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Anti-candida efficacy of Silver Nanoparticles biosynthesized using *Sphaeratnhus amaranthoides* aqueous extract on *Candida albicans* -An *In-vitro* Study

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Abstract:

Objective: The Present study focuses on the efficacy of silver nanoparticles biosynthesized using the aqueous leaf extract of *Sphaeranthus amaranthoides* on *Candida albicans* ATCC 10231 and its biofilms. **Methods:** Silver nanoparticles of *Sphaeranthus amaranthoides* were synthesized and characterized by UV, FT-IR, Zeta Potential, and SEM. Anti-*Candida* efficacy of synthesized silver nanoparticles was assessed on *Candida albicans* by disc diffusion and Minimum inhibitory concentration method. The biofilm formation inhibition effect of *Sphaeranthus amaranthoides* nanoparticles was assessed. Further mechanistic studies were also studied by evaluating the cell wall components of *Candida albican*s and its virulence factor like secreted aspartyl proteinase assay. **Results:** *Sphaeranthus amaranthoides* silver nanoparticles formation was characterized. The in-vitro disc diffusion method showed a zone of inhibition of 12±0.15 mm at 100 µg/mL concentration. Further mechanistic studies revealed that the biosynthesis of silver nanoparticles of *Sphaeranthus amaranthoides* acted on the cell wall of *Candida* thereby breaking the integrity of glucan moiety and proteins present in the cell wall. It also can inhibit the virulence factor such as Secreted Aspartyl Proteinase **Conclusion:** It is evident that silver nanoparticles of *Sphaeranthus amaranthoides* have anti-*candida* potency against *Candida albicans* and can be used as a therapeutic to treat candidiasis.

Keywords: Dental Materials, Microbiology/Immunology, Dental Public Health, Oral Health.

Introduction:

Green synthesis is an emerging field in biomedicine due to its high stability, non-toxic and eco-friendly. Metallic nanoparticles have shown promising activity in treating several diseases, in which silver nanoparticles (AgNp's) have a multi-functional biological activity such as anti-bacterial, antifungal, and anti-cancer activities [1,2]. In the traditional medicine system, medicinal plants have been the basis for the treatment of various ailments which possess a variety of bioactive compounds and /or phytochemicals [3]. The phytochemical-rich constituents from the plant extracts with several health benefits can act as capping and reducing agents for AgNPs. The phytomolecules can provide stability to the nanoparticles and electrostatic effect in the suspension [4]. Using medicinal plants for greener routes will serve the dual purpose of inhibitory effect against pathogens as well as the synthesis of metallic nanoparticles.

Candida albicans, a eukaryotic opportunistic dental pathogen of humans and the etiological agent of candidiasis infection has increased dramatically over the past three decades causing severe morbidity and mortality ranking fourth place in most commonly encountered dental pathogens causing dental plaques [5]. *C. albicans* can form a biofilm on teeth which is a major virulence mechanism adapted by the organism to escape from host immune cells, antimicrobials, and cleaning agents. In the protective environment, *C. albicans* enter a transition state from yeast cells to hyphae which involve the penetration of the epithelial cells of the skin surface and leads to invasive chronic skin infection [6]. Secreted Aspartyl Proteinase (SAP), an extracellular enzyme produced by

Candida albicans plays a vital role in the pathogenesis by distorting the host cell membrane and facilitating microbial adhesion and invasion into host tissues [7]. Under suitable predisposing conditions, it can colonize by forming biofilms and provide the basis for *Candida* proliferation, invasion, and dissemination [7]. Multiple Drug resistance has developed against many microbial infections including candidiasis in this modern technology. Haphazard use of available synthetic drugs is often associated with adverse effects on the host including hypersensitivity, immunosuppressant and allergic reactions. Researchers are paying much attention to phytochemicals and or plant-based active compounds as an alternative strategy to treat infections as they are much less toxic and with no side effects.

