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Synthesis of Silver Nanoparticles using *Rosmarinus officinalis* Leaf Oil and its Antimicrobial Activity against Vaginal Candidiasis

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Abstract

Biosynthesis of nanoparticles is under exploration is due to wide biomedical applications and research interest in nanotechnology. In the present work, we describe the synthesis of silver nanoparticles (Ag NPs) using leaf oil extract of *Rosmarinus officinalis* and its fungal activity. UV–visible spectroscopy, XRD, FTIR, SEM, TEM and EDX analyses were performed to ascertain the formation of Ag NPs. The synthesized Ag NPs were characterized by a peak at 450nm in the UV–visible spectrum. XRD confirmed the crystalline nature of the nanoparticles of 82.46 nm size. The XRD peaks at 38.06°, 44.35°, 64.51° and 77.36° can be indexed to the (111), (200), (220) and (311) Bragg's reflections of cubic structure of metallic silver, respectively. The FTIR result clearly showed that the extracts containing OH as a functional group act in capping the nanoparticles synthesis. SEM shows the 3D topological characteristic of Ag NPs. This reveals that the powder particles are slightly agglomerated but its size range of 82.46nm and the closed view of needle nanoparticle. TEM images revealed that all Nanoparticles observed from the micrograph majority are spherical with a small percentage of elongated particles and ranged in size of 35–60nm with an average size of 45nm. Antifungal activity of Ag NPs was tested against Vaginal Candidiasis such as *Candida albicans, Candida tropicalis* and *Candida kefyr* using standard well diffusion method. The maximum zone of inhibition was observed in the Ag NPs against *Candida tropicalis* (22.23 mm) and *Candida kefyr* (16.13 mm). The results suggest that the synthesized Ag NPs act as an effective antifungal agent. It is confirmed that Ag NPs are capable of rendering high antifungal efficacy and hence has a great potential in the preparation of drugs used against fungal diseases.

Keywords

Silver nanoparticles, fungus, *Rosmarinus officinalis* leaf oil extract.

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INTRODUCTION

The field of nanotechnology is one of the most active areas of research in modern materials science. New applications of nanoparticles (NPs) and nanomaterials are emerging rapidly. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, easily scaled up for large-scale synthesis and further there is no need to use high pressure, energy, temperature and toxic chemicals. Synthesis of NPs using biological entities has great interest due to their unusual optical, chemical, photo electrochemical and electronic properties [1]. One of the most considered methods is the production of metal NPs using biological systems such as microbes, fungi and several plant extracts. NPs produced by plants are more stable and the rate of synthesis is faster in the case of micro-organisms. Moreover, the NPs are more various in shape and size in comparison with those produced by other organisms [2].

Nanoparticles, generally considered as particles with a size of upto 100 nm, exhibit completely new or improved properties as compared to the larger particles of the bulk material that they are composed of based on specific characteristics such as size, distribution and morphology. Among the various types of metal, nanoparticle silver is by far the most studied due to its wide and rapidly growing application in a number of scientific areas and in consumer products [3] and its known toxic effects on the environment and human health [4].

In recent years, the biosynthetic method using plant extracts has received more attention than chemical and physical methods, and even than the use of microbes, for the nano-scale metal synthesis due to the absence of any requirement to maintain an aseptic environment. Nanoparticles have attracted considerable attention owing to their various applications. The silver nanoparticles are reported to possess anti-bacterial [5], antiviral [6], anti-fungal activity [7]. Synthesis of nanoparticles using plants or microorganisms can potentially eliminate this problem by making the nanoparticles more bio-compatible. Indeed, over the past several years, plants, algae, fungi, bacteria, and viruses have been used for low-cost, energy-efficient, and nontoxic production of metallic nanoparticles [8]. Use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes by eliminating the elaborate process of maintaining cell cultures. Recently green silver nanoparticles have been synthesized using various natural products like *Nelumbo nucifera* [9], Ocimum *sanctum* [10], *Pongamia pinnata* [11] and *Cinnamon zeylanicum* [5]. Gold nanoparticles (Au NPs) have larger surface area and higher dispersion owing to their very small size. Moreover, colloidal gold solutions are the subject of an increased interest for investigation of their cytotoxicity properties for applications in pharmacology, medicine, food industry, and water purification, etc. Au NPs are biocompatible materials and they have various biological applications including labeling, drug-delivery, photo thermal therapy and tissue/tumor imaging, and sensing [12].

