentration.

***Key words*:** *Cladosporium cladosporioides*, Onychomycosis, silver nanoparticals, non-dermatophyte.

Introduction

Onychomycosis is an infection of the nail equipment caused by dermatophytes, Yeasts and non-dermatophyte mold. It can cause nail plate discoloration, thickening and onycholysis (Welsh, 2010). Most cutaneous infections are the work of the homogeneous group of keratinophylic fungi known as dermatophytes. Traditionally referred to a non dermatophytic infection of the nail but is now used as a general term to denote any fungal nail infection (tinea unguium specifically describes a dermatophytic invasion of the nail plate (Neupane, 2009). The a superficial fungal is keratinophylic meaning use the keratin found in the nail and hair for growth and development because it's contain enzyme called keratinize able utilize the keratin and caused infection. The factors that increase the prevalence of onychomycosis include increasing age, female gender, underlying conditions such as diabetes, immune deficiency, peripheral arterial disease and psoriasis, environmental and behavioral factors such as sporting and religious practices and certain professions. Genetics has also been identified as a factor governing the epidemiology of Onychomycosis (Neupane, 2009). Nanotechnology is one of the application most thoroughly investigated nanomaterials and owes its popularity to its biocidal properties (Kalaiselvam, 2013) silver nanoparticales (Nano-Ag) have long been known to have strong inhibitory and fungicidal effect as well as a broad spectrum of antimicrobial activities. It is expected that the high specific surface area and high fraction of surface atoms of silver nano particles will lead to high antimicrobial activity compared to bulk silver metal (Noorbakhsh *et al*., 2011). This study was aimed to biosynthesis of silver nanoparticles and study antifungal effect against onychomycosis fungi consistency (Soltani *et al*., 2015, Ellis *et al*., 2011).

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Material and Method

Fungal isolates

In the period of November 2017 to March 2018, a total of 70 patients attending In Baghdad/Iraq. All isolates Cultivation on different media Sabouraud's dextrose agar (SDA) with chloramphenicol and cycloheximide for isolation dermatophyte fungi (*Trichophytonrubrum* and *Trichophyton mentagrophytes*; SDA with chloramphenicol for mould (*Aspergillus niger, Mucor* spp. *Penicllium* spp. *Syncephalastrum* spp. and *Cladosporium* spp.) and yeast isolates *Candida* spp and *Rhodotorula*. The culture were incubated at 28°C for 5-7 days to isolate pure colonies to examine their shape, size and color.

Biosynthesis of silver Nanoparticles using Biomass

The mycelia of *Cladosporium cladosporioides* were inoculated in 250mL Erlenmeyer flasks, each containing 100 mL of potato dextrose broth (PDB) medium and incubated at 25±2°C for 7 days. Later, mycelia were harvested by filtration through whatman filter paper no. 1 and washed thrice with sterilized distilled water to remove the traces of medium on fungal biomass. Two gram of wet biomass were treated with 50ml (1mM) of silver nitrate and incubated at 28°C in darkness and light at 200 rpm for 3 days. A control experiment containing only 1mM of silver nitrate solution was also performed. All experiments were carried out in triplicates.

