

Full Length Research Paper

Green synthesis of silver nanoparticles using *Ocimum sanctum* (Tulashi) and study of their antibacterial and antifungal activities

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In the present research program, cost effective and environment friendly silver nanoparticles were synthesized from the leaf extract of *Ocimum sanctum*, as reducing agent. The nanoparticles were characterized by using UV-visible, SEM and XRD methods. The absorption peak at 430 nm broadens with increase in time indicating the polydispersity nature of the nano particles. The SEM image showed relatively spherical shape nanoparticles. The XRD results suggested that the crystallization of the bio-organic phase occurs on the surface of the silver nanoparticles or vice versa. The antibacterial and antifungal activities of the nanoparticles have also been investigated.

Key words: Silver nano particles, *Ocimum sanctum*, antibacterial, antifungal.

INTRODUCTION

In the recent years controllable synthesis of noble metal nanoparticles has attracted much attention due to their potential applications in many areas (Yang and Cui, 2008). They have been extensively exploited for use in biomedical areas, such as targeted drug delivery (Jayanth and Vinod, 2003), imaging (Ji-Ho et al., 2009), sensing (Yıldız and Ibtisam, 2010) and antimicrobial activity (Pallab et al., 2008). Among these metal nanoparticles, silver nanoparticles have attracted intensive research interest because of their important applications as antimicrobial, catalytic, and antifungal activity (Yang and Cui, 2008; Jayanth and Vinod, 2003; Ji-Ho et al., 2009). Silver has been used as an antimicrobial agent for centuries, the recent resurgence in interest for this element particularly focuses on the increasing threat of antibiotic resistance, caused by the abuse of antibiotics (Yıldız and Ibtisam, 2010; Pallab et al., 2008). It is generally recognized that silver nanoparticles may attach to the cell wall, thus disturbing cell-wall permeability and cellular respiration.

The nanoparticles may also penetrate inside the cell causing damage by interacting with phosphorus- and sulfur containing compounds such as DNA and protein. Another possible contribution to the bactericidal properties of silver nanoparticles is the release of silver ions from particles (Pallab et al., 2008).

Ag nanoparticles can be successfully synthesized traditionally by chemical and physical methods. However, these methods strongly depend on severe reaction conditions, for example, aggressive agents (sodium borohydride, hydrazinium hydroxide, cetyltriethylammonium bromide), harmful solvent system to environment and ecology, higher temperature and higher pressure, and so on. To pursue a healthy life and space, it is imperative to develop a clean synthetic approach ("green chemistry") to obtain nanomaterials targeted on different applications, especially in biomedical fields.

In recent years, plant-mediated biological synthesis of nanoparticles is gaining importance due to its simplicity and eco-friendliness. Although biosynthesis of gold nanoparticles by plants such as alfalfa (Shetty et al., 2006; Ahmed et al., 2002), *Aloe vera* (Gupta et al., 2006), *Cinnamomum camphora* (Singh et al., 1996), *Azadirachta indica* (Samjon et al., 2007), *Emblca officianalis* (Sood et

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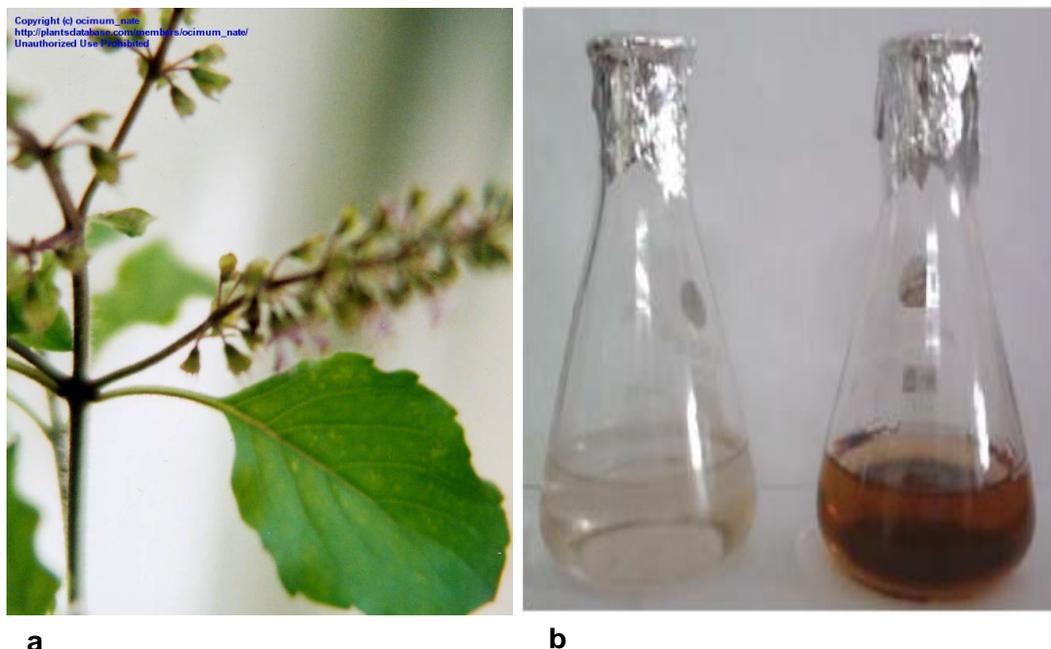