Using plants for NPs synthesis can be advantageous over other biological processes because it eliminates the elaborate process of maintaining cell cultures and can also be suitably scaled up for large-scale NPs synthesis [13]. Extracellular nanoparticle synthesis using plant leaf extracts rather than whole plants would be more economical owing to easier downstream processing.

Biosynthesis of Au NPs by plants such as *Aloe vera* [14], tamarind [15] and *Cinnamomum camphora* [16] has been reported. Green synthesis of silver NPs from the seed extract of pomegranate fruit was demonstrated and the biologically synthesized nanoparticles were found to highly toxic against different multi-drug resistant human pathogens [17]. The biosynthesis of silver NPs was carried out using *Syzygiumcumini* seed extract as reducing agent [18]. The biosynthesis of stable and nearly spherical Au NPs using the seeds extract of *Benincasa hispida* as reducing and capping agents [19].

The considerable antimicrobial activities of inorganic metal oxide NPs, such as ZnO, MgO, TiO_2 and SiO_2, and their selective toxicity to biological systems suggests a potential application as therapeutics, diagnostics, surgical devices and nanomedicine based antimicrobial agents [20]. Extracellular biosynthesis of Au NPs using flower extracts of Plumeria alba as reducing agent and appears to have significant antimicrobial capacity resembling a broad spectrum antibiotic against *A. niger, A. flavus* [21]. There are many reports on the synthesis and antimicrobial activity of silver NPsbut in the case of Au NPs very few studies have been conducted, and it also has been reported as non-toxic compared to other metallic NPs such as silver and platinum NPs [22]. So far, there is no report on the synthesis of NPs by utilizing the leaf oil extract of *Rosmarinus officinalis* and used to synthesize Ag NPs without addition of any external surfactant, reducing agent, capping agent or template. The present study was designed to synthesize and characterize Ag NPs and to investigate the antifungal activity [23].

MATERIAL AND METHODS

Materials

Silver Nitrate ($AgNO_3$) was purchased from Sigma Aldrich, India. All other reagents used in the reaction were of analytical grade with maximum purity. The plant leaf essential oils compounds were purchased from Commercial center Aromax Trading Company, Chennai, Tamil Nadu (India). The silver nitrate was purchased from HiMedia (Mumbai, India).

The clinically isolated vaginal candidiasis fungus spp used for the test were *Candida albicans (C.albicans), Candida tropicalis (C. tropicalis)* and *Candida kefyr* (*C. kefyr*). All the stock cultures were obtained from Microlabs, Institute of Research and Technology, Vellore, Tamil Nadu, India. The microorganisms were grown overnight at 37°C in Mueller-Hinton Broth at pH 7.4.

Biosynthesis of silver nanoparticles

For the biosynthesis silver nanoparticles 10 ml of plant leaf oil compound was added into 90 ml of 1 mM silver nitrate aqueous solution and incubated at room temperature for 24 h. Formation of brown colour was indicates synthesis of silver nanoparticles (Plate 1A&B). The control leaf oil extract of *Rosmarinus officinalis* aqueous broth without auric silver nitrate did not show any change in color. The optimum time required for the completion of reaction was 1hr. Different concentration of $AgNO_3$ solution was used to get maximum Ag NPs. The overall optimized reaction condition was observed in 1mM $AgNO_3$ solution and neutral p^H.