Characterization of silver Nanoparticals

1. XRD Analysis: The structure and composition crystallite size of synthesis of silver nanostructure determine by X-Ray Diffraction spectroscopy (Philips PNA analytical). The synthesis AgNPs were studies with cu-ka radiation at voltage of 30 KV and current of 20 mA with scan rate of 0.030/s.
2. UV-Visible Spectrophotometer: Biotransformation of silver ions was monitored by UV-visible spectroscopy measurement of the reaction medium. Three milliliters of supernatant were taken and absorbance was scanned by Labomed, UV-Vis double beam (Labomed, Inc, USA) within the wavelength ranging from 300 to 900 nm. The absorption of the visible depends directly on color of the chemicals in solution (Husseiny *et al.*, 2015).
3. Scanning electron microscopy: Morphological characterization and elemental analysis of Bio-AgNPs were performed with an ultrahigh-resolution field emission scanning electron microscope (Nano SEM-FEI Nova 200-FEG/SEM: OR, USA), with integrated microanalysis X-ray system (energy dispersive spectrometer (EDS)) and electron backscatter diffraction (EBSD, EDAX-Pegasus X4M (EDS/EBSD)). For SEM analysis, a portion of freeze-dried of each C Bio-Ag-NPs was loaded in the specimen holder of the equipment and afterwards analysed (Pereira *et al*., 2014).
4. Fourier Transform Infrared Spectroscopy (FTIR): FTIR analysis of the dried powder of AgNPs was carried out by scanning the spectrum in the range 400-4,000 cm^{-1} at a resolution of 4 cm-1 (8400S/Shimadzu / Japan). FTIR measurements were made to locate the possible biomolecules, which are responsible for the reduction of silver ions to AgNPs and stabilization of AgNPs. To prepare dried powder of AgNPs, the fungal treated broth was centrifuged at 12000 g for 15 minutes. Supernatants were discarded and pellets of Ag NPs were washed three times with autoclaved distilled water. The dried powder of AgNPs was subjected to FTIR analysis.

Antagonistic activity of Silver Nanoparticles against fungi

1. Inhibitory effect of silver Nanoparticales against Dermatophyte and Non-dermatophyte by food poising Method.

The antifungal activity of silver nanoparticles on most resistance isolates of dermatophytes fungi (*Trichophyton rubrum* and *T. mentagrophytes*) and non dermatophyte mold (*Aspergillus niger*, *penicillium* spp. *Cladosporium cladosporioides*, *Mucor* spp. and *Syncephalastrum* spp.) were assay by food poising method. SDA disk (5mm diameter) of a pure culture of fungal isolates taken from the margins of (4-7days -old culture, dermatophyte and non dermatophyte respectively were placed on to the critter of AgNPs at concentration (25, 50, 100) µg/ml (3ml of each concentration mixed with 27ml of SDA). Control petri plates contained only SDA. All plates were incubated at 28°C for 4-7 days. Then growth measurements were taken using ruler. Three petri dish for each treatment were considered this evolution.

2. Inhibitory effect of Silver Nanoparticles against yeasts by agar plate well diffusion assay.

The antifungal activity of the silver nanoparticles on four most resistance isolates of *Candida* spp. (*C. albicans, C. famata, C. guilliermondii* and *C. lusitaniae*) and one isolate of *Rhodotorula* spp were assayed by agar plate well diffusion assay. Method described by Abood, (2014) was followed to detect of AgNPs inhibition activity by spreading 0.1 ml of the yeast suspension on the surface of SDA and left to dry at room temperature. 100 µL of an AgNPs solution, 25, 50 and 100 µg/mL concentrations were added into 5mm diameter wells,

Later that incubated for 24 hrs. at 37°C then the zone of inhibition has been measured using ruler.

Statically analysis

Minitab software version 6 was used for analyzing data the ANOVA-test has been done to calculate the P value between the control and test groups in the previous studies .least significant difference -LSD test was done also to compare means between groups in this study the results were presented as mean ± SD. AP value equal or less than 0.05 was considered as the level of statistical significance.

Results and Discussion

Properties of silver Nanoparticles

1. X-Ray Diffraction: Further confirmation of synthesized AgNPs was examined by the XRD Diffraction spectroscopy. In fig. 1, showed one peaks. Strong diffraction peaks were: 38°C refer to the silver nanoparticals at 60°C condition when use biomass of *Cladosporium cladosporioides*.

Pereira *et al*., (2014) show the XRD pattern of diffraction peaks showed at 2θ values of 32.32°, 45.99°, 66.72° and 75.76° assigned to the planes of (111), (200), (220) and (311) faced centre cubic (fcc) of silver were obtained ranging from 10 to 80. The values agree well with those reported for silver (face centric cubic) by Joint Committee on Powder Diffraction Standards File No. 04-0783.

