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Rosemarinus officinalis Mediated Synthesis of Silver Nanoparticles and its Activity against Pathogenic Fungal Strains

Amna Qayyum[1], Tajwar Malik[1], Syeda H. Ali[1]*, Mohammad Mushtaq[1], Syeda A. Ali[2]

[1]*Department of Microbiology, Baluchistan University of Information Technology Engineering and Management Sciences, Quetta, Baluchistan, Pakistan.*
[2]*Department of Biochemistry, Sardar Bahardur Khan Women University, Quetta, Balochistan, Pakistan.*

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ABSTRACT

Nanotechnology is advancing rapidly with applications in various fields. Silver (Ag) nanoparticles are known for their pronounced impact in pharmaceutical industry owing to its strong antimicrobial activity. Antimicrobial resistance of drugs has shifted the focus of the scientific community to the search of novel bioactive compounds from medicinal plants with more potential to treat various ailments. *Candida* species are widely recognized as pathogens accountable for high morbidity and persistent infection. In this study we elucidated the robustness of biogenic silver nanoparticles synthesized using *Rosemarinus officinalis* against pathogenic *Candida* species. We tested and compared the impact of 161 µg/mL, 270 µg/mL concentrations of silver nanoparticles and characterized the particles using UV-VIS Spectrophotometer and Scanning Electron Microscopy (SEM). The antifungal activity of the Silver nanoparticles was tested against *Candida albicans, Candida tropicalis, Candida glabrata* and *Candida krusei* using Agar well diffusion method. The synthesized nanoparticles were spherically shaped with size ranging from 75 – 98 nm. The results revealed high efficacy of 161 µg/mL silver nanoparticles against *C.* albicans, *C. tropica*lis, and *C. krusei* compared to 270 µg/mL Ag nanoparticle. Silver nanoparticles can be used as a therapeutic drug instead of synthetic drugs due to its reduced toxicity and adverse effects. Search of naive plants as novel therapeutic agents can open new avenues in drug development to combat antibiotic resistance.

Keywords: *Rosemarinus officinalis*, Silver nanoparticles, *Candida* species, Antifungal activity.

Introduction

Nanoparticles are structures with size range of 0.1 to 100 nm.[1] Nanotechnology has acquired worldwide consideration owing to its broad applications in biomedical and pharmaceutical industry. Recently, biologically synthesized nanoparticles are widely gaining attention due to its pharmacologically dynamic properties than physicochemically synthesized nanoparticles.[2] Whereas, the development of eco-pleasing strategies in the production of nanoparticles is an indispensable advantage in the field of nanotechnology which is rapidly bringing advancement in medical diagnosis and treatment.[3]

Nanoparticles are considered as solid particle that can easily be dissolved, trapped or linked with a drug. Nanoparticles can be prepared physically, biologically and chemically but biological procedures are considered safe and effective as compared to the other methods. Silver is known for its antimicrobial properties and in recent times its use as biocide nanoparticle has revolutionized the medicinal field.[4] Silver nanoparticles (AgNPs) are critical due to its synthetic, physical, natural properties and applications.[3] Various techniques are available to synthesize AgNPs such as electrochemical, chemical reduction and via metabolites of living organisms such as plants and microbes. However, physical and chemical techniques cause assimilation of toxic substances. Whereas, the preference of environmentally friendly procedures is to avoid toxic chemicals produced during the synthesis. Moreover, green synthesis of AgNPs is a rapid and safe method with symmetric morphology under controlled conditions.[5]

Antimicrobial resistance (AMR) is the resistance of pathogens toward antibiotics. High dose of antibiotics due to overuse or misuse of drugs has not only escalated the number of such infections but also has potential to cause harm to the host. The lack of effective drugs by pharmaceutical companies possess huge challenge to the scientific community. Therefore, the researchers are playing vital role to discover medicinal plants for novel antimicrobial metabolites to use against resistant pathogens. Discovering novel antibiotic is vital to combat emerging resistance of pathogens to older drugs.[6]

