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Spatoglossum asperum J. Agardh mediated synthesis of silver nanoparticles, characterization and evaluation antifungal activities

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Abstract
We explored the possible use of the aqueous extract of marine brown alga *Spatoglossum asperum* as the medium for the synthesis of silver nanoparticles (AgNPs) and evaluated their antifungal activity. The silver nanoparticles synthesized using the algal extract were characterized by means of UV-Vis spectroscopy, FT-IR, XRD and HR-TEM. The antifungal activity against *Aspergillus flavus*, *Candida albicans*, *Candida tropicalis*, *Trichophyton mentagrophytes* were investigated and the results compared with standard Flucanazole as a control. The synthesized AgNPs exhibited a potent antifungal activity against tested fungal strains. The results of this study indicated that AgNPs have remarkable potential as an antifungal agent in treating infectious diseases.

Keywords: Silver nano particles; marine algae; *Spatoglossum asperum*; antifungal activity

Introduction
Nanoparticles are considered to be the fundamental building blocks of nanotechnology [1]. Nanoparticles obtained from plants can successfully replace chemical reduction processes and are considered to be eco-friendly; the plants sources are safe to handle and possess a wide spectrum of metabolites that may aid reduction [2-6]. The utilisation of plant extracts for the nanoparticle synthesis would be the sustainable alternative for environmentally benign green nanotechnology, and this methodology could be more beneficial over other biological processes as it eliminates the elaborate process of maintaining cell culture [3].
Silver nanoparticles have also been synthesized using microorganisms, which have the capacity to reduce metal ions via resistance and detoxification mechanisms [6]. Silver nanoparticles, owing to their powerful bioactivity against bacteria, fungi, protozoa and viruses, are considered to be the most promising of any antimicrobial agent [1].
The use of marine natural products in the synthesis of metallic nanoparticles has the wide range of applications in the field of nanotechnology. Marine macroalgae having capacity to serve as a effective metal-reducing and capping agents to provide a robust coating on the metal nanoparticles in a single step because of the presence of active metabolites such as flavonoids, alkaloids, steroids, phenols, polysaccharides, saponins that are having hydroxyl, carboxyl, and amino functional groups [7-11]. The present study intended to synthesise, characterize and the evaluation of antifungal activity of the nanoparticles from marine brown alga *Spatoglossum asperum* J. Agardh.

Experimental
Collection and preparation of algal material: The marine brown alga *Spatoglossum asperum* J. Agardh was collected from 2.5 meters depth in the rapid Island, Gulf of Mannar, a Mandapam Coastal area in South India. Collected brown seaweed was washed with sea water to remove the epiphytes and sand particles. After dried, 1 g of fresh materials was cut into small pieces; grind with 50 mL of distilled water with mortar and pestle and these extracts were boiled for 5 min. The boiled extract was filtered through Whatman No.1 filter paper and the supernatant was used and stored at 4°C for further process.

Characterization of green synthesized *Spatoglossum asperum* silver nanoparticles
UV–Visible spectrometric analysis of silver nanoparticles: The UV−visible spectra of the synthesized silver nanoparticles were recorded as a function of wavelength using a UV−Vis spectrophotometer (UV-3000 PC spectrometer) operated at a resolution of 0.5 nm. The reduction of silver was measured periodically at 300–700 nm. A spectrum of silver nanoparticles was plotted with the wavelength on the *x*-axis and absorbance on the *y*-axis**.**

Fourier transform infrared (FTIR) analysis of silver nanoparticles: FTIR measurements of the silver nanoparticles were carried out to identify the major functional groups of the synthesized compounds. FTIR measurements were carried out using a JASCO FT-IR 4100 by employing the KBr disc technique. The FTIR spectra were collected from 50 scans at a resolution of 4 cm^{-1} in the transmission mode (4000–440 cm^{-1}).

Transmission electron microscopy (TEM): The TEM technique was used to visualize the morphology of the synthesized silver nanoparticles. The TEM micrographs were obtained using a Zeiss LIBRA®120 TEM operating at 80 kV. A drop of the silver nanoparticles in methanol was loaded on a carbon-coated copper grid, allowed to dry at room temperature and later analyzed.