2. UV-Visible Spectrophotometer: The presence of nanoparticles was confirmed by obtaining a spectrum in the visible range of 30nm-600nm using UV-visible spectrophotometer. In fig. (2a, b) showed, absorbance peak 450nm which specific for the AgNPs. There was a single peak indicating synthesis of sepherical nanoparticles in biomass condition. These results agree with Husseiny *et al*., (2015). AgNPs synthesized shown maximum absorbance peak at 420 nm by UV-visible spectroscopy.

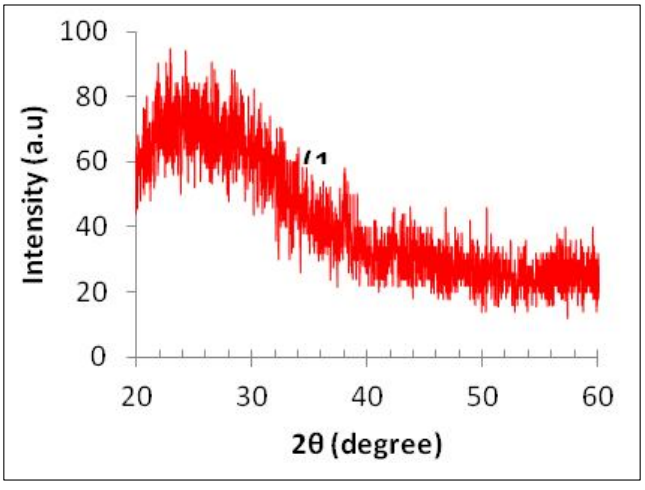

Fig. 1: X-Ray pattern of AgNPs synthesis by *Cladosporium cladosporioides* biomass in pH 9 (C. biomass).

3. SEM Analysis: Morphological characterization and elemental analysis of Bio-AgNPs were performed with an ultrahigh-resolution field emission scanning electron microscope (Nano SEM-FEI Nova 200-FEG/SEM: OR, USA), in fig. 3, showed a aggregation surface morphology. The particle sizes were ranging from 5-50nm. (Rathna *et al*., 2013) show the SEM images and their size distributions revealed that, the mean diameters and standard deviation of silver nanoparticles were about 20 -80 nm with the different morphologies. Electron microscopy data indicate that the extracellular particles produced .

4. Fourier Transform Infrared Spectroscopy (FTIR): FTIR measurements of the samples of dried powder were carried out to identify the probable interactions between silver and bioactive molecules, which may be responsible for synthesis and stabilization (capping material) of AgNPs. In fig. 4, shown FTIR spectrum of AgNPs synthesis by *Cladosporium cladosporioides* in biomass (*C. biomass*) 3750.44 cm1 with a shoulder at 3735.57 cm1, which were assigned to the stretching vibrations of primary and secondary amines. The two bands observed

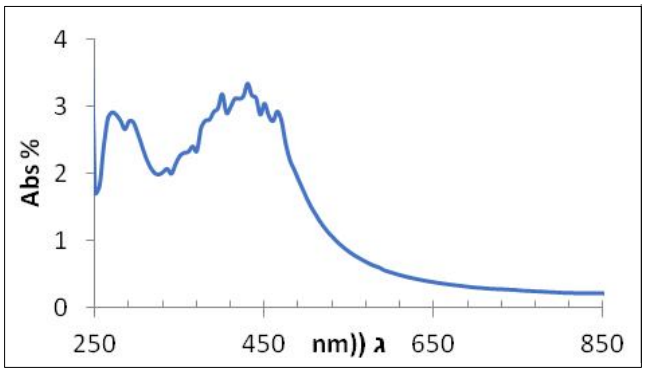

Fig. 2a: Absorptions spectrum of biosynthetic AgNPs using *Cladosporium cladosporioides* biomass in pH 9.