Figure 1. (a) *Ocimum sanctum* L, (b) Picture of aqueous solution of 1 mM AgNO₃ with *Ocimum* leaf extracts before adding the leaf extract and after addition of leaf broth.

al., 2006), lemongrass (Sharma et al., 2002), and *Tamarindus indica* LIn (Kantak and Gogate, 1992) have been reported, the potential of the plants as biological materials for the synthesis of nanoparticles is yet to be fully explored. *Ocimum sanctum*, a wild herbaceous plant is very common in all tropical countries, including India. The stems are slender and often reddish in color, covered with yellowish bristly hairs especially in the younger parts. The leaves are oppositely arranged, lanceolate and are usually greenish or reddish, underneath measuring about 5 cm long. The stem and leaves produces white or milky juice when cut (Panda and Kar, 1998). The plant has been widely acknowledged for the treatment of cough, coryza, hay asthma, bronchial infections, bowel complaints, worm infestations, kidney stones in traditional medicine (Sood et al., 2005; Bauer et al., 1966).

In the present research program green synthesis of silver nanoparticles were carried out using the leaf extract of *O. sanctum* plant. The silver nanoparticles were characterized by using UV-visible spectra, scanning electron microscopy and XRD. The antibacterial and antifungal activities of the nano particles have been investigated using a number of bacteria and fungus and the results seem to be very encouraging.

MATERIALS AND METHODS

Plant material collection

O. sanctum leaves were collected from the local garden. The leaves

were air dried for 10 days, and then kept in the hot air oven at 600°C for 24 to 48 h. The leaves were ground to a fine powder.

Solvent extraction

Ten grams of air dried powder was placed in 100 ml of organic solvent (90% methanol) in a conical flask, plugged with cotton and then kept on a rotary shaker at 180 to 200 rpm for 24 h. After 24 h, it was filtered through 4 layers of muslin cloth and centrifuged at 5000 × g for 10 min. The supernatant was collected and the solvent was evaporated. The crude extract diluted with 5% of DMSO to make the final volume one-tenth of the original volume and stored at 4°C in air tight bottles for further studies.

Synthesis of silver nanoparticles

1 mM silver nitrate was added to plant extract to make up a final solution of 200 ml and was centrifuged at 18,000 rpm for 25 min. The collected pellets were stored at -4°C. The supernatant was heated at 50 to 95°C. A change in the color of solution was observed during the heating process.

Reduction of silver ion into silver particles during exposure to the plant extracts could be followed by color change. Silver nanoparticle exhibited dark yellowish – brown color in aqueous solution due to the surface Plasmon resonance phenomenon (Figure 1). The results obtained in this investigation were very interesting in terms of identification of potential weeds for synthesizing the silver nanoparticles. UV-Vis spectrograph of the colloidal solution of silver nanoparticles was recorded as a function of time. Absorption spectra of silver nanoparticles formed in the reaction media at 10 min showed an absorbance peak at 430 nm, broadening of peak indicated that the particles are polydispersed (Figure 2). The SEM image showed relatively spherical shape nanoparticle formed with diameter range of 40 to 50 nm (Figure 3). Similar phenomenon was reported by Bauer et al. (1966). Further