X-ray diffraction analysis of silver nanoparticles: The x-ray diffraction of the nanoparticles was recorded on a Brucker D8 Advanced, equipped with a proportional Cu-Kα radiation counter (λ = 1.5405 Å, nickel filter) operated at a voltage of 45 kV and a current of 30 mA.

Antifungal activities: Pure culture of *Aspergillus flavus* (ATCC 20048), *Candida albicans* (ATCC 20408), *Candida tropicalis* (ATCC 2090), *Trichophyton mentagrophytes* (ATCC 28185) were purchased from M/S LGC Promochem India Pvt. Ltd, Bangalore, India. Stock cultures were prepared and maintained in Sabouraud Dextrose Agar (SDA) slants at 4 °C. Prior to the experiment, SDA broth was prepared, inoculated and incubated with pure culture at 25 °C for 3 days. A disc diffusion method was adopted to evaluate the antifungal activity of Ag nanoparticles [12] (Perez *et al*., 1990). About 150 CFU/mL of innoculum was swabbed onto SDA plates uniformly and allowed it to dry in a sterile environment. A sterile disc of 6 mm (HIMEDIA) was loaded with 30 µL of test solution (Seaweed extract, 1 mM $AgNO_3$ and AgNPs were incubated at 25 °C for 2-3 days to measure zone of inhibition. The mean was calculated by performing the experiments in triplicates.

Determination of MIC and MFC of silver nano particles: The Minimum Inhibitory Concentration (MIC) for antifungal activity was determined by using *C. albicans*. The green synthesized silver nano particles of *S. asperum* showed more activity against *C. albicans* among the tested dermatophytes. The MIC was 20 µg/mL, while the Minimum Fungicidal Concentration (MFC) was 30 µg/mL, when compared with standard antibiotic Flucanazole, which showed MIC at 12.5 µg/mL and MFC at 25 µg/mL. From the above results, it can be concluded that the silver nano particles synthesized by the brown alga *S. asperum* seems to be more potent against the Gram negative bacteria and the dermatophytic fungi.

Statistical analysis: The data were analyzed by using the one way ANOVA with equal sample size by using SPSS 17.0. The difference was considered significant when $p<0.005$. All the values were expressed as mean ± standard deviation (S.D). Triplicate assays were performed for each set of test conditions.

Results and Discussion

Characterization of silver nano particles: The reduction of silver ions to silver nanoparticles by exposing the silver nitrate to seaweed extract was tracked by monitoring the changes in the color with UV/Vis spectroscopy. *S. asperum* extract showed a color change from brown to reddish-yellow, this may be due to the excitation of surface plasmon vibrations and it provides a convenient spectroscopic signature to indicate the formation of silver nano particles (Fig. 1). The strong surface plasmon resonance centred at 420 nm clearly indicated an increase in intensity with time and stability after 2 h of reaction (Fig. 2). The metal particles were observed to be stable in solution on long term storage.

(A) **(B)**

Fig 1: The aqueous extract of *S. asperum* (A) before (B) after synthesis of AgNPs.