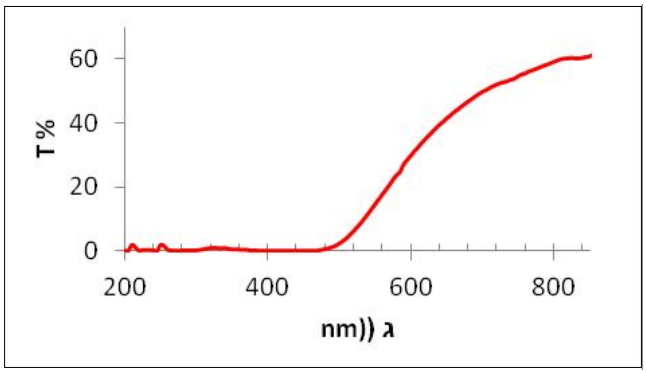

Fig. 2b: Absorptions and translation spectrum of biosynthetic AgNPs using *Cladosporium cladosporioides* biomass in pH 9.

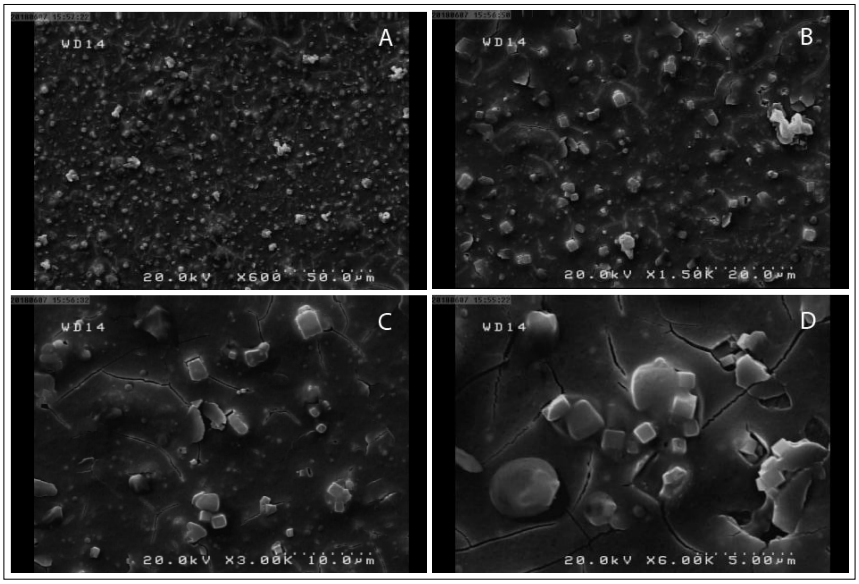

Fig. 3: Scan Electron microscope (SEM) image of silver nanoparticals synthesis by *Cladosporium cladosporioides* in biomass condition with size (5-50) nm .

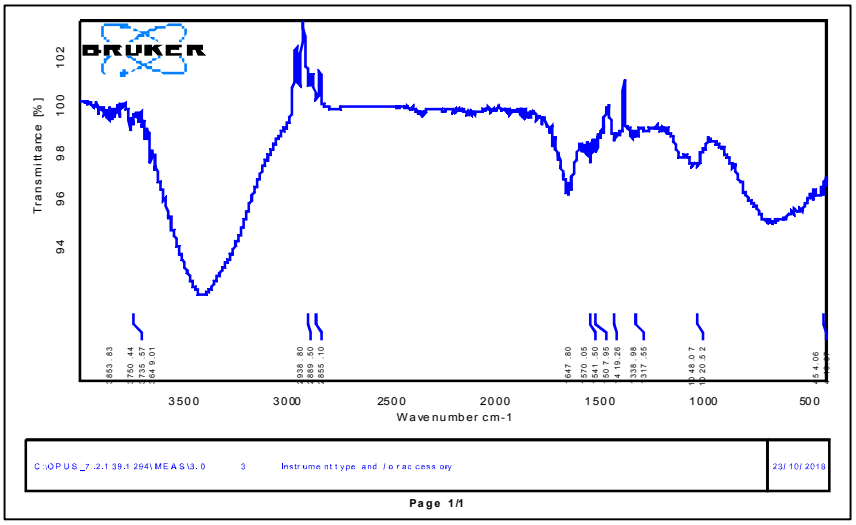

Fig. 4: Fourier transform infrared (FT-IR) spectrum of Bio-AgNPs synthesized by cladosporium Biomass in pH9.