According to World Health Organization (WHO), approximately 2000 medicinal plants have been identified in 91 countries.[7] Medicinal plants are replacing synthetic drugs due to the potential side effects of drugs in treating various ailments.[8] Recently, medicinal plants are employed to produce AgNPs, as a natural capping agent and reducing agent. Various medicinal plants such as *Azadirachta indica*, *Aloe vera Ocimum tenuiflorum, Syngonium podophyllum*,and *Ficus benghalensis* are reported for the synthesis of AgNPs against various pathogenic microbes.[9-11] *Rosemarinus officinalis* (commonly called Rosemary) is a medicinal plant belonging to the family *Labiatae or Lamiaceae* is widely used throughout the world due to its remarkable therapeutic properties such as antimicrobial, anti-inflammatory, anti-cancer and diuretic activities.[12] Here, we aimed to identify the antifungal property of Rosemary mediated silver nanoparticles against pathogenic yeast strains. We further determined the minimum concentration-dependent activity of the biosynthetic silver nanoparticles against the respective strains.

*Corresponding author. E mail: s.hafsaali@yahoo.com
Tel: +923468378508

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Materials and Methods

Plant Selection
Dried leaves of *Rosemarinus officinalis* were obtained from Baluchistan Agricultural Research and Development Centre Quetta (BARC).

Synthesis of Silver Nanoparticles
Plant Extraction
The plant extract was prepared using dried leaves of *Rosemarinus officinalis*, washed thrice with distilled water, and dried in a dark place. Dried leaves (2.5 g) were boiled in 50 mL of distilled water, cooled and filtered at room temperature for further use.[13]

Silver Nitrate Solution
Silver nanoparticles were synthesized according to Jain *et al* with some modifications. The concentrations of 1.5 mM and 2.5 mM of silver nitrate ($AgNO_3$) was prepared in Erlenmeyer flask and covered with aluminum foil to avoid photodegradation. This solution was constantly stirred at 1200 rpm.[14] About 10 mL of plant filtrate was added dropwise into the beaker containing silver nitrate solution. The reaction was conducted overnight until the colour changes to dark brown. Further, synthesized silver particles were purified by centrifuging at 15000 rpm for 15 mins, and the pellets were washed twice with distilled water. Finally, silver nanoparticles in the pellets were collected for further characterization.[13]

Characterization of Silver nanoparticles
Visual identification
Silver nanoparticle solution was observed visually. The colloidal solution was observed from the beginning of the reaction to assure reduction of Ag^+ ions to Ag particles, with a shift from colourless to dark brown colour.[15]

UV-VIS Spectrophotometric analysis
Synthesized silver (Ag) nanoparticles were further characterized using UV-VIS Spectrophotometer (Jenway, Model no. 6305) to monitor bio-reduction of Ag^+ ions at a wavelength ranging from 300 - 700 nm, whereas distilled water was used as blank. Initial and final readings were taken to confirm the synthesis of silver nanoparticles.[16]

Scanning Electron Microscopy (SEM)
To determine the shape and size of biogenic silver nanoparticles, SEM was performed. The sample of silver nanoparticles were dried in hot oven at 80°C for 3 days. The morphology of the nanoparticles was identified using SEM.

Antifungal Activity
The synthesized silver nanoparticles were tested against *C. albicans, C. krusei, C. glabrata, and C. tropicalis*. Yeast strains were grown overnight in an incubator at 24°C and compared with 0.5 McFarland solution. 100 µL of inoculum containing 10^5 CFU/mL was spread onto Yeast and Mold agar-containing Petri dishes. About 100 µL of water (negative control), 50 mg/mL of plant extract (positive control), 100 µL silver nitrate (1.5 mM, 2.5 mM) and 161 µg/mL, 270 µg/mL silver nanoparticles (treatment) was added in each well, respectively. The plates were incubated at 24°C for 48 hours.[17] Each experiment was repeated thrice, with three replicates. Zone of inhibition was measured in cm.

Statistical Analysis
The results were statistically analyzed by evaluating zones of inhibition in cm and data expressed as mean ± standard error mean. The data was analyzed using Graphpad Prism (version 5.01).