Characterization of Ag NPs

The bioreduction of silver nanoparticles was monitored by sampling the reaction mixture at regular intervals and the absorption maxima was scanned by UV–Vis spectra, at the wavelength of 200–700nm in Schimadzu 1601 spectrophotometer operated at a resolution of 1 nm. Silver (Ag) nanoparticles exhibit unique and unable optical properties on account of their surface plasmon resonance (SPR), dependent on shape, size and size distribution of the nanoparticles [24]. The reduction of Ag+ ions was monitored by measuring the UV–visible spectra of the solutions after diluting a small aliquot (0.2mL) of the sample 20 times. The solution mixture was subjected to centrifugation at 10,000rpm for45 min; resulting pellet was dissolved in de ionized water and filtered through 0.22µm Millipore filter. An aliquot of this filtrate containing silver nanoparticles was used for XRD, FTIR, SEM and TEM analysis. X-ray diffraction (XRD) measurements of the *R.officinalis* leaf oil broth reduced Ag nanoparticles were carried out on films of the respective solutions drop-coated onto glass substrates on a Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30mA with CuKα1 radiation. For Fourier transform infrared (FTIR) spectroscopy measurements, dry powders of the nanoparticles were obtained in the following manner. The AgNPs synthesized after 1hr of reaction of leaf oil extract of *Rosmarinus officinalis* broth were centrifuged at 10,000rpm for 15 min, following which the pellet was re-dispersed in sterile distilled water to get rid of any uncoordinated biological molecules. The process of centrifugation and re-dispersion in sterile distilled water was repeated three times to ensure better separation of free entities from the metal nanoparticles. The purified pellets were then dried and the powders subjected to FTIR spectroscopy measurement. Characterization involved FTIR analysis of the dried powder of AgNPs, by scanning it in the range350–3000cm−1 at a resolution of $4cm^{-1}$. These measurements were carried out on a Perkin–Elmer Spectrum-One instrument in the diffuse reflectance mode at a resolution of $4cm^{-1}$ in KBr pellets and the pellets was mixed with KBr powder and pelletized after drying properly. The pellets were later subjected to FTIR spectroscopy measurement. For electron microscopic studies, 25µlof sample was sputter coated on copper stub and the images of nanoparticles were studied using SEM (JEOL, Model JFC-1600) and for transmission electron microscopy (TEM) analysis were prepared on carbon-coated copper TEM grids. TEM measurements were performed on a JEOL model 1200EX instrument operated at an accelerating voltage of 120 kV and later with an XDL 3000 powder.

Antifungal assay

Agar well diffusion method

In this study standard agar well, diffusion method was followed [25], [26], [27]. Each fungal isolate was suspended in Potato Dextrose broth Himedia Mumbai, Maharashtra (India) broth and diluted to approximately 105 colony forming unit (CFU) per mL. They were “flood inoculated onto the surface of Potato Dextrose agar and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer and 25µl of the sample’s solutions were delivered into the wells. The plates were incubated for 48 h at room temperature. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Ethanol was used as solvent control, Ketoconazole was used for yeast like fungi. The tests were carried out in triplicate.

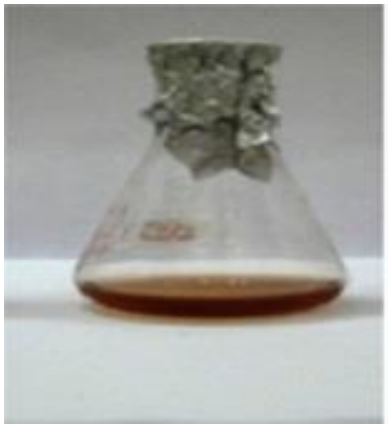
RESULTS AND DISCUSSION

UV spectrophotometry study

The UV–visible absorption spectra finding demonstrates a novel technique for the preparation of the Au NPs. The scale of wavelength was fixed between 200 and 700 nm, the surface Plasmon resonance (SPR) of the Ag NPs formed corresponded to 420 nm and there was an increase in intensity till 1hr as a function of time without any shift in the peak wavelength (Fig. 1). It can be observed that the reduction of Silver ions reaches saturation within1hr of reaction, and after that, only slight variations can be noted in the intensity of SPR bands. This result indicates that the reaction is completed in 1hr.