S. amaranthoides is a tropical herbal plant in Southeast Asia and South Africa that is widely used for the treatment of various human ailments like skin disease, wound healing, rejuvenation, and eczema [8]. *S. amaranthoides* contains rich phytoconstituents like terpenoids, flavonoids, and alkaloids which are rich in antioxidant, antimicrobial, and antihelminthic properties [9]. Since *S. amaranthoides* have effective anti-microbicidal properties the leaf extract from this plant is widely used as wound healing medicine in the Indian Ayurvedic medicine system [10]. In the present study, *Sphaeranthus amaranthoides* plant extracts and their green process-mediated silver nanoparticles were synthesized, characterized, and subjected to their anti-*candida* activity by in-vitro methods.

Materials and methods

Chemicals and Reagents: All the necessary chemicals and reagents for this experimental work were procured from Sigma Aldrich (USA). All the chemicals used are of analytical grade with 99% purity.

Plant material Collection: *S. amaranthoides* were purchased from a local herbal market, in Chennai. The plant material was identified to be *S. amaranthoides* from the Department of Botany, Sri Sankara Arts and Science College, Chennai. A voucher specimen of the plant (SU45715) was deposited at the Department of Botany, Sri Sankara Arts and Science College for future reference.

Plant Extraction: The leaf of *S. amaranthoides* (520 grams) was shade-dried and the dried leaf materials were subjected to organic extraction with methanol solvent (w:v) [8]. The extracted solvent system was then filtered and evaporated using a rotary evaporator. The obtained crude dried extract of 416 milligrams was used for nanoparticle synthesis.

Biosynthesis of Silver nanoparticles

Silver nanoparticles were synthesized from *S. amaranthoides* using the methodology of [11] with slight modifications. In brief 100 mL of 1 mM Silver nitrate ($AgNO_3$) solution was mixed with the 20 mL of whole plant extract and stirred thoroughly for 30 min using the magnetic stirrer. The mixture was then boiled at 50ºC

for 30 min and stored under dark conditions at room temperature. The reduction of silver ions and synthesis of silver nanoparticles was confirmed by measuring the optical color change through the UV-2550 Shimadzu Spectrophotometer (Shimadzu). The silver nanoparticle synthesized through the greener method was obtained by centrifugation at 12000 rpm for 5 min and rinsed with sterile distilled water to remove the debris. The samples were then concentrated by placing them under a rotary vacuum evaporator (RVC 2-18 CD plus).

Characterization of Silver nanoparticles

The absorption spectrum of silver nanoparticles was analyzed through a UV/Vis spectrophotometer (Enspire, PerkinElmer, USA) in the wavelength of 400-700 nm. The structural characterization of the synthesized nanomaterial was analyzed through Fourier Transform Infrared spectroscopy (FTIR, Thermo Scientific Nicolet 6700), in the wavelength of 400–4500 cm^{-1}. The structural morphology of silver nanoparticles was assessed through Field emission scanning electron microscopy (FE-SEM) (Supra-55, Carl Zeiss). The particle size and stability of the silver nanoparticles were evaluated through dynamic light scattering (DNS, Nano Zetasizer).

In vitro Anti *Candida* studies

Fungal strains and growth conditions

Candida albicans (ATCC 10231) was used for biofilm studies. *C. albicans* were maintained in Potato dextrose broth/agar (PDB/PDA) medium (Hi-media) at 37°C. For biofilm studies, yeast cells were collected after centrifugation at 2500 rpm for 20 min. The cells were then washed and resuspended in 100 mM phosphate buffer saline (PBS, pH 7.2).

In-vitro Biofilm Inhibition Study

A glass slide of 0.3 cm^2 was placed inside the 96-well microtiter plate (Tarsons Inc, India) containing 100 µL of PDB medium with *Candida albicans* inoculum with 10^7 CFU/mL. The test wells were mixed with biofilm inhibitory concentration (BIC) of silver nanoparticles and incubated at 37 °C for 24 h. Following the incubation, the slides were removed from the wells and the loosely bound cells were rinsed with distilled water. The slides were then stained with crystal violet dye and dried for 15 minutes then washed with distilled water to remove excess stain. The slides were allowed to dry in air and observed under a light microscope (Leica, DM2000 LED) at 100 X magnification. The images were processed through light microscope LAS-X life science software program 4.9 [12]. Another set of slides was examined under FE-SEM to reveal their morphology in the presence and absence of AgNPs.

