



ENHANCED EFFICACY OF KETOCONAZOLE COATED SILVER NANOPARTICLES AGAINST THE FUNGUS *MALASSEZIA FURFUR* A DANDRUFF CAUSING AGENT

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ABSTRACT

The present study describes simple and effective method of silver nanoparticles (AgNp's) synthesis via green root using the fungus *Aspergillus pseudodeflectus*. The synthesized AgNp's were characterized by visual observation, UV-Spectroscopy which showed maximum absorption at 422.5nm, FTIR showed bands at 3418.59, 1636.52, 1384.27, 1075.84cm⁻¹, TEM revealed the formation of spherical nanoparticles with size ranging between 20-60nm, SEM showed the formation of well dispersed AgNp's, XRD revealed the crystalline nature of AgNp's, EDS showed the optical absorption peak at 3kev, which confirmed the presence of elemental silver. The

efficacy of AgNp's against dandruff causing *Malassezia furfur* (Anti-dandruff activity) was performed. The disc diffusion method and In Vitro activity of Ketoconazole alone, AgNp's and Ketoconazole coated AgNp's was done. The zone of inhibition for Ketoconazole alone was 22 mm, while that of AgNp's alone was only 18mm. Surprisingly we could observe the zone of inhibition of 30mm which was enhanced to a maximum extent when Ketoconazole was coated with AgNp's produced by the fungus *A. pseudodeflectus*. The minimal inhibitory concentration was found to be 0.062 for Ketoconazole and 0.025 for AgNp's alone, where as it was 0.0108 for Ketoconazole coated AgNp's which clearly reveals the enhanced efficacy of AgNp's with minimum concentration.

KEYWORDS: *Aspergillus pseudodeflectus*, Silver Nanoparticles, TEM, SEM, FTIR, XRD, EDS, Anti dandruff.

1. INTRODUCTION

Nanotechnology is emerging field with its applications in science and technology for the purpose of synthesis and development of nanomaterials at nanoscale level (M.A. Albrecht *et.al.*,2006). Silver nanoparticles are at the edge of rapidly developing field of nanotechnology. Due to its unique properties it is widely used in catalysis, chemical sensing and bio-sensing photonics, electronics, optics, DNA sequencing, Surface Enhanced Raman spectroscopy and pharmaceuticals (Varshwy R *et.al.*, 2009). Various techniques such as chemical, physical and mechanical methods have been developed for synthesis of metal nanoparticles. As these methods are costly, toxic and non-eco friendly (Mahendra Rai *et.al.*, 2009) a great deal of effort has been put into biosynthesis of inorganic materials especially metal nanoparticles using micro-organisms (Mandal.*et. al.*, 2006).

Investigators concerned with use of microbes for the deliberate synthesis of nanoparticles of different chemical compositions include use of bacteria and algae for gold (Beveridge T.J *et.al.*,1980, Fortin D *et.al.*,2000), silver (Waus-Joerger *et.al.*,2001, Nair B *et.al.*,2002), Cds (Kowshik M *et.al.*,2002), Zns (Labrenz M *et.al.*, 2000) and magnetite (Roh Y *et.al.*,2001) nanoparticles, and use of fungi for Ag, Pbs and Au nanoparticles (Duran N *et.al.*, 2005). The nanoparticles synthesized using fungi present good polydispersity, dimensions and stability. Fungi can accumulate metals by physico and biological methods which includes extra cellular binding by metabolites and polymers, binding to specific polypeptides and metabolism dependent accumulation. Certain fungi can rapidly synthesize metal nanoparticles extra cellularly using high levels of secreted proteins and or enzymes that not only stabilize particles but allows for an improved yield (Aniket Gade *et.al.*, 2010).