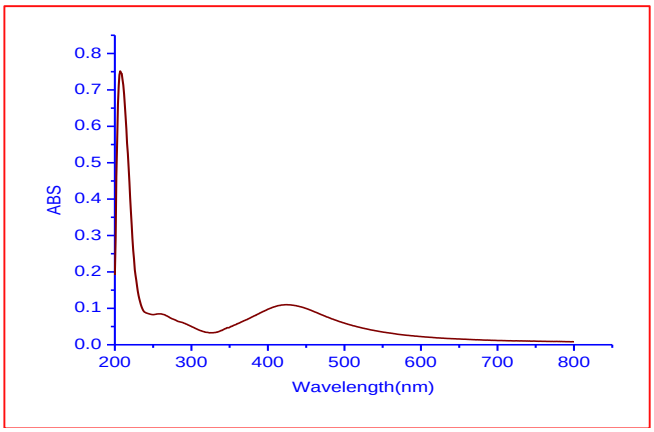

Fig 2: UV Spectrum of AgNPs using aqueous extract of *S. asperum*

FT-IR spectrum was used for confirming the presence of chemical functional groups present other than silver nano particles. The FT-IR peaks seen at about 3467 cm^{-1} (N-H stretching, primary amines), 2426 cm^{-1} (N-H stretching of amino ions), 2277 cm^{-1} (-N=C=O (aliphatic cynanide/nitrile), 1694 cm^{-1} (C=O stretch amides), 1468 cm^{-1} (-C–H stretching of alkyl), 1384 cm^{-1} (-CH_3 bending vibrations alkyl), 1057 cm^{-1} (S=O stretch sulfone), 1015 cm^{-1} (C=S stretch thiocarbonyl), 848 cm^{-1} (S-OR stretch esters), and 647 and 603 cm^{-1} (C-H bending deformation) respectively (Fig. 3). The results revealed that the capping ligand of the AgNPs may be an aliphatic compound containing amino, sulfo and amide groups. The biological molecules such as secondary metabolites could possibly play a major role in the synthesis and stabilization of the metal nano particles was proved [7, 13].

High Resolution Transmission Electron Microscopy provided further insight into the morphology and size details of the silver nano particles. TEM images clearly revealed that the nano particles are spherical shaped (Fig. 4). The majority of the nano particles observed from the micrograph are spherical with a small percentage of elongated particles ranged in size of 1050 nm. The average mean size of silver nano particle was ~ 28.8 nm. Control of the size and structure of the resultant nanoparticles can be related to the interactions between bio-compounds such as polysaccharides, proteins, polyphenols and phenolic compounds and metal atoms [14].

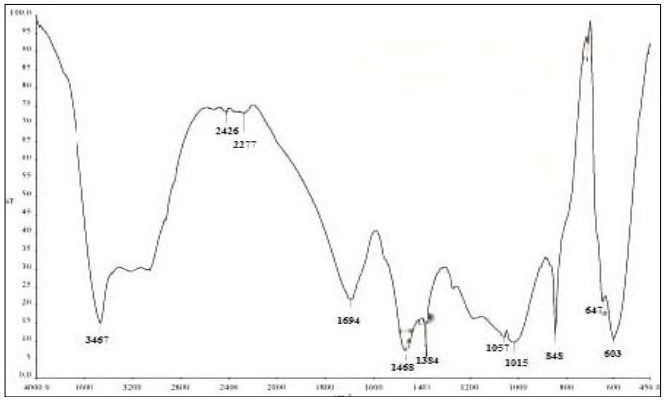

Fig 3: FT-IR spectrum of *S. asperum* mediated synthesized silver nanoparticles

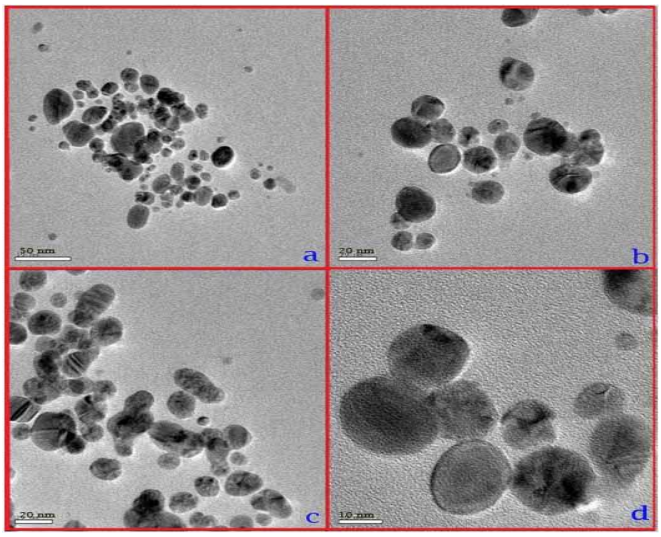

Fig 4: HR-TEM images of the silver nano particles synthesized by using *S. asperum.* a) 50 nm (b) 20 nm (c) 20 nm (d) 10 nm.