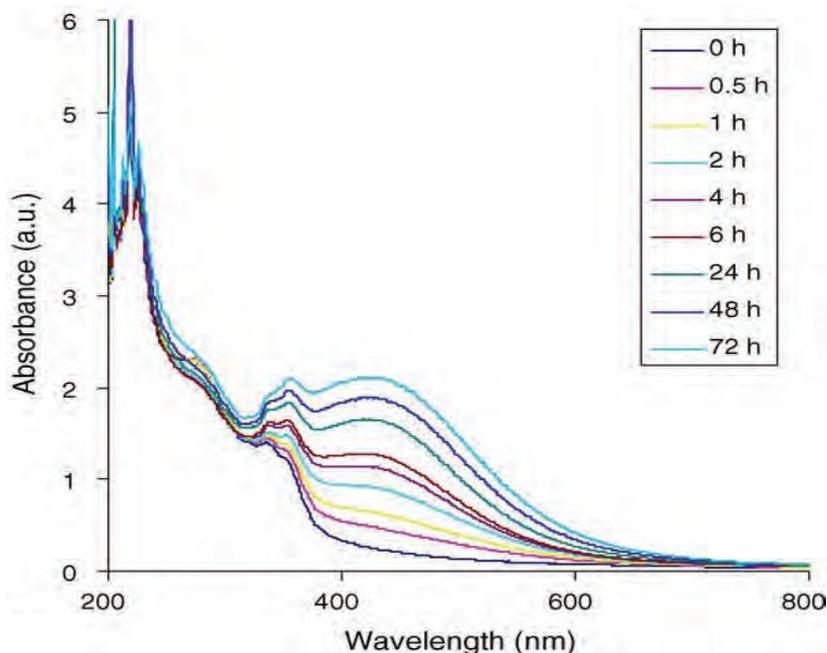


Figure 2. Uv-visible spectra of silver nano particles at different times.

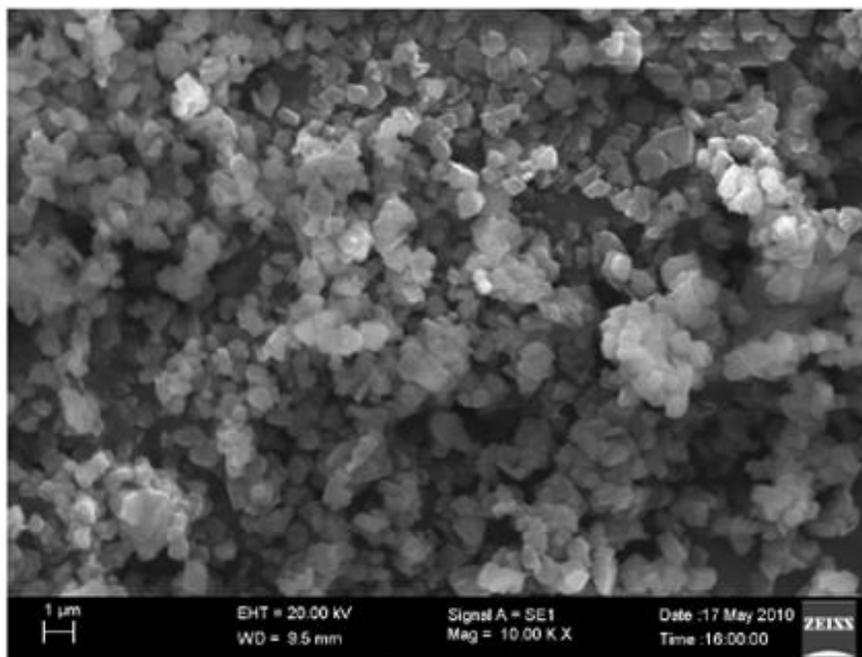


Figure 3. SEM image of silver nanoparticles formed by *Ocimum sanctum*.

the nanoparticle synthesis by green route was found highly toxic against 7 clinically isolated fungal species. At a concentration of 50 μ l silver nanoparticles revealed a higher activity against *Candida tropicalis*, *Candida krusei*, *Aspergillus flavus*, *Aspergillus fumigatus*. The inhibitory activities of all the silver nanoparticles reported in Table 1 are comparable with standard antimicrobics Ketoconazole

(30 mg) and Itraconazole (30 mg).