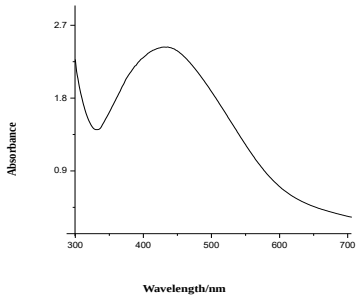
Plate 1. Green synthesis of silver nanoparticles using *Rosmarinus officinalis* oil

A: $AgNO_3$ solution



B: Synthesized silver nanoparticle

Figure 1 - UV–Vis spectrum analysis of silver nanoparticle reduced by *Rosmarinus officinalis* plant leaf oil at 450 nm



The XRD patterns of vacuum dried silver nanoparticles synthesized using leaf extract of *R. officinalis* leaf oil. A number of Bragg reflections with 2θ values of 38.06°, 44.35°, 64.51° and 77.36° sets of lattice planes are observed which may be indexed to the (111), (200), (220) and (311) facts of silver respectively in (Fig.2). It suggests that the prepared silver nanoparticles are biphasic in nature. The slight shift in the peak positions indicated the presence of strain in the crystal structure which is a characteristic of nanocrystallites. While the absorption spectra provide solid evidence of nanoparticle formation and their growth kinetics, the shape and size of the resultant particles were elucidated with the help of TEM. Nanoparticles observed from the micrograph majority are spherical, particles size ranged in size of 35–60nm with an average size of 45nm in Fig. 3. Thus the XRD pattern proves to be strong evidence in favor of the UV–vis spectra and TEM images for the presence of silver nanocrystals. FTIR spectroscopy analysis were carried out to identify the biomolecules responsible for the reduction of Ag+ ions and capping of the bioreduced silver nanoparticles synthesized by using plant extract. Fig. 4 shows the synthesized AgNPs using *R.officinalis* leaf oil aqueous extract where the absorption peaks were located at 1079, 1383, 1627, and 1729 in the region 500–3000cm−1. The peaks corresponding to presence of fatty acids, carbonyl groups, flavanones and amide I band of proteins.

Figure 2 - XRD patterns of silver nanoparticles synthesized by plant leaf essential oil *Rosmarinus officinalis*

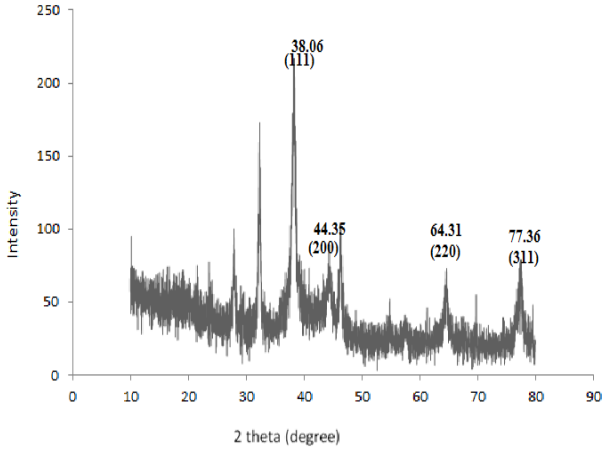


Figure 3- Transmission electron microscope image of silver nanoparticle synthesized using plant leaf oil *Rosmarinus officinalis*