In recent years, resistant to commercially available anti-microbial agents by pathogenic bacteria and fungi have been increasing at alarming rate and have become a serious problem (Wright G D *et.al.*, 2000). People who are infected with drug resistant micro organisms usually spend more time in the hospital and require a form of treatment that uses two or three different antibiotics which are less effective more toxic and more expensive (Ingle A *et.al.*,2008). There is a pressing need to search for new anti-microbial agents from natural and inorganic substances (Kim T N *et.al.*,1998, Cho K H *et.al.*,2005). Among inorganic anti-microbial agents, silver has been employed most widely. The researchers are moving towards nanoparticles especially AgNp's to solve the problem of emerging pathogens which have developed resistance to 'n' number of antibiotics (C.G. Gemmel *et.al.*,2006). AgNp's

exhibits very strong bactericidal activity against both gram +ve and -ve bacteria including multi drug resistant strains (Morones J R *et.al.*,2005). In addition, AgNp's can be considered as potential anti-fungal agent (Ales Pana C *et.al.*,2009). Nano silver has fastest and broad spectrum fungicidal activity which makes it a good candidate to eradicate fungal infection without recurrence (Wright B *et.al.*,1999). Drug nanoparticle hybrid system have been widely used in the enhancement availability, bio activity and stability of drugs used in various infections. One such hybrid is Ketoconazole complexed with AgNp's enhanced the activity against *Malassezia furfur* which is recognized as a causative agent of dandruff (T.Devasena *et.al.*, 2009).

Dandruff is a common embarrassing scalp disorder effecting the large chunk of population. It effects the half of the post pubertal of any ethnicity of both genders (Saint D. L *et.al.*,1990). It is a condition that produces the irritating white and itchy scalp. It is a dander with dry, white or greyish appearing as small patches especially at the top of hair. The severity of dandruff may fluctuate with season and often worsen in winter (Pitard C F *et.al.*,2006). In this fast growing, competitive and colorful world, people are more conscious of their personality in terms of facial beauty which reflects through hairstyles. The severity of dandruff may lead to loss of hair thus projecting the facial appearance. Therefore they are running after cosmetic products to enhance their beauty. There is a long standing use of Ketoconazole as an anti-dandruff agent. Due to the resistance developed by the fungal strains, here we report that Ketoconazole coated AgNp's have a powerful effect in completely eradicating the dandruff causing *Malassezia furfur*.

Ketoconazole is reported to be effective in the treatment of severe dandruff (Pierard-Franchinont C *et.al.*, 2001). In spite of several commercially available keto based Anti-dandruff shampoos dandruff occurrence is more frequent. Further, resistance of dandruff to antifungal agent is also of immense interest due to development of resistant strain. A novel approach to rule out the problems of dandruff is to complex Ketoconazole with AgNp's which has influence in reducing the dandruff causing agent. Reports revealed that AgNp's enhance the activity of Ketoconazole, due to the fact that Ketoconazole acts on fungi at the level of cell wall but AgNp's powerfully penetrate through the membrane leading to the complete eradication of the fungus.

2. MATERIALS AND METHODS

2.1 Isolation of Fungi

Soil samples were collected from different areas of Kalaburagi District, Karnataka, for the isolation of fungal strain and cultured on Potato Dextrose Agar medium. Isolates were identified based on colony morphology and lactophenol cotton blue staining. The fungal isolates were sub-cultured and preserved for further work.

2.2 Extracellular synthesis of Silver Nanoparticles

The fungus was grown in 250ml Erlenmeyer flask, containing 100ml of MGYB broth (A.Banu *et.al.*, 2011) containing Malt extract 0.3%, Glucose 1%, Yeast extract 0.3% and Peptone 0.5% at 29⁰C for 72 hours in static position. After incubation the biomass was washed with distilled water. This is repeated 2-3 times to remove any traces of medium contents. This biomass was taken into flasks containing 100ml of distilled water and incubated in same position for 48 hours. The suspension was filtered with the help of Whatman filter paper no.1, as obtained filtrate was challenged with 1mM AgNO₃ at 29⁰C for reduction (Dattu Singh *et.al.*,2014).