Fig. 5 shows the XRD patterns of silver nanoparticles synthesized using marine brown alga, *S. asperum*. A number of Bragg reflections with 2θ values of 11.83°, 29.39°, 44.13° and 64.30° sets of lattice planes are observed which may be indexed to the {1 0 0},{1 1 0}, {2 0 0}, and {2 2 0} facets of silver respectively.

X–ray diffraction pattern thus clearly illustrates that the silver nanoparticles formed in this present synthesis are crystalline in nature. The metallic silver nano-crystals showed typically optical absorption peak approximately at the 3 KeV due to surface plasmon resonance [15].

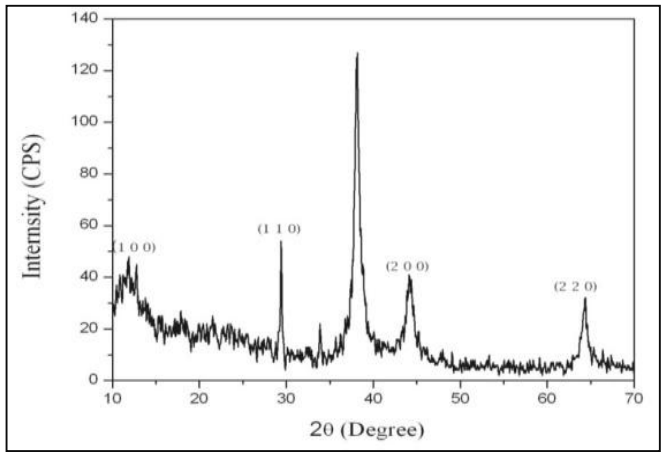

Fig 5: X-ray diffraction pattern of silver nano particles using *S. asperum.*

Antifungal activities of biosynthesized silver nano particles The antifungal activity of the silver nano particles synthesized from *S. asperum* was compared with that of standard antibiotic Flucanazole. The fungal cultures of dermatophytes *Candida albicans, Candida tropicalis, Trichophyton mentagrophytes* and non dermatophytes *Aspergillus flavus* were tested (Table. 1; Fig. 6).

The biosynthesized silver nano particles were found highly active against all the dermatophytic pathogens at a concentration of 100 µg/mL. The results that higher activity against *C. albicans* (20.67 ± 0.88 mm), whereas, a moderate activity against *C. tropicalis* 17.67 ± 0.33 mm and *T. mentagrophytes* (17.33 ± 0.88 mm), when compared with standard antibiotic. But in the case of a non dermatophytic fungus *A. flavus* (12.67 ± 0.33 mm), whereas, the standard drug and green synthesized AgNPs showed lesser activity. The zone of inhibition clearly showed that the fungal strains tested were susceptible to silver nano particles.

Table 1: Antifungal activities of silver nano particles synthesized from *S. asperum*

S. No	Name of the microorganisms	Zone of inhibition (mm)				
		25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL	Flucanazole
1	*Candida albicans*	12.67 ± 0.33 (50.68%)	14.67 ± 0.33 (58.68%)	17.67 ± 0.88 (70.68%)	20.67 ± 0.88 (82.68%)	25.00 ± 1.00 (100%)
2	*Candida tropicalis*	10.33 ± 0.66 (37.79%)	12.67 ± 0.33 (46.35%)	14.33 ± 0.88 (52.43%)	17.67 ± 0.33 (64.65%)	27.33 ± 0.66 (100%)
3	*Trichophyton mentagrophytes*	10.67 ± 0.33 (45.73%)	13.67 ± 0.33 (58.59%)	15.33 ± 0.33 (65.70%)	17.33 ± 0.88 (74.28%)	23.33 ± 0.66 (100%)
4	*Aspergillus flavus*	-	8.00 ± 1.00 (57.14%)	10.00 ± 0.57 (71.42%)	12.67 ± 0.33 (90.50%)	14.00 ± 1.00 (100%)