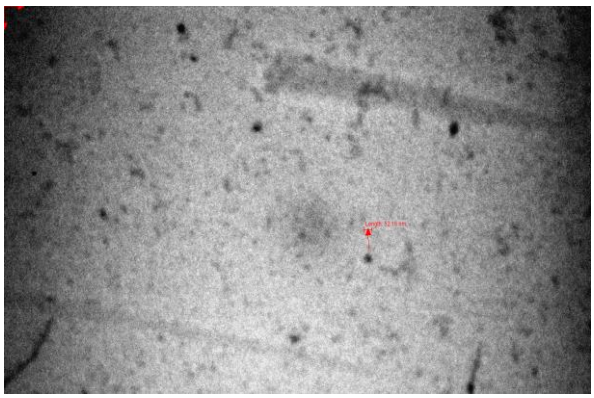
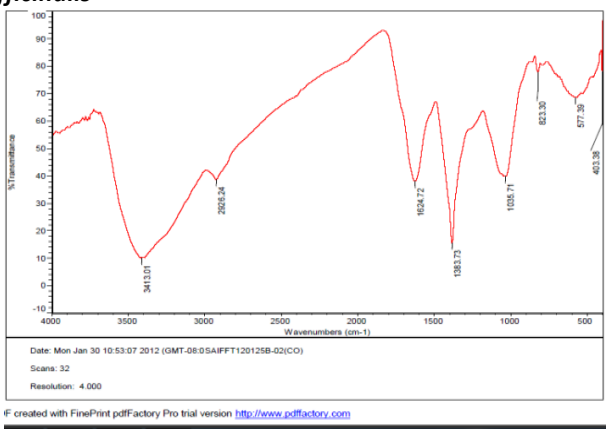


Figure 4- FTIR spectrum of vacuum dried powder of silver nanoparticles synthesized from plant leaf essential oil *Rosmarinus officinalis*



SEM analyses of the synthesized silver nanoparticles were clearly distinguishable measured 35–60nm in size. It is clear that the triangles, pentagons and hexagons structures with sizes of up to 45nm in (Fig.5). The co-existence of Ag NPs in smaller and larger size was due to the Ag NPs formed in early and later stages of the reaction, which shows that both nucleation to form new NPs and aggregation to form larger particles happened consecutively. Fig. 5A shows the spot-profile EDX of Ag NPs showed strong signals for silver atoms along with weak signals from carbon and oxygen. These weak signals could have been arisen from X-ray emission from

macromolecules like proteins/enzymes bound to the NPs or in the vicinity of the particles.

Figure 5A - Scanning electron microscope image of silver nanoparticle synthesized using plant leaf oil *Rosmarinus officinalis*

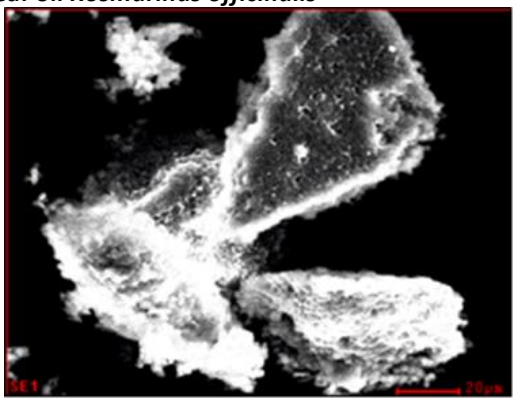
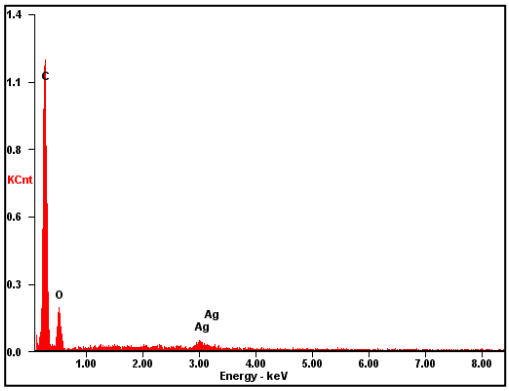