Results and Discussion

Characterization of Biosynthetic Silver Nanoparticles.
Colour Characterization
The change in the colour of the colloidal solution validated the reduction of Ag^+ ions to Ag nanoparticles.[18] The colloidal solution of silver nanoparticles turned pale yellow, pale brown, and ultimately dark brown at the end of the reaction (Plate 1). Neem leaf extract comprises of reducing agents such as phenolics, flavanones, and terpenoids which are known to reduce silver nitrate to silver nanoparticles.[19] Medicinal plant extracts are rich in antioxidants and play a vital role to hinder oxidative damage of cellular components by preventing initiation of oxidative chain reaction. Phenolic compounds are known for their anti-oxidative ability to neutralize free radicals due to its redox property.[20] *Ananas comosus* extract used to synthesize silver nanoparticles confirmed that ferulic acid due to its phenolic nucleus served as a potent antioxidant to scavenge free radicals, thereby acting as capping and reducing agent to the synthesis of silver nanoparticles. Similarly, *Rosemary* extract comprises of phenolic compounds; rosemarinic acid, caffeic acids, hydrocinnamic acids with high scavenging ability to suppress the formation of free radicals and may be involved in reducing $AgNO_3$ to the silver nanoparticle.[21]

UV-VIS Spectrophotometry
UV-Visible spectrophotometric analysis confirmed the synthesis of biosynthetic silver nanoparticle with surface plasmon absorption maxima at ~425 nm. The yellowish-brown colour of the colloidal silver solution was due to the excitation of surface plasmon vibrations of silver particles.[22] Initial absorbance reading showed the synthesis of silver nanoparticle, while the final value confirmed the presence of silver nanoparticle as depicted by a sharp peak between 420 - 450 nm for both concentrations of the colloidal silver solutions (Figure 1). The observed absorption peak depends on the size and refractive index of the the colloidal solution.[23]

Scanning Electron Microscopy (SEM)
SEM was performed to determine the morphology of biogenic silver nanoparticles. The results confirmed spherical shaped nanoparticles in a range between 75 - 98 nm (Plate 2).

Plate 1: Colour change of the colloidal silver nanoparticle from pale yellow to dark brown as silver nanoparticles are formed.

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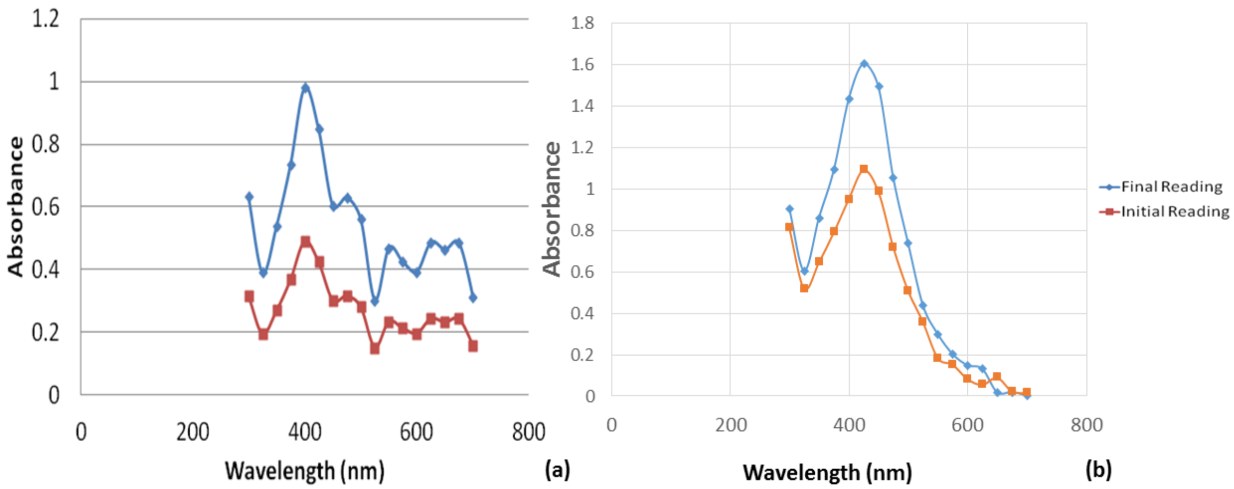

Figure 1: Characterization of silver nanoparticles using UV-VIS Spectrophotometer. The colloidal solutions of AgNP were characterized using UV-VIS spectrophotometer. Absorbance of the colloidal solution was measured from 300 nm to 700 nm. A sharp peak between 400 - 450 nm of 161 µg/µL Silver nanoparticle (a) and 270 µg/µL silver nanoparticle (b) confirmed conversion of Ag^+ to Ag nanoparticles.