Values are expressed as Mean ± SEM, n=3

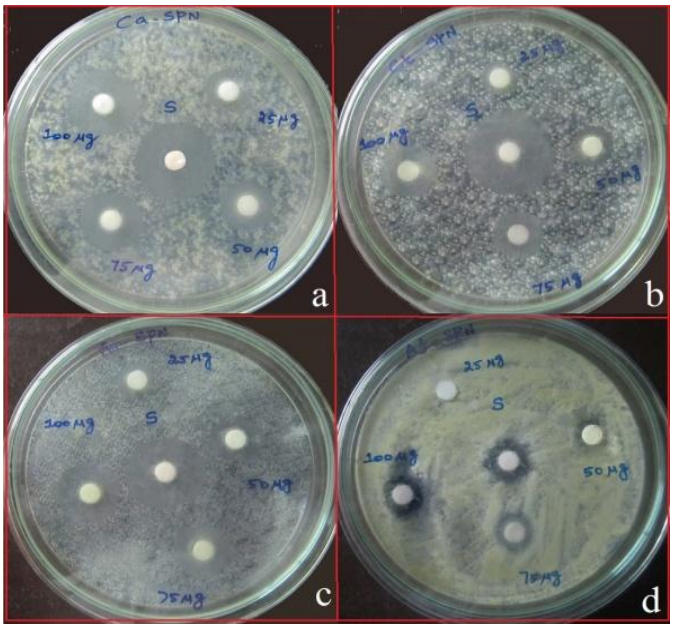

Fig 6: Antifungal activities of various concentrations with silver nano particles of *S. asperum.* **a)** *Candida albicans* **b)** *Candida tropicalis* **c)** *Trichophyton mentagrophytes* **d)** *Aspergillus flavus*

Moreover, the silver nano particles showed the highest antifungal efficacy. But, the exact possible inhibitory mechanism displayed by the silver nano particles is unclear and requires further investigation. It is a well-known fact that the antimicrobial activity of silver nano particles is likely to be well correlated with its decreased size and shape owing to an increased surface area with enhanced antimicrobial effect [8, 16].

Recently, Hwang *et al.,* (2012) have reported that an increase in the hydroxyl radicals by silver nano particles causes apoptotic cell death in *Candida albicans* [17] Altogether, the fungal cells when exposed to silver nano particle solution results in the formation of 'pits' on the surface of membrane resulting in the destruction of its cellular integrity [18].

The AgNPs exhibited high antimicrobial activity and this property can be very useful, especially against microorganisms resistant to conventional antimicrobials [19]. *C. albicans* and *C. tropicalis* showed high sensitivity to AgNPs. Smilarly, Panácek *et al.,* (2009) and Kumar *et al.,* (2013) have highlighted the antifungal activity of AgNPs against *Candida* sp., The evaluation of antibiotic resistant pathogenic fungi has stimulated the search for an effective antifungal agent from alternative sources [20, 21]. Many studies have shown the antifungal effects of silver nano particles [20, 22, 23]. However, only limited literatures supports the effects of AgNPs against fungal pathogens.

The primary significance of this study is the observation that Nano-Silver can inhibit the growth of dermatophytes, which cause superficial fungal infections. Secondly, the fact that the preparation method of nano-silver described here is cost-effective is also important. Recently, due to the emergence of antibiotic-resistant fungi and limitations of the use of antibiotics, clinicians have returned to using silver wound dressings, containing varying levels of silver. For these reasons, the antifungal activity and its mechanism of silver nanoparticles specifically, were investigated [8].

Conclusion

The present study demonstrated that the rapid synthesis of Ag nano particles using marine brown alga *S. asperum* for its antifungal activity. The presence of functional bioactive compounds in seaweed extract is responsible for the formation of Ag nano particles as revealed by FTIR. The average size of spherical-shaped Ag nano particles ranges between 5 and 50 nm. This work also demonstrates the use of Ag nano particles as a potential antifungal agent against *C. albicans.*

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