Figure 5B - SEM- EDS spectrum showed the presence of silver signal



3.2 Antifungal assay of Ag NPs

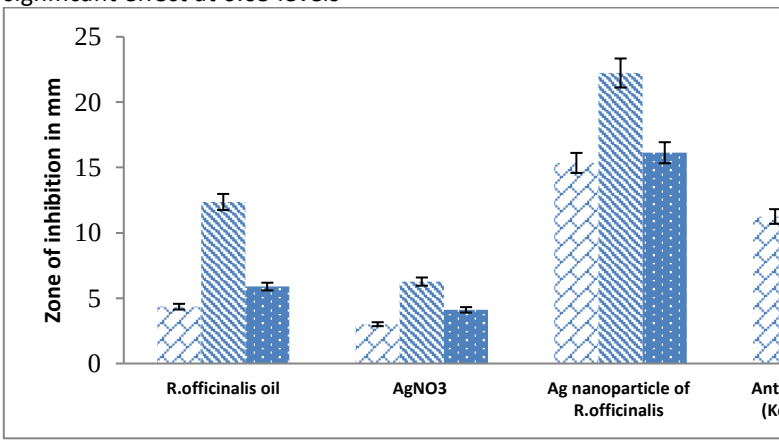
The plant essential oil *Rosmarinus officinalis* showed notable antifungal activity against *Candida albicans, Candida tropicalis* and *Candida kefyr* in Table-1. The essential oil *Rosmarinus officinalis* was very highly active against *Candida tropicalis* (12.36±0.48) and least against *Candida albicans* (4.35±0.35). Silver nitrate solution was highly active against *Candida tropicalis* (6.27±0.44) and least against *Candida albicans* (3.01±0.33). The silver nanoparticle *Rosmarinus officinalis* was also highly active against *Candida tropicalis* (22.23±0.78). All fungi were found to be sensitive and mostly comparable to the standard reference antifungal drug Ketoconazole to some extent. The results are shown in Graph 1.

Table 1 - Antifungal activity of synthesized silver nanoparticle *Rosmarinus officinalis* oil

Microorganisms	*R.officinalis* oil	$AgNO_3$ solution	*R.officinalis* oil + AgNP	Control (Ketoconazole)
Candida albicans	4.35±0.35^{c}	3.01 ±0.33^{c}	15.35±0.05^{c}	11.25± 0.19^{c}
Candida tropicalis	12.36 ±0.48^{d}	6.27±0.44^{d}	22.23±0.78^{d}	13.04±0.21^{d}
Candida kefyr	5.89± 0.59^{e}	4.11±0.67^{e}	16.13 ±0.24^{e}	12.08±0.88^{e}

The values are represented as the Mean ± SD of essential oil *Rosmarinus officinalis* and synthesized silver nanoparticle *Rosmarinus officinalis*. These essential oil *Rosmarinus officinalis* and synthesized silver nanoparticle *Rosmarinus officinalis* have significant effect at 0.05 levels

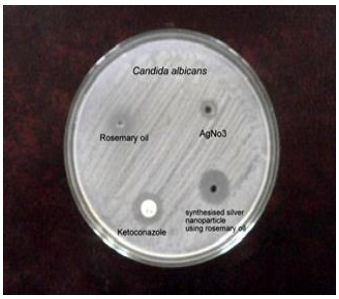
Figure 1. Antifungal activity of synthesized silver nanoparticle *Rosmarinus officinalis* oil



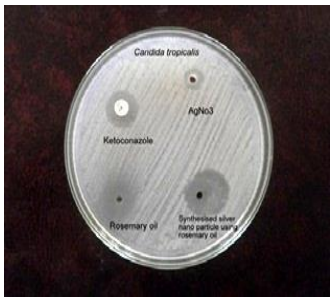
The silver nanoparticles show efficient antimicrobial activity compared to other salts. Therefore, silver is ideally suited for effective control of germs, molds and fungus. Its benefit over the use of antibiotics can be used as a powerful strategy to combat the increasing spread of multidrug resistance resulting from broad use of antibiotics. Therefore, clinical efficiency of antibiotics has been compromised [28].