Test microorganisms

The bacterial strains studied were *Staphylococcus aureus* (ATCC

Table 1. Minimal inhibitory concentration (MIC) of *Ocimum sanctum* methanolic extract.

Microorganism	MIC (mg/ml)
<i>Escherichia coli</i>	33.02±1.96
<i>Enterobacter cloacae</i>	24.86±1.05
<i>Enterococcus faecalis</i>	17.66±0.75
<i>Proteus vulgaris</i>	34.08±1.72
<i>Klebsiella pneumoniae</i>	22.83±1.20
<i>Staphylococcus aureus</i>	16.60±0.90
<i>Staphylococcus saprophyticus</i>	11.10±0.04

25923), *Staphylococcus saprophyticus* (ATCC 35552), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 15380), *Enterococcus faecalis* (ATCC 10741), *Enterobacter cloacae* (ATCC 10699), and *Proteus vulgaris* (ATCC 12454). All microorganisms were maintained at 4°C and were obtained from Central Rice Research Institutem, Cuttack.

Antimicrobial susceptibility test

The disc diffusion method was used to screen the antimicrobial activity. *In vitro* antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile Petri plates. The plates were allowed to solidify for 5 min and 0.1% inoculum (0.5 McFarland standard) suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. 50 µl concentration of test extract was loaded on 0.5 cm sterile disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 h. At the end of incubation, inhibition zones formed around the disc were measured with transparent ruler in millimeter. For each bacterial strain, negative controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter and the result obtained was tabulated. For positive control, three antibiotics, namely Cefotaxime (30 mcg/disc), streptomycin (10 mcg/disc) and Ampicillin (10 mcg/disc) were used. These studies were performed in triplicate.

Antifungal activity study

Antifungal activities of the synthesized silver nanoparticles were determined, using the agar well diffusion assay method (NCCLS, 2000). Approximately 20 ml of molten and cooled media (SDA) was poured in sterilized Petri dishes. The plates were left overnight at room temperature to check for any contamination to appear. The fungal test organisms were grown in dextrose broth for 24 h. A 100 ml sabouraud dextrose broth culture of each fungal organism (1×10⁵ cfu/ml) was used to prepare fungal lawns. Agar wells of 5 mm diameter were prepared with the help of a sterilized stainless steel cork borer. Three wells were prepared in the agar plates. The wells were labeled as A, B, C. 'A' well was loaded with 50 µl of silver nanoparticles suspended 'hydrosol', 'B' well was loaded with 50 µl of distilled water and 'C' well loaded with 50 µl of positive control drugs. Various fungicides (Table 1) were used as positive controls. The plates containing the fungal and silver nanoparticles were incubated at 37°C. The plates were examined for evidence of zones of inhibition, which appear as a clear area around the wells (Nweze et al., 2004). The diameter of such zones of inhibition was

measured using a meter ruler and the mean value for each organism was recorded and expressed in millimeter.

UV-VIS spectra analysis

The reduction of pure Ag⁺ ions was monitored by measuring the UV-Vis spectrum of the reaction medium up to 72 h after diluting a small aliquot of the sample into distilled water. UV-V is spectral analysis was done by using UV-VIS spectrophotometer UV-2450 (Shimadzu).

SEM analysis of silver nanoparticles

Scanning electron microscopic (SEM) analysis was done using Hitachi S-4500 SEM machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

X-ray diffraction studies

The formation and quality of compounds were checked by X-ray diffraction (XRD) spectrum. The XRD pattern was measured by drop coated films of AgNO₃ on glass plate and employed with X-ray diffractometer (INEL X-ray diffractometer) of characteristic Co-ka1 radiation ($\lambda = 1.78 \text{ \AA}$) in the range of 20 to 90° at a scan rate of 0.05°/min with the time constant of 2 s.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) was determined using micro-broth dilution methods. Concentrations of extract (1 to 9 mg ml⁻¹) dilutions were prepared using tubes containing 9 ml of double strength broth. The tubes were inoculated with the suspension of standardized inocula (0.5 McFarland standard) and were incubated at 37°C for 24 h. MICs were recorded as the lowest concentration of extract showing no visible growth in the broth.

