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Research Article

GREEN ALLOY OF SILVER NANOPARTICLES FROM ENDOPHYTIC EXTRACTS OF *WITHANIA SOMNIFERA* AND STUDIES OF ANTIBACTERIAL AND ANTIMITOTIC ACTIVITY

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ABSTRACT

Objectives: The main aim is to elaborate a cost-effective and environmentally friendly synthesis of silver nanoparticles (AgNPs) by endophytic extracts isolated from *Withania somnifera* as a reducing and capping agent, which has proven antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella* sp.

Methods: Characterization of AgNPs was carried out employing ultraviolet-visible spectrophotometry, scanning electron microscopy (SEM), and X-ray diffraction studies (XRD). Antibacterial activity of AgNPs was conducted by disc diffusion method antimitotic activity was also evaluated by determining mitotic index in *Allium cepa* root tips.

Results: Ultraviolet-visible spectroscopy was given a peak at 400 nm confirmed the AgNPs. The images of the SEM have confirmed the formation of AgNPs with an average size of 40 nm. XRD results were remarkable in confirmation of synthesized AgNPs with distinct XRD peaks at 2θ values of 38, 44, 64, and 77 lattice planes were observed which indexed the facts of silver (111), (200), (220), and (311), respectively. The AgNPs showed effective antibacterial activity against tested microorganisms at 100 µg/discs concentrations. A significant mitotic index (22.8 ± 1.4^{b} and 26.9 ± 0.9^{b}) was observed in *A. cepa* root tips at 10 mg/ml, 5 mg/ml concentration, respectively.

Conclusion: It can be concluded that the endophytes of *W. somnifera* can be a good source for AgNP synthesis and showed a significant antimicrobial activity against tested microorganisms, especially *E. coli* followed by *S. aureus* and *P. aeruginosa.* Suggestive results were found in antimitotic activity which one of screening methods for development of anticancer drugs. An important outcome of our study will be the extension of value-added products for the industries of biomedical and nanotechnology based.

Keywords: Silver nanoparticles, Antibacterial, Antimitotic.

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INTRODUCTION

Endophytes may be bacteria or fungal organisms colonizing inter- or intra-cellular inside the tissues of host plants, causing no discernible symptoms of disease to plants [1]. These endophytes will be having the same characteristics of the plants in which it fosters [2]. Endophytic fungi are an unexplored group of organisms with abundant biodiversity and present in most of the plant parts especially in the tissues of evidently healthy leaf, with expansive potentials for imminent pharmaceutical substances [3,4].

Biological synthesis of silver nanoparticles (AgNPs) with these endophytes is plain sailing and eco-friendly. Nanotechnology is a luxuriate and blossomed the human life in the field of physical, chemical, and biological sciences and is mainly concerned with the synthesis of nanoparticles of different sizes, chemical composition, shapes [5,6] and their potential uses for the welfare of human kind [7]. Nowadays nanoparticles are of appreciable importance with the mechanism of action from that of antibiotics, easy proliferation into cells with limited side effects, thus rendering safe, cost effective [8] and economical to combat the future challenges. Biological synthesis of nanoparticles is gaining attention compared to the chemical and physical method of synthesis [9]. Nanoparticles synthesized with these endophytes shows distinguished results in antitumor [10], antibacterial [11], antibiotic [12], antiseptic [13], antioxidant [14], anti-inflammatory [15], antifungal [16], antiapoptosis [17], etc.

Withania somnifera is a member of family *Solanaceae* and