2.3 Characterization Studies for Silver Nanoparticles

2.3.1 UV-Visible Spectroscopy

The formation of AgNp's was preliminarily confirmed by visual inspection of color change from pale yellow to reddish brown. Synthesized AgNp's were characterized using UV-Visible Spectroscopy (T90+ UV-Vis Spectrophotometer) shows specific surface plasmon resonance.

Transmission Electron Microscopy (TEM Hitachi H7500, Japan) reveals the size and shape of the nanoparticles. The samples were prepared by drop coating of AgNp's solution on to the carbon coated copper grid and were loaded on to a specimen holder. TEM micrographs were taken and then size and shape of AgNp's were confirmed. SEM (Scanning Electron Microscopy) is done to record surface morphology of the AgNp's.

AgNp's synthesized were air dried at room temperature and was subjected to FT-IR. The interaction between protein and AgNp's were analyzed by this method. Presence of elemental silver was detected by employing Energy Dispersive Spectroscopy (EDS – JOEL Model JED-2300). X-Ray diffractometer (XRD) provide the crystalline nature of the particles.

2.4 Determination of Anti-Fungal (Anti-Dandruff) Activity of Synthesized AgNps

2.4.1 Disc Diffusion Method

The Anti-dandruff activity of synthesized AgNp's was evaluated using Agar diffusion method. The microbial culture *Malassezia furfur* was procured from National Centre for Industrial Micro organisms (NCIM) Pune, India were subcultured in Sabouraud Dextrose Agar(SDA) medium plates and further stored in slants as stock culture. The stock culture was prepared by inoculating each culture from slant to flask in sterile Sabouraud Dextrose Broth supplemented with olive oil and incubated at 28⁰C for 48 hours. The stock culture is adjusted to 0.5 Mc. Farland standard turbidity and used for assay.

The SDA plates with Streptomycin(50mg/ml) and 0.1ml of inoculum of standardized culture of test organism is spread uniformly. Wells were prepared using a sterile borer of diameter 10mm and 50 μ l volume.

The wells were added with 10 μ l of Ketoconazole(5mg/ml), AgNp's(2mg/ml), Ketoconazole(5mg/ml) + AgNp's(2mg/ml) separately. The plates were placed at 4⁰C for one hour to allow the diffusion of test solution into the medium. Later the plates were incubated at 37⁰C for 48 hours. The plates were observed for inhibition zones, diameter of the zones were measured in millimeters.

2.4.2 In Vitro studies

In Vitro inhibitory activity of Ketoconazole were determined by serial tube two fold dilution method Rajarajan *et.al.*, 2002 .A standard concentration of Ketoconazole was serially diluted with distilled water in a row of 6 tubes. The concentration in the tubes was 5.0, 2.5, 1.25, 0.625, 0.3125 and 0.156 mg/ml. From each tube 0.1ml of solution was transferred to another set of 6 tubes followed by 0.9ml of *Malassezia furfur* culture broth inoculum.

The above procedures were also followed by Ketoconazole coated AgNp's. AgNp's were mixed with Ketoconazole solution and stirred vigorously for 2 hours in a magnetic stirrer. The tubes were then serially diluted with distilled water in a row of 6 tubes. The Ketoconazole concentration in the tubes was 5.0, 2.5, 1.25, 0.625, 0.3125 and 0.156. From each tube, 0.1ml of solution was transferred to another set of 7 tubes followed by 0.9ml of *Malassezia furfur* culture broth inoculum. The 7th tube containing 0.1ml of distilled water and 0.9ml of inoculum served as control.

All the tubes were incubated at 35⁰C for 24 hours. The test tubes were observed for the presence of turbidity. The tubes were compared with that of control. The lowest concentration of the drug inhibited the growth of fungus was inferred by the lack of visual turbidity and further confirmed by plate assay and recorded as inhibitory value of the drug.