RESULTS AND DISCUSSION

Reduction of silver ion into silver particles during exposure to the plant extracts was observed as a result of the color change. Silver nanoparticle exhibits dark yellowish – brown color in the aqueous solution due to

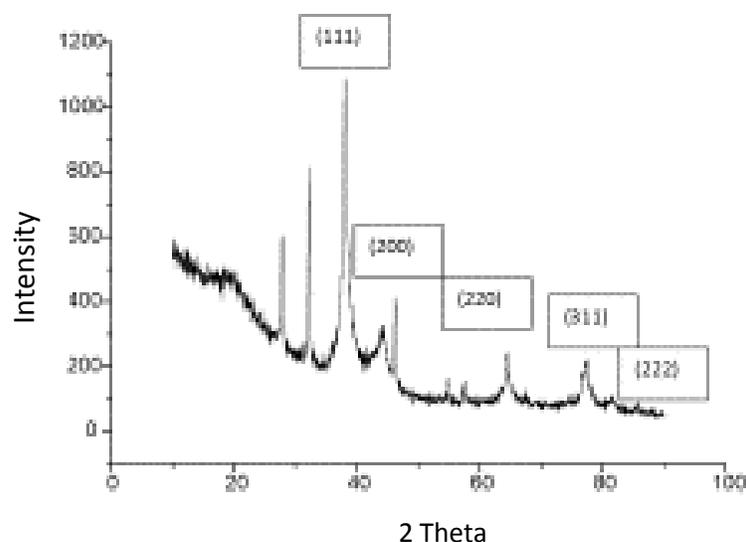


Figure 4. XRD of silver nanoparticles.

the surface Plasmon resonance phenomenon (Figure 1). UV-Vis spectrograph of the colloidal solution of silver nanoparticles has been recorded as a function of time. The absorbance peak at 430 nm show broadening of peak with increase of time, indicating that the particles are polydispersed in nature (Figure 2).

The SEM image showed relatively spherical shape nanoparticle formed with diameter range 0 to 50 nm (Figure 3). Similar phenomenon was reported by Bauer et al. (1966).

Figure 4 shows the XRD pattern of silver nanoparticles obtained. The peaks could be assigned to the five different facets of silver namely (1 1 1), (2 0 0), (2 2 0), (3 1 1) and (2 2 2) planes. A few unassigned peaks were also noticed in the vicinity of the characteristic peaks. These sharp Bragg peaks might have resulted due to the capping agent stabilizing the nanoparticle. Intense Bragg reflections suggest that strong X-ray scattering centres in the crystalline phase and could be due to capping agents. Independent crystallization of the capping agents was ruled out due to the process of centrifugation and redispersion of the pellet in millipore water after nanoparticles formation as a part of purification process. Therefore, XRD results also suggested that the crystallization of the bio-organic phase occurs on the surface of the silver nanoparticles or vice versa. Generally, the broadening of peaks in the XRD patterns of solids is attributed to particle size effects. Broader peaks signify smaller particle size and reflect the effects due to experimental conditions on the nucleation and growth of the crystal nuclei.

Antibacterial activity

The results for antibacterial activity of silver nano-

particles are shown in Table 1. The most potent effect, related to methanol extract of *O. sanctum* showed inhibition effect against *S. aureus* as well as *S. saprophyticus* in similar degree of inhibition zone diameter (20 ± 1.32 and 20 ± 1.51 mm). MIC values for the active extract are indicated in Table 2. As shown in Table 2 methanol extract of *O. sanctum* with the MIC value of 10.19 ± 0.08 mg/ml revealed that *S. saprophyticus* was the most susceptible bacterium which confirmed the results of disc diffusion method.