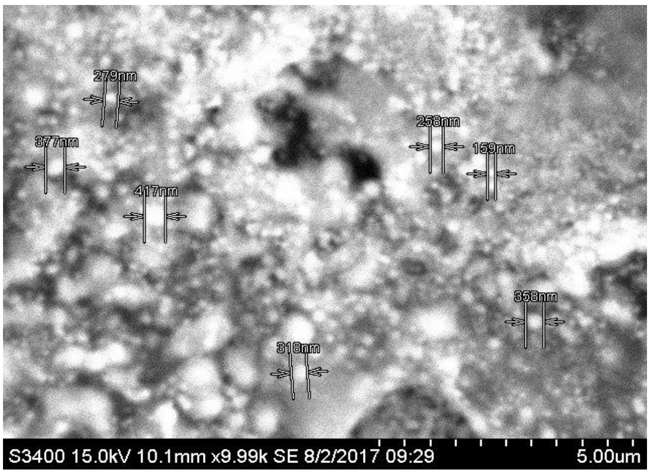

Plate 2: Synthesized silver nanoparticles (2.5 mM) characterized using SEM were spherical in shape with a size in range between 75 - 98 nm.

Antifungal Activity of Biogenic Silver Nanoparticle

Antimicrobial susceptibility of the biosynthetic silver nanoparticles was investigated against *Candida albicans*, *Candida krusei*, *Candida glabrata*, *and Candida tropicalis* using agar well diffusion method.

Lower concentration of silver nanoparticle was effective against Candida albicans.

Candida albicans is a commensal yet an opportunistic fungus and a primary cause of candidiasis. Our results revealed that biogenic silver nanoparticles at 161 µg/µL exhibited significant zone of inhibition with mean value of 1.60 ± 0.40 cm against *Candida albicans*. This activity was followed by 2.5 mM silver nitrate with zone of inhibition of 1.317 ± 0.208 cm and 270 µg/mL silver nanoparticle with zone of inhibition of 0.983 ± 0.224 cm (Figure 2). Whereas, the control groups i.e. water and plant extract did not inhibit the growth of *C. albicans*. *C albicans* makes up healthy microbiota of the gastrointestinal tract (GIT), reproductive tract, oral cavity, and the skin. It is among the few fungal species that cause diseases in human under certain circumstances. Infections usually range from superficial skin infections to life-threatening systemic infections such as *Candidiasis* and Vaginal infections.[24] Several factors contribute to its pathogenicity such as certain molecules that mediate adhesion to host cell and causes invasion by secreting enzymes like hydrolases.[25] Several studies report the mechanism of action of AgNPs such that its small size easily enters fungal cell and impairs intracellular processes like DNA, RNA, and protein syntheses.[26] Increased in morbidity rate related to candidiasis has called for effective novel remedial solutions.[27] Previously, silver nanoparticles biosynthesized with *Glycyrrhiza glabra* root and *Amphipterygium adstringens* bark extracts inhibited the growth of *Candida albicans* suggesting that biosynthesis of silver nanoparticles could bring breakthrough research in the field of biomedical sciences. Recently, *S. cerevisiae* used to synthesize silver nanoparticles inhibited growth of fluconazole-susceptible and fluconazole-resistant *Candida albicans* isolates.[27]

Higher concentration of Silver nanoparticles was effective against Candida glabrata

C. glabrata infections are difficult to treat due to its resistance to many antifungal agents. *Candida glabrata* inhabits the mouth, intestinal, esophageal, and vaginal mucosal surfaces as commensals, yet interaction with the host and defense mechanisms is poorly known.[28] Our results showed that the zone of inhibition against *C. glabrata* at concentration of 2.5 mM was 1.833 ± 0.2108 cm and at 1.5 mM Silver nitrate the zone of inhibition was 1.15 ± 0.15 cm, while for the silver nanoparticles, the zones of inhibition were 1.617 ± 0.2587 cm and 0.85 ± 0.15 cm at 270 µg/µL and 161 µg/µL, respectively. Whereas, the controls (water and plant extract) were ineffective against the respective strain (Figure 3). A higher dose of silver nitrate against *C. glabrata* reveals its resistant nature as compared to other strains of *Candida spp.* Previous research demonstrated that the antibacterial action of AgNPs is more compelling than the antifungal activity.[29] This activity is due to the unpredictability of the eukaryotic yeast cell which requires a higher dose of silver nanoparticles to cause ramifications in a cell.[30]

Higher concentration of silver nanoparticles was effective against Candida krusei