Biofilm quantification using XTT

C. albicans biofilms grown in the presence and absence of Au-NPs for XTT assay in the microtitre plates were incubated for 12h at 37°C. After incubation, the microtiter plate was rinsed with Milli-Q to remove the loosely attached cells [13]. The

plates were added with 100 µL of XTT menadione solution and incubated in the dark for 5 h at 37°C. The change in the color formation by water-soluble formazan product was measured at 450 nm using a microtitre plate reader (BioRad, USA).

Percentage of Biofilm Inhibition = (Treated OD/ Control OD X 100 - Control OD)

Disc Diffusion method

The anti-*Candida* activity of *Sphaeratnhus amaranthoides* plant extract was tested using the Disc diffusion method [14,15]. *Candida* strains were maintained on Potato dextrose agar (PDA) medium and by using an aseptic technique where a sterile swab was dipped into fungal inoculum and spread plated over PDA agar plate. A 6 mm disc was prepared by using Whatman No.2 filter paper and gently pressing each disc to the agar to ensure that the disc was attached to the agar. 50 µL (1mg/mL) of the plant extracts were applied in each of the discs and incubated for 48 h at 28ºC. Results were recorded by measuring the zone of inhibition around the discs.

Minimum Inhibitory Concentration (MIC):

Minimum Inhibitory Concentration (MIC) was determined by the micro-broth-dilution method [16]. The minimum concentration, at which there was no visually detectable fungal growth, was taken as MIC. Extract concentrations of 0.02-0.200 mg/mL were evaluated for the MIC study. Specifically, 0.1mL of standardized inoculum (1-2 x 10^7 CFU/mL) cultured in PDB broth medium was added to each test tube. The tubes were incubated aerobically at 28ºC for 48-72 hours. Two controls were maintained for each test sample. PDB broth with fungal inoculum culture without SaAgNPs treatment was used as negative control and the Flucanozole drug added group was used as a standard positive control. The lowest concentration (highest dilution) of the extract that produced no visible signs of fungal growth (no turbidity) when compared with the control tube was regarded as MIC.

Autofluorescence studies

Candida albicans cultures control without SaAgNPs treatment and treated with SaAgNPs were grown separately in the yeast extract peptone dextrose (YEPD) medium for 16 hrs at 37ºC in an orbital shaker and 250rpm. 100µL of culture was placed in 96 well-black coated plates. The excitation wavelength was set at 450 nm in a multimode reader to detect the autofluorescence in Spectra Max fluorimeter and emission was observed at 530 nm.

Mechanistic studies

Cell wall preparation

Log phase culture cells were taken and treated with different concentrations of SaAgNP were added and incubated for 32 h at 28°C and cells were centrifuged at 8000 rpm for 15 min, followed by suspension of cellular pellet in 1mM Tris-HCl buffer pH6.8, and

kept in ice bath for 30min. Cells were centrifuged at 8000rpm for 10 min. The supernatant was taken and estimated for sugars by the method of Dubois [17] and protein estimation by the Bradford method [18].

Isolation of Secreted Aspartyl proteinase (SAP)

SAP enzyme was isolated according to the protocol [19]. *C. albicans* log phase culture was grown on YEPD broth and the cell pellets were washed with sterile distilled water. 10^{-6} cells were then inoculated into flasks containing 20 mL of brain heart infusion (BHI) broth and incubated on a shaker at 37ºC for a total of 65 hours. Culture was centrifuged at 3000 rpm for 10 minutes at 25°C to obtain the culture supernatant which serves as a source of SAP.