at 2889.50cm^{-1} and 2885.10cm1 can be assigned to the C-N stretching vibrations of aromatic and aliphatic amines, respectively. The overall observation confirms the presence of protein in samples of silver nanoparticles from *Cladosporium cladosporioides* it has also been reported earlier that protein can bind to silver nanoparticles through their free amine groups or cysteine residues (Gole *et al*., 2001 and Jeevan *et al*., 2012), or through free amide groups (Bansal *et al*., 2004, Saravanan *et al*., 2013). So that the protein could most possibly to form a coat covering around AgNPs. And it stabilize the aqueous synthetic medium.

Inhibitory effect of AgNPs Synthesis *C. cladosporioides* Against Onychomycosis fungi

1. Inhibitory effect of AgNPs Against dermatophytes by poising food assay.

The inhibitory effects of various concentrations of AgNPs (25, 50 and 100µg/mL) were assayed on two dermatophyte isolates (*T. rubrum, T. mentagrophytes*) by poising food assay. The effect of AgNPs synthesis by (*C. biomass*) the high inhibition effect of AgNPs which presented in *T. mentagrophytes* with growth rate (20.0mm) but found the low effect in *T. rubrum* with growth rate (25.0mm). But when the 50µg/ml concentration not found any effect of AgNPs in *T. mentagrophtes* and *T. rubrum* with growth rate (30.0mm). But in 25µg/ml concentration not found any effect of AgNPs in *T. rubrum* with growth rate (30.0mm). The results of statistical analysis showed significant ($P \leq 0.05$) between *Trichophyton* spp. isolates, like in table 1.

This results agree with the results were done Pereira *et al*., (2014) find Bio-AgNPs produced by the fungal cell-free filtrate showed an antifungal activity higher than fluconazole but less than terbinafine, itraconazole and Chem-AgNPs. For non-dermatophyte fungi the high inhibition effect of AgNPs synthesis by (*C. biomass*) AgNPs which presented in *A. niger* with growth rate (18.0mm). While the growth rate for *Cladosporium* spp. and *Penicllium* spp. are (20.0mm). While the low effect in *Mucor spp.* and *Syncephalastrum* spp. with growth rate (25.0mm). The results of statistical analysis showed significant ($P \leq 0.05$) between Mold spp. isolates. While 50µg/ml the hight inhibition effect of AgNPs which present in *A.niger* at growth rate (20mm). While the growth rate

Table 1: Effect of silver nanoparticles synthesis by *Cladosporium cladosporioides* against Dermatophytes fungi by poising food assay.

Fungi Type	Mean colony diameter (mm) ± SD			
	Concentration of nanoparticles (µg/ml)			
	Control	Clado.biomass		
		100	50	125
T.rubrum	30.0± 0.0	25.0±0.0	30.0±0.0	30.0±0.0
T.mentagrophytes	30.0±0.0	20.0±0.0	30.0± 0.0	30.0± 0.0
P value	0.05	0.05	0.05	0.05

Table 2: Effect of silver nanoparticles synthesis by *Cladosporium cladosporioides* against Non-Dermatophytes fungi by poising food assay.

Type of Molde	Mean colony diameter (mm) ± SD				
	Concentration of nanoparticles(µg/ml)				
	Control	Clado.biomas			
		100	50	125	LSD
Cladosporium spp.	30.0±0.0	20.0±0.0	25.0±0.0	30.0±0.0	3.4
Aspergillus niger	27.1±0.2	18.0±0.0	20.0±0.0	30.0±0.0	2.1
Penicllium spp.	35.0±0.0	20.0±0.0	25.0±0.0	30.0±0.0	5.1
Mucor spp.	30.0±0.0	25.0±0.0	30.0±0.0	30.0±0.0	2.9
Syncephalastrum spp.	35.0±0.0	25.0±0.0	30.0±0.0	30.0±0.0	4.8
LSD	0.3	6.1	2.4	NS	
P value	0.05	0.05	0.05	NS	