Antifungal activity relies upon the size and shape of silver nanoparticles. Small-sized nanoparticles have a high surface area which maximizes antimicrobial impact. The biosynthetic silver nanoparticles showed significant activity against *C. krusei* compared to all other treated groups. Our results showed that 2.5 mM silver nitrate was active against *C. krusei* as shown by the zone of inhibition with mean value of 1.40 ± 0.14 cm, while the silver nanoparticles at 161 µg/µL showed zone of inhibition of 0.85 ± 0.15 cm and at 270 µg/µL, the zone of inhibition was 0.93 ± 0.25 cm. None of the control groups (water and plant extract) showed any impact against this strain

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(Figure 4). The activity of silver nanoparticles has similar effects as silver ions. Positively charged silver ions may attach with negatively charged cell membranes of microbes by electrostatic attraction.[31] Silver nanoparticles make pits in the cell wall and damages cell permeability and induce proton leakage by the formation of Reactive Oxygen Species (ROS) in the membrane and ultimately resulting in cell death.[32] These outcomes demonstrated that Nano-Ag influenced yeast cells by assaulting their layers. Moreover, Nano-Ag capture cell cycle at the G2/M stage in *C. albicans* to such an extent that Nano-Ag hindered some cellular processes which are engaged in the typical bud development.[33]

Lower concentration of silver nanoparticles was effective against Candida tropicalis

Candida tropicalis is a normal flora of the skin and stomach lining in humans. *C. tropicalis* causes bloodstream infections and is highly pathogenic than *C. albicans*. Fungal cells maintain their membrane potential by establishing multiple ion gradients across the cytoplasmic membrane. Support of intracellular components is imperative for *Candida* viability such as glucose and trehalose act as shielding agents to prevent the inactivation or denaturation caused by stressful conditions.[34] Our results demonstrated a higher activity of 161 µg/µL and 270 µg/µL of biogenic silver nanoparticles with zones of inhibition of 1.90 ± 0.10 cm and 0.933 ± 0.25 cm, respectively against *C.* tropicalis. While 2.5 mM silver nitrate showed zone of inhibition of 1.40 ± 0.14 cm (Figure 5). Whereas, the control and plant extract were ineffective against *C. troplicalis* (Plate 3). Previously, silver nanoparticles synthesized using the fungus *Arthroderma fulvum* showed a significant antifungal activity against *Candida tropicalis*. *Candida tropicalis* have been distinguished as the most common pathogenic yeast types of candida species.[35]

Antifungal resistance is an emerging problem with *Candida* infections. *C. glabrata* causes diseases that are hard to treat and are regularly impervious to numerous antifungal ointments.[31] The development of free radicals by AgNPs makes it a potential biocide against resistant strains.[36] Over 90% of fungal diseases such as athletes foot, nail contaminations, parasitic contaminations are caused by *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata*.[24,31] A few kinds of *Candida* are progressively susceptible to first and second-generation antifungal drugs such as fluconazole and the echinocandins (anidulafungin, caspofungin, and micafungin). *Candida glabrata* and *Candida krusei* comprises 70% of all resistant fungal species. A few types of fungal species are vulnerable to treatment with an antifungal drug, whereas many fungal species are developing resistance against drugs due to misuse of antifungal drugs.[24]

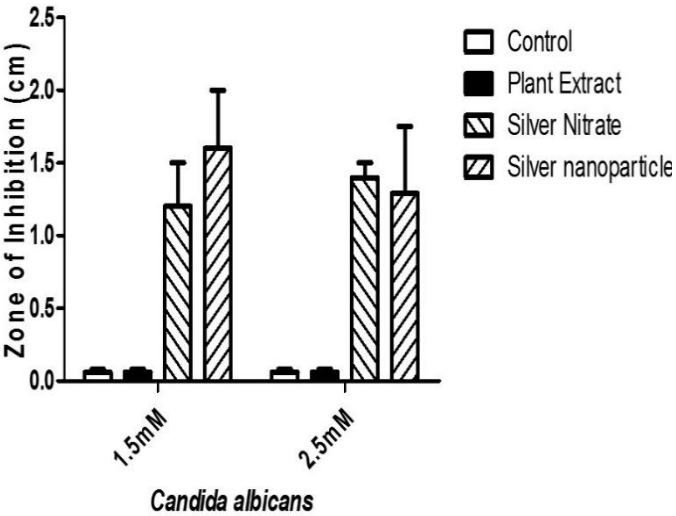

Figure 2: Antifungal activity of silver nanoparticles against *C. albicans* showed significant difference *$p < 0.05$* as compared to $AgNO_3$ and control groups.