Enzyme Kinetics:

Different concentrations of bovine serum albumin (BSA) substrates (0.75 to 4.25 µM) were taken and the velocity of the Sap was measured at 280 nm. A graph was plotted by taking the concentrations of substrates on the X-axis and the velocity of enzyme or reaction rate on the Y-axis. Kinetic studies of Sap enzyme were studied using Graph pad Prism.5 software free trial version. Hanes-Woolf plot was plotted and Km and Vmax were determined.

Results

Characterization of Silver Nanoparticles

The color change from brown to black indicated the synthesis of silver nanoparticles. The characterization of synthesized silver nanoparticles through UV spectroscopic studies revealed the peak of surface plasmon resonance (SPR) at 452 nm (Figure 1A). FTIR analysis revealed the presence of functional groups in the extract. Functional groups are responsible for the reduction of silver metal as well as for the stabilization of the synthesized nanomaterials. Figure 1B represents the FTIR absorption spectra in the IR range of 400-4000 cm^{-1}. The silver nanoparticles showed major stretching frequencies at 633.32 cm^{-1}, 1088.57 cm^{-1}, 1120.54 cm^{-1}, 1421.21 cm^{-1}, 1637.38 cm^{-1}, 2074.67 cm^{-1}, and 3439.67 cm^{-1}, indicating the presence of functional groups in the plant extract. The FTIR stretching frequency around 3439.67 cm^{-1} is attributed to the O–H group. The stretching frequency around 1088.57 cm-1 and 1120.54 cm^{-1} is attributed to the C–O and C=C groups, respectively. The bonding frequency at 1421.21 cm^{-1} indicates the C−H vibration. The presence of phenolic groups, ether linkage, and flavonoids are evident from these results.

The particle size and stability of the silver nanoparticles were evaluated through dynamic light scattering (DNS, Nano Zetasizer). The size ranges from 17 nm to 46 nm and the mean average size of the silver nanoparticles was found to be 32 nm (Figure 1C). DLS analysis emphasizes the size of the synthesized nanoparticles with an average size of 90.1nm with a polydispersity index (PI) of 0.389. Zeta potential was found to be -25.6mv (Figure 1D).

In vitro *Anti-candida* studies

Sphaeranthus amaranthoides synthesized silver nanoparticles exerted an effective antibiofilm effect against the *C.albicans* fungi. The control group shows the agglomerated biofilm formation and subsequent treatment with *S.amaranthoides* treated silver nanoparticles with 50 and 100 µg/mL diminished the biofilm formation by the *C.albicans* fungi (Figure 2A). From the results, it has been proven that biosynthesis of silver particles of *Sphaeranthus amaranthoides* possesses the efficacy of inhibiting the *Candida albicans* which is visualized through electron microscopy where the morphological changes in fungi cell were observed (Figure 2B). Bio quantification of biofilm inhibition using XTT assay showed the percentage of inhibition by 89.78%. Disc diffusion studies showed a zone of inhibition of 12 ± 0.15 mm at 100 µg/mL concentration (Figure 2C).

Autofluorescence

Autofluorescence can study the morphological and biochemical changes when treated with any drug or inhibitor. In the present study, the autofluorescence effect on *Candida albicans* treated with SaAgNPs exhibited different wavelength patterns respectively. The control group of *C.albicans* showed maximum absorption at 530nm which shows log phase fungal culture without any obstruction (Figure 3). Followed by it *C.albicans* cultures treated with *S.amaranthoides* nanoparticles of 25µg and 50 and 75µg showed gradually low absorption patterns. There is a significant growth reduction curve observed in the *S.amaranthoides* nanoparticles 100µg treated group which is higher than the standard amphotericin drug treated group. Flavins and flavoproteins likely contribute to the fungal autofluorescence that we observed around 530 nm. There is a decrease in the intensity of auto fluorescence when *Candida* treated with SaAgNP is observed.