3. RESULTS AND DISCUSSION

3.1 Isolation of Fungi.

The fungal isolate screened from soil samples after morphological and microscopic studies was identified as *Aspergillus pseudodeflectus*. (Fig. 1a & 1b)



Fig.1a. *Aspergillus pseudodeflectus* on PDA plate. **Fig.1b.** Microscopic image of *Aspergillus pseudodeflectus*

3.2. Extracellular Synthesis of AgNp's.

After 72hrs the biomass of the fungus (Fig.2a) was separated by filtration. The fungal filtrate (Fig.2b) was treated with equal volume of 1mM AgNO₃ solution. After 24hrs of incubation appearance of color change from pale yellow to brown is a clear indication of the formation of AgNp's in a reaction mixture (Fig.2c). The appearance of brown color was due to the excitation surface plasmon vibrations.



(Fig 2a.)

(Fig 2b.)



(Fig.2c.)

Fig.2.2a Fungal biomass 2b.Fungal filtrate of *A.pseudodeflectus* 2c.Color change to reddish brown after exposure to 1mM AgNO₃.

3.3. Characterization of AgNp's

3.3.1. UV-Visible Spectroscopy

Visual observation of color change from light yellow to brown after addition of AgNO₃ to enzyme filtrate is the primary indication of AgNp's biosynthesis. UV Visible absorption spectroscopy is one of the most widely used technique for structural characterization of AgNp's from *A. pseudodeflectus* which showed the maximum absorbance at 422.5nm (Fig.3) confirmed the production indicating the specific surface plasmon resonance. It is reported that the absorption spectrum of AgNp's presents a maximum between 420-450 nm (Maliszewska K *et. al.*,2008). Similar results were observed by Ninganagouda *et. al.*,2014 revealed plasmon resonance of AgNp's between 380-450nm and Dattu Singh *et.al.*2014, also revealed the absorption peak at 425nm by an endophytic fungi *Penicillium sps* isolated from the leaf of *Curcuma longa* medicinal plant.



Fig.3. UV-Vis spectroscopy of AgNp's.

3.3.2. Transmission Electron Microscopy (TEM)

A drop of AgNp's solution was placed on a carbon coated copper grid and kept under vacuum before loading them on to the specimen folder. TEM-Micrograph revealed that the particles are spherical and well dispersed. The particle size of AgNp's synthesized by *A.pseudodeflectus* ranges from 20-60nm (Fig.4) Afreen *et. al.*,2011 using *Rhizopus stolonifer* reported the size of particles between 20-35nm. Shivaraj *et.al.*,2014 also reported the Size of AgNp's between 20-55 nm by using *Aspergillus niger*.

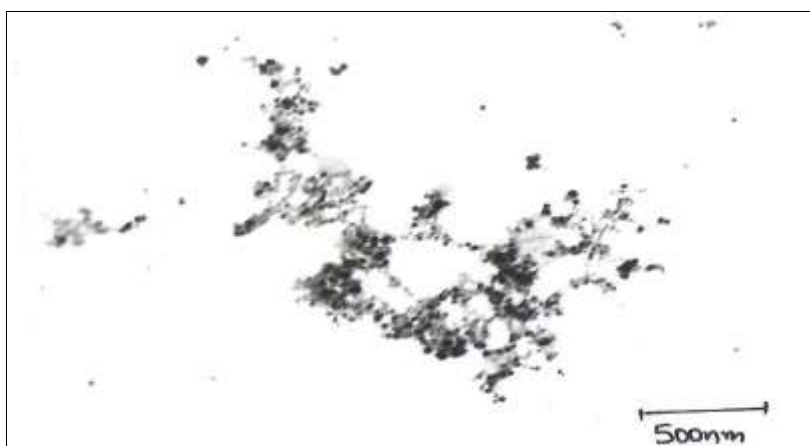


Fig.4. TEM micrograph of AgNp's.

3.3.3. Scanning Electron Microscopy (SEM)

Thin films of the sample were prepared on carbon coated copper grid by dropping a very small amount of the sample. Extra solution was removed using a blotting paper and the thin film on the SEM grid were allowed to dry for analysis. SEM photographs shows the formation of well dispersed AgNp's (Fig.5).