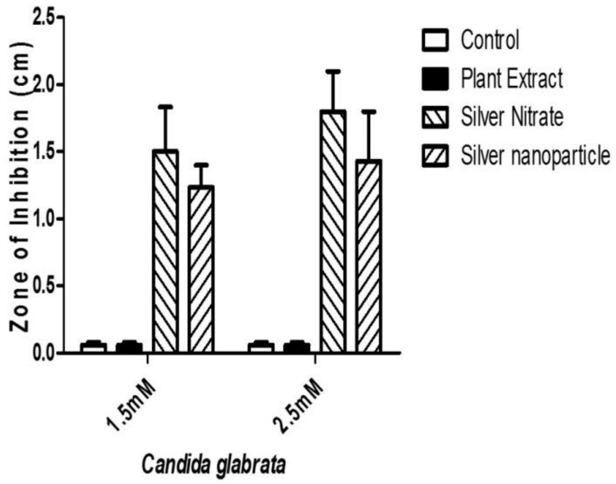

Figure 3: Antifungal activity of silver nanoparticles against *Candida glabrata* demonstrated huge distinction *$p < 0.05$* when contrasted with controls.

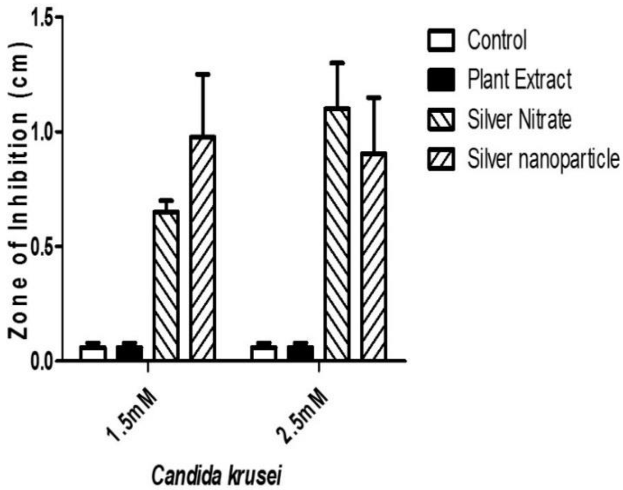

Figure 4: Antifungal activity of silver nanoparticles against *Candida krusei* showed significant difference *$p < 0.05$* as compared to control groups.

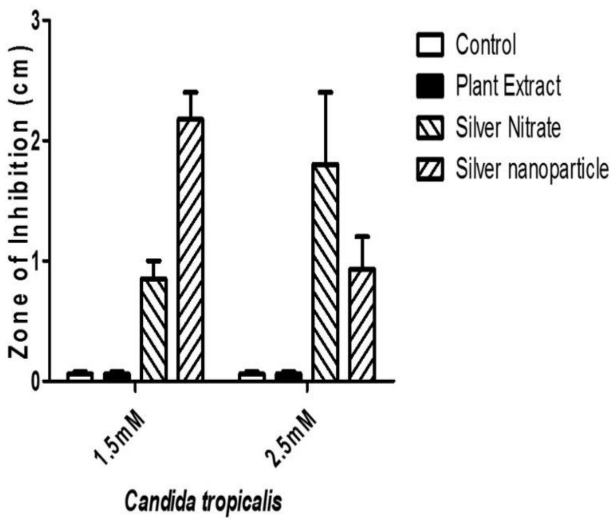

Figure 5: Antifungal activity of silver nanoparticles against *Candida tropicalis* showed significant difference *$p < 0.05$* in a zone of inhibition as compared to controls.