Mechanistic studies:

The fungal cell membrane is made of exopolysaccharides with Glucan moiety and extracts cellular proteins. Antifungal drugs lyse the fungal cell membrane and proteins into simple sugars and amino acids. Based on this mechanism in the present experimental findings the SaAgNP reacts with the intact glucan moiety and proteins of the cell wall of *Candida albicans*. The mechanistic study of *C.albicans* treated with SaAgNP's shows that the sugar concentration increases in the culture medium with increasing concentration of SaAgNP's (Figure 4A). Eventually, the protein concentration tends to reduce with increasing dose concentration of SaAgNP's (Figure 4B). These reciprocating mechanistic results suggest that SaAgNP interacts with fungal cell walls thereby bringing the lysis of *C.albicans*.

Kinetic Studies

Hanes-Woolf model was plotted by taking substrate concentration on the X-axis and [s]/[v] on the Y-axis. From the graph it was observed the Vmax of SAP activity was found to be 0.062 µM/mg/min and Km found to be 0.18 µM (Figure 5). When SaAgNP

was treated with the culture of *Candida albicans* we did not observe any values. It showed that the SaAgNP can act on virulence factors of *Candida albicans* thereby destroying it.

Discussion

This result indicated the biotransformation of Ag^{+} to Ag^{0}. It was reported that a broad range of characterization peaks (200-500 nm) for silver nanoparticles synthesized from the medicinal plant *Ocimum Sanctum* leaf extract [20]. Similarly [21], has reported a characterization peak of silver nanoparticles synthesized from the ethanolic extract of the traditional medicinal plant *Pelargonium sidoides* at 456 nm. Furthermore, morphological studies through SEM analysis revealed the shape of synthesized silver nanoparticles with a spherical shape as the predominant one. The amorphous nature of the nanoparticles was revealed through SAED. A study by [22] reported the spherical shape of the green synthesized silver nanoparticles from the medicinal plant *Acanthospermum australe* with an average size of 14±2 nm. Strikingly, a study by [23] revealed the approximate size of silver nanoparticles from the extracts of wild and tissue-cultured *Ceropegia juncea* as 3–32 μm and 8.28–13.82 μm, respectively. The presence of phenolic groups, ether linkage, and flavonoids is evident from these results [24,25]. Thus, the biomolecules from the medicinal plant extract could have served as a reducing agent to form silver nanoparticles. DLS analysis emphasizes the size of the synthesized nanoparticles. Therefore, the silver nanoparticles synthesized by biogenic methods were examined for DLS to examine the size of the fabricated silver nanoparticles. Typically, the Polydispersity Index measures the heterogeneity of the samples based on the size of the particles. Here, the lower PI value suggests a lower rate of agglomeration or aggregation of silver nanoparticles. Thus, the lower values of PI indicate the higher stability of silver nanoparticles [26]. The electric charge on the surface of nanoparticles can be measured in terms of Zeta potential. Generally, the zeta potential of silver nanoparticles should have a value of higher than +30mv or lesser than -30mv. The negative potential values confirm the negative charge on the surface of colloidal nanoparticles and columbic repulsion forces induced by surface negative charge minimize the aggregation and thus contribute to the stability of nanoparticles synthesized.

In-vitro, evaluation of ant biofilm efficacy through CV assay revealed the antibiofilm potential of silver nanoparticles synthesized from the medicinal plant extract. The CV absorbance values of silver nanoparticles treated samples were found to be reduced in comparison to untreated samples, indicating the hindrance activity of silver nanoparticles in biofilm development. Silver nanoparticles at 100 μg displayed maximum biofilm inhibitory concentration (89.78%) against *C. albicans*. The results were further corroborated through light microscopic analysis. In which silver nanoparticles treated slides displayed a lesser number of cells adhered to the surface in comparison to untreated slides which have a cluster of biofilm cells. The significant reduction in the adherence property implies the antibiofilm efficacy of synthesized silver nanoparticles. Also, silver nanoparticles inhibited the biofilm formation at the