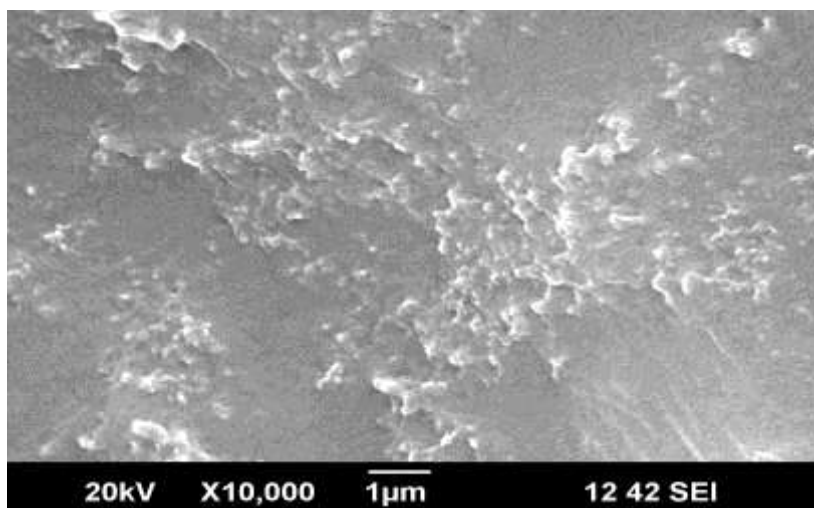


Fig.5. SEM Micrograph of AgNps

3.3.4 Fourier Transform Infrared Spectroscopy(FT-IR)

The synthesized AgNp's were dried at room temperature and were subjected to FT-IR analysis in the range of 500 to 4000 cm^{-1} . The probable biomolecules responsible for reduction, capping and effective stabilization of the AgNp's were recorded using FT-IR spectrophotometer at diffuse reflectance mode. The aim of FT-IR spectroscopic analysis is to determine chemical functional groups in the sample. FT-IR representative spectra in the region of 500 to 4000 cm^{-1} revealed the presence of different functional groups such as 3418.59 secondary amide (N-H stretch), 1636.57 – tertiary amide (C=O stretching), 1384.27 alkane (C-H stretching), 1075.84 primary alcohol (C-O stretching), 524.80 halogen compounds (C-Br stretching) respectively [Fig.6].