penicillium spp. and *Cladosporium* spp. with growth rate (25.0mm), while low effect of AgNPs was on *Mucor* spp. and *Syncephalastrum* spp. with growth rate (30mm). The results of statistical analysis showed significant ($p \leq 0.05$) between Mold spp. isolates. But in 25µg/ml not found any effect of AgNPs on all Mold spp. with growth rate (30.0mm). The results of statistical analysis showed non significant. Xu *et al*., (2013) showed that the activity of nano-silver against *Aspergillus* spp. is tow times greater than of Amphotericin B. Also Agree with Pulit (2013), showed that even Low concentration of Nano silver particals makes it possible to achieve a high percentage of growth inhibition. Generally, there are three theories for the antimicrobial mechanisms of nanometal toxicity (Pinto *et al*., 2017, Ouf *et al*., 2015) they can be genotoxicions that can destroy DNA, by DNA loses its ability to replicate which leads to cell death. The cytotoxicity and genotoxicity of AgNPs are size, concentration and exposure time dependent, release of toxic ions (Ag+) that can bind to sulpher containing proteins this accumulation prevents the proteins from properly functioning in the membrane and interfere in cell permeability, Interruption of electron transport, protein oxidation and membrane potential collapse resulting in activated expression of ribosomal subunit proteins, as well as certain other cellular proteins and enzymes essential to ATP product.

2. Inhibitory effect of AgNPs Against Yeast by Agar plate well Diffusion assay: In table 2, (100, 50, 25µg/ml) concentration of AgNPs synthesis by (*Clado. biomass*), found all *Candida* spp. and *Rhodotorula* spp. are resistance effect to AgNPs. The results of statistical analysis showed non-significant between *Candida* spp. and *Rhodotorula* spp. isolates. This results partially agree with the results were done Nasrollahi *et al*., (2011) show the antifungal effects of silver nanoparticles on *C. albicans*. By attach AgNPs with cell membrane and penetrate in the fungi then produce a site witch little molecular weight in center of fungi and then AgNps attach to respiratory sequence and finally cell division stop lead to cell death. This results agree with the results were done Kalaiselvam, (2013) who find efficacy of synthesized nanoparticles was tested against *T. rubrum, E. floccosum* and *T. mentagrophytes*. For non dermatophyte fungi Xu *et al*., (2013) showed that the activity of nano-silver against *Aspergillus* spp., this results partially agree with the results were done Nasrollahi *et al*., (2011) show the antifungal effects of silver nanoparticles on *C. albicans*. Generally, there are three theories for the antimicrobial mechanisms of nanometal toxicity they can be genotoxicions that can destroy DNA, by DNA loses its ability to replicate which leads to cell death. The cytotoxicity and genotoxicity of AgNPs are size, concentration and exposure time dependent, release of toxic ions (Ag+) that can bind to sulpher containing proteins this accumulation prevents the proteins from properly functioning in the membrane and interfere in cell permeability, Interruption of electron transport, protein oxidation and membrane potential collapse resulting in activated expression of ribosomal subunit proteins, as well as certain other cellular proteins and enzymes essential to ATP product.

Table 3: Effect of silver nanoparticles synthesis by Cladosporium cladosporioides against yeast by cell diffusion method.

Type Yeast	Inhibition zone (mm) mean ± SD				
	Concentration of nanoparticles (µg/ml)				
	Control	Clado.biomas			
		25	100	50	25
Candida albicans	6.0±0.0	10.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Candida famata	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Candida guilliermondii	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Candida lusitaniae	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
Rhodotorula.spp	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0
LSD	0	3.4	0	0	0
P value	NS	0.01	NS	NS	NS

Conclusion

In conclusion, the biomass of locally isolates of *Cladosporium cladosporioides* had ability to Biosynthesis of AgNPs, Moreover AgNPs proved excellent antimicrobial activity against pathogenic fungi causing onychomycosis.

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