early stages of *C. albicans* biofilm. Generally, *C. albicans* biofilms have different stages starting from adherence, yeast cell colonization, followed by EPS production, maturation, and finally dispersion. Remarkably, during yeast cell formation it has been reported to switch their morphology from yeast cell to hyphal development and elongation. This morphological switching is associated with invasive infection in *C. albicans* [27]. Some earlier reports have recorded the antibiofilm efficacy of silver colloidal nanoparticles against *C. albicans* and *C. glabrata* biofilms [28]. In which the authors have treated preformed *Candida* biofilms (formed on denture acrylic) with colloidal silver nanoparticle suspensions at 54 mg/L. Similarly, [29] have reported antibiofilm activity of silver nanoparticles biosynthesized using *Streptomyces griseorubens* against *C. albicans* and bacterial species. Likewise, [30] have reported antibiofilm activity against *Candida* and bacterial species using the silver nanoparticles biosynthesized using the leaf extract of *Terminalia catappa*. Overall, the *in vitro* assay revealed the antibiofilm potency of silver nanoparticles in biofilm development.

Autofluorescence in fungi is commonly due to native fluorophores because of flavins which emit around 530 nm with an excitation of 450 nm. Flavin molecules are associated with the plasma membrane and also intracellular/granules and mitochondria of *Candida* [31]. From the results, it was evident that Sa AgNP extract acts on the cell wall of *Candida albicans* thereby damaging the integrity of the cell wall. Earlier studies report that plant extracts like *Syzygium jambolanum, Cassia siamea, Odina wodier, Momordica charantia,* and *Melia azedarach* exhibited prominent anti-*Candidal* effect against *Candida glabrata* and *Candida tropicalis* [32]. Similarly, medicinal plant extracts of *Lawsania inermis*, and *Portulaca oleracea* exhibited significant anti-*Candidal* effects with a MIC value of ~10 µg/mL [33]. A traditional herb of India *Rosa centifolia* and *Curcuma longa* glycolic extracts were effective against *C. albicans*, *C. dubliniensis,* and *C. tropicalis* biofilms over different periods [34]. Based on these results further studies were carried out to study the cell wall components of *Candida albicans*. *C.albicans* is of immense importance to understanding the actual mechanism of infection leading to probable prevention or treatment of the disease [35]. This plays an important pathogenic factor in invading and colonizing the host tissue.

Conclusion:

In the present study, *S. amaranthoides* plant leaf-mediated green synthesis of silver nanoparticles and its anti-candida efficacy were scrutinized by an in-vitro method. The antifungal effect of SaAgNP is significant at the concentration of 100µcg/mL compared to that of the standard drug Amphotericin. The biofilm inhibition properties of the SaAgNP are also promising enough that prevent the agglomeration of *C.albicans* fungal growth. The physical characterization studies of SaAgNP through FTIR and Zetane potential analysis reveal that the SaAgNP is stable. Further application-oriented research aspects of these SaAgNP in terms of cellular level and animal model toxicity will enhance the biomedical efficacy of the SaAgNP which can be applied for antimicrobial treatment of *C.albicans* especially in dental applications.

Author's Contributions: XXX, XXX, XXX: Conceptualization, Methodology, Software, Validation, Formal Analysis, and Investigation. **XXX, XXX**: Resources, Data Curation, Writing - Original Draft, Writing - Review and Editing. **XXX:** Visualization, Supervision, Project Administration, Funding Acquisition.

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Figure captions:
Figure 0: Graphical Abstract

Figure 1: A) UV Surface Plasma Resonance Spectroscopic pattern of SaAgNPs; B) FTIR spectroscopy of SaAgNPs; C) Zetane Potential analysis of SaAgNPs; D) DLS analysis of SaAgNPs.

Figure 2: A) Biofilm inhibition pattern of SaAgNPs; B) SEM image of Anti-*candida* effect of SaAgNPs; C) Antimicrobial Zone of Inhibition of SaAgNPs against *C.albicans*.

Figure 3: Autofluorescence anti-*candida* effect of SaAgNPs.

Figure 4: Cell membrane mechanistic study of SaAgNPs against *C.albicans*.

Figure 5: Hanes-Woolf plot Kinetic study of SaAgNPs against *C.albicans*.