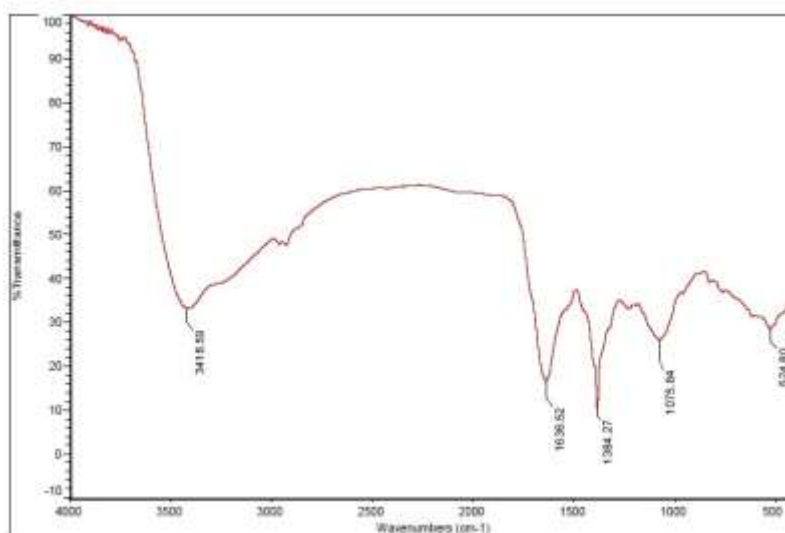


Fig.6. FT-IR Spectrum of AgNp's

3.3.5 X-Ray Diffractometer Analysis(XRD)

XRD analysis reveals the crystalline nature of AgNp's. The diffracted intensity were recorded from 0 to 80 $^{\circ}$ (2 θ). The AgNp's exhibited peaks of silver at 2 θ =29.232 $^{\circ}$, 32.151 $^{\circ}$, 37.917 $^{\circ}$ and 46.058 $^{\circ}$ that can be indexed to the (211), (264), (111), (220) facets of silver respectively (Fig.7). Biosynthesized nanoparticles were highly stabilized. Prema Kulkarni *et.al.*,2014, reported the XRD pattern which showed the presence of sharp reflections at 111, 200, 220 and 311 using *Aspergillus terreus*. While Guangquan *et.al.*,2012, revealed the intense XRD peaks corresponding to (111), (200), (220), (311) planes at 2 θ angles of 38.28 $^{\circ}$, 44.38 $^{\circ}$, 64.54 $^{\circ}$ and 77.64 $^{\circ}$ respectively. Our results correlate with the above said authors.

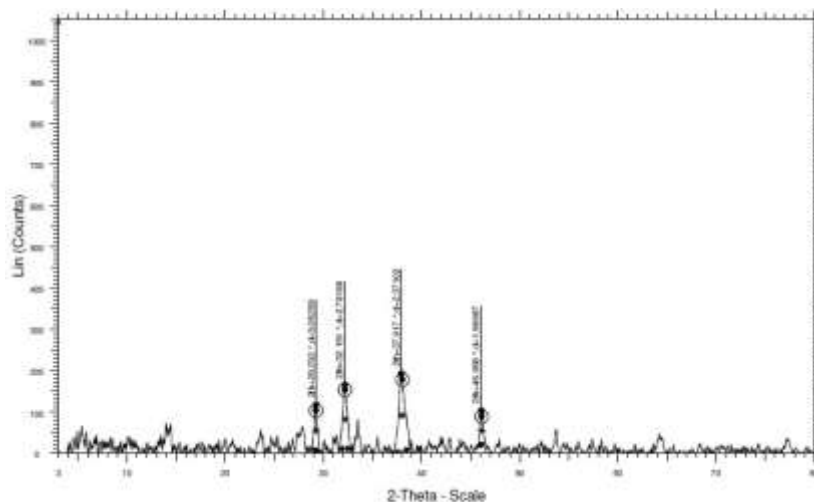


Fig.7. X-Ray Diffraction of AgNp's synthesized from *A. pseudodeflectus*

3.3.6 Energy Dispersive Spectroscopy(EDS)

The reduction of Ag^+ ions to elemental silver by *A. pseudodeflectus* is characterized by EDS analysis. The spectrum shows the optical absorption peak approximately at 3Kev which reveals the presence of pure metallic AgNp's (Fig.8). Similar results of EDS optical absorption peak was also reported by Shivraj Ningana gouda *et.al.*,2014 by using *A niger* and Afreen *et.al.*,2014 by using *R stolonifer*.