Antibacterial activity of silver nanoparticles was assessed by using disc diffusion method against *Candida albicans* and *Candida tropicalis*. The results of this study also clearly indicated that silver nanoparticles synthesized from plant extracts of *Rosmarinus officinalis* has many pharmaceutical applications for the control of pathogens [29].

Plate 1A-C. Photographs showing the anti-fungal activity of synthesized silver nanoparticle from plant rosemary oil against selected fungi



A: *Candida albicans*



B: *Candida tropicalis*

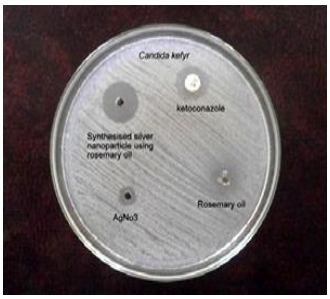


Plate -*Candida kefyr*

The significant and higher antifungal activity of against *Candida albicans* are probably due to the presence of flavonoids in the plant [30]. Chemical synthesis methods lead to the presence of some toxic chemical absorbed on the surface that may have adverse effect in the medical applications. Biosynthesis of nanoparticles by plant extracts is currently under exploitation. Green synthesis provides advancement over chemical and physical method as it is environment friendly, cost effective, easily scaled up for large scale synthesis and in this method there is no need to use high pressure, energy, temperature and toxic chemicals. Gold in its bulk form has long been considered an inert, noble metal with some therapeutic and even medicinal value hence Au NPs are thought also to be relatively non-cytotoxic [31]. [32] have reported that the reduction process of Ag+ to Ag nanoparticles occurs possibly in the presence of enzyme NADPH-dependent dehydrogenase. The exact route in which the electrons are shuttled is matter of investigation. Also the information regarding environment responsible for high stability of metal nanoparticles is not comprehensively available. [33] demonstrated the synthesis of Au NPs using *Anacardium occidentale* within 10 min. Au NPs synthesis was evaluated at different contact time taking absorbance with UV–vis spectroscopy and the intensity of SPR bands were observed in 2, 4, 6, 8 and 10 min. In the present study, the optimum time required for the completion of reaction was 10 min and yield of 0.3±0.1 g. The size and shape of the Au NPs synthesized using the seed extract of *Nigella sativa* were found to be very sensitive to the quantity of the extract and the amount of extract is increased, the stronger the interaction between the extract biomolecules and nascent nanoparticles, thus the yield of nanoparticles increased as shown by surface plasmon resonance bands in the UV–vis–NIR spectra. [34] have reported that the development of an antimicrobial gel formulation containing silver nanoparticles in the size range of 7–20 nm synthesized by a proprietary biostabilization process exhibited good antifungal activity against *A. niger* and *C. albicans*.The modified denture base acrylic combined with silver nanoparticles displayed significant antifungal properties against *C. albicans* strain [35]. The antimicrobial activity of N-cholylamino acid capped Ag NPs reported effective antifungal agent against *C. albicans, Candida krusei* and *Candida tropicalis* using RPMI broth were determined by MIC studies [36]. Nano titanium dioxide particles immobilized onto polycaprolactone (5wt %) was quite effective killing of *C. albicans* after only 60 min exposure with a near visible light source [37]. The results thus obtained lend strong evidence that could warrant the consideration of Au NPs as antifungal agent that could circumvent the side and passive immune effects of other medications.

CONCLUSION

The present green synthesis method is a low-cost approach, capable of producing Ag NPs at room temperature. The size and structures of the obtained NPs were characterized by SEM and XRD. Moreover, this plant mediated synthesis method represents a considerable improvement for the preparation of Ag NPs because of it allows better control over their nanostructures. The synthesized Ag NPs had significant antifungal activity. This kind of study may also make a possible platform in future for preparing nanomedicines for Vaginal Candidiasis related diseases. It is confirmed that Ag NPs are capable of rendering high antifungal efficacy and hence has a great potential in the preparation of drugs used against Vaginal Candidiasis diseases.

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