Inhibition zone values were obtained from the synthesized silver nanoparticle suspension and were tested against *E. coli*, *E. coli* 0157:H7, *K. pneumoniae*, *S. aureus*, and MRSA. The results and images of inhibition zones are presented as the average values in Table 2. Table 2 shows that the Ag NPs suspension gave high and similar antibacterial activity against Gram-negative and Gram-positive bacteria. Because of their size, Ag NPs can easily reach the nuclear content of bacteria and they present a large and impressive surface area, enabling broad contact with bacteria. This could be the reason why they give the best antibacterial effect. For solid support systems, some researchers have argued that Ag⁺ ions released from the surface of Ag NPs are responsible for their antibacterial activity. For aqueous phase systems, the results show that the antibacterial test of Ag⁺ ions is high at the concentration levels reached by releasing, and that the presence of Ag NPs is very important, which reinforces the idea that the larger the surface area the stronger the antibacterial activity. The diameters of inhibition zone in the agar plate are given in mm. The tests were repeated three times for each treated sample, and the results are presented in Table 2. The suspension of antibacterial activity of *E. coli* 0157:H7 is higher than *E. coli* and the antibacterial activity of *K. pneumoniae* is also higher than *S. aureus*.

Table 2. Antibacterial activity.

Microorganism	Cef	Str	Amp	Plant extract	Control
<i>E. coli</i>	38±1.80	42±2.10	—	14±0.33	0.0
<i>E. cloacae</i>	-	-	-	16±0.9	0.0
<i>E. faecalis</i>	-	28±0.77	-	12±0.07	0.0
<i>P. vulgaris</i>	13±0.67	28±0.66	-	13±0.02	0.0
<i>K. pneumoniae</i>	-	27±0.55	-	11±0.04	0.0
<i>S. aureus</i>	26±1.03	30±1.99	34±1.44	19±1.30	0.0
<i>S. saprophyticus</i>	40±1.73	46±2.06	42±2.22	20±1.40	0.0

Table 3. Antifungal activity.

Microorganaisms	Zone of inhibition in mm		
	Silver nanoparticle	Control 1	Control 2
<i>C. albicans</i>	15.22±0.04	22.15±0.11	ND
<i>C. tropicalis</i>	11.03±0.22	23.03±0.05	ND
<i>C. krusei</i>	11.15±0.11	20.00±0.02	ND
<i>C. Kefyr</i>	13.00±0.05	ND	17.05±0.03
<i>A. niger</i>	12.24±0.03	16.01±0.05	ND
<i>A. flavus</i>	06.04±0.11	17.00±0.06	ND
<i>A. fumigatus</i>	07.05±0.02	17.04±0.03	ND

Antifungal activity

Further the nanoparticles synthesized by green route were found to be highly toxic against clinically isolated fungal species. At a concentration of 50 µl silver nanoparticles revealed a higher antifungal activity against *C. albicans*, *C. kefy*, *A. niger* whereas intermediated activity were showed against *C. tropicalis*, *C. krusei*, *A. flavus*, *A. fumigates* (Table 3). The inhibitory activities of all the silver nanoparticles are reported in Table 1. The data results were compared with the standard antimicrobics of Ketoconazole (30 mg) and Itraconazole (30 mg).

Conclusion

The biosynthesis of silver nanoparticles using leaf broth of *O. sanctum* provides an environmental friendly, simple and efficient route for synthesis of benign nanoparticles. The bioreduced silver nanoparticles were characterized using UV-Vis, SEM, XRD, techniques. These reduced silver nanoparticles were surrounded by a faint thin layer of proteins and metabolites such as terpenoids having functional groups of amines, alcohols, ketones, aldehydes and carboxylic acids. The antibacterial and antifungal activities of the nanoparticles have been evaluated. Our current study revealed that the minimum inhibitor concentration (MIC) of *S. saprophyticus*

(10.19±0.08 mg/ml) is lesser than the other test microorganisms and which was followed by *S. aureus* (18.68±0.95 mg/ml). *S. saprophyticus* is an uropathogenic *staphylococcus* frequently isolated from young female outpatients with uncomplicated urinary tract infections. *S. saprophyticus* is a true urinary tract pathogen causing both upper and lower urinary tract infections. Further, the aforementioned silver nanoparticle was revealed to possess an effective antifungal property against *C. albicans*, *C. kefy*, *A. niger*. The present study emphasizes the use of plant medicinal for the synthesis of silver nanoparticle with antifungal effect. Further studies with other plant-mediated synthesis of silver nanoparticles are in progress.

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