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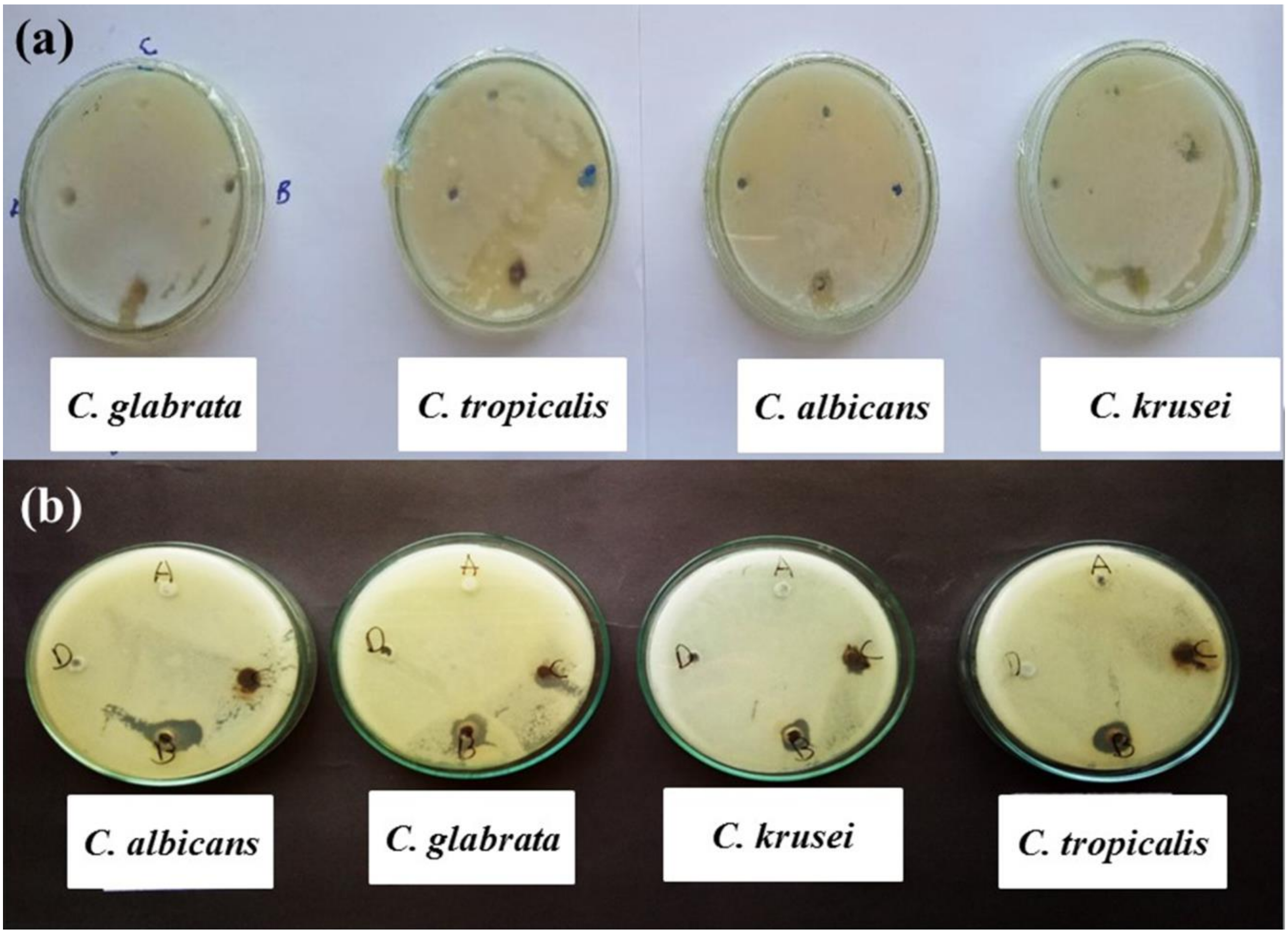

Plate 3: Synthesized biogenic silver nanoparticles (a) 161 µg/µL and (b) 270 µg/µL were tested on the growth of *Candida albicans, Candida glabrata, Candida krusei and Candida tropicalis* using agar well diffusion method. Well labelled D containing Silver nanoparticles showed significant impact on the growth of pathogenic fungi as compared to the control groups.

Conclusion

Silver nanoparticles synthesized from *Rosmarinus officinalis* showed significant antifungal activity. We found 161 µg/µL AgNPs was more effective as compared to 270 µg/µL. These findings suggest that nanoparticles at lower concentration are more symmetric in shape with high surface area that acts vigorously against pathogenic *Candida* species. The biosynthesis of Ag nanoparticles from *Rosemary* is an eco-friendly option in contrast to chemical and physical techniques. We conclude that synthesized AgNPs enhances therapeutic efficacy and enhances the medicinal potentials of medicinal plant. Further investigations can be conducted to reveal non-toxic, anticancer property, and anti-protozoal activity of biogenic nanoparticles.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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