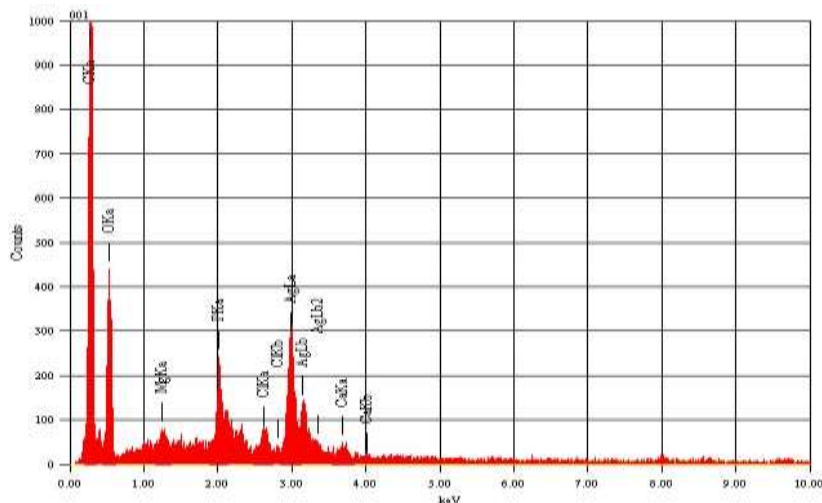


Fig.8. Energy Dispersive Spectra of AgNp's

3.4 Determination of Anti-dandruff activity of the synthesized AgNp's

The Anti- dandruff activity of the synthesized AgNp's from *Aspergillus pseudodeflectus* were studied. Results were compared with the antibiotic Ketoconazole and studies were also extended to know the synergistic effect of Ketoconazole + AgNp's and AgNp's alone. Results were encouraging with the synergistic effect.

3.4.1 Disc Diffusion Method

Disc diffusion method was carried out to check the inhibitory action of independent Ketoconazole, AgNp's and Ketoconazole + AgNp's. Results in Table 1 revealed that 5mg/ml Ketoconazole showed 22mm zone of inhibition, while 2mg/ml AgNp's alone showed 18mm zone of inhibition. When synergistic effect of Ketoconazole + AgNp's were studied, AgNp's are so effective that most probably might have enhanced the activity of Ketoconazole or individually have shown the inhibitory effect by denaturing the disulphide bridge in the cellular proteins thereby destroying the tertiary structure and function of cellular proteins, thus increasing the zone of inhibition to 30mm.

Table 1: Anti-dandruff activity of Ketoconazole coated silver nanoparticle (AgNp's)

Drug Tested	Diameter of zone of inhibition (mm)
Ketoconazole (5mg/ml)	22
AgNp's (2mg/ml)	18
Ketoconazole (5mg/ml)+AgNp's (2mg/ml)	30

3.4.2 In Vitro Studies.

In Vitro inhibitory activity of Ketoconazole coated AgNp's on *Malassezia furfur* as depicted in Table 2 revealed that Ketoconazole alone at concentrations from 0.5 to 0.062 were effective. The lowest dosage of Ketoconazole that completely inhibited the fungus *Malassezia furfur* was 0.062, still lowering the concentration there was no effect. AgNp's alone showed complete inhibition of the fungus at 0.025 while In Vitro studies revealed that a combination or synergistic effect of Ketoconazole + AgNp's showed complete inhibition at a concentration of 0.01087 which clearly indicates that Ketoconazole coated AgNp's are more effective at lower concentrations too.

Table 2: In vitro inhibitory activity of Ketoconazole coated silver nanoparticles (AgNp's) on *Malassezia furfur*

Antifungal drugs	Concentration of the test drugs in µg								MIC
	1	2	3	4	5	6	7	8	
Ketoconazole	0.5	0.25	0.125	0.062	0.031	0.015	0.007	C	-
	+	+	+	+	-	-	-	-	0.062
AgNp's	0.2	0.1	0.05	0.025	0.0125	0.0062	0.0031	C	-
	+	+	+	+	-	-	-	-	0.025
Ketoconazole coated AgNp's	0.7	0.35	0.175	0.087	0.0435	0.0217	0.01087	C	-
	+	+	+	+	+	+	+	-	0.01087

“+” Complete inhibition

“-” No inhibition

“C” Control

CONCLUSION

Most of the organisms are developing resistance to antibiotics. One such example is *Malassezia furfur*, a dandruff causing agent, at time was susceptible to the antibiotic Ketoconazole is now resistant. Therefore, we tried a nano agent i.e, AgNp's coated onto the antibiotic Ketoconazole which showed an encouraging result. Thus, it can be suggested that Ketoconazole capped with AgNp's can prove as a best Anti-dandruff agent against *Malassezia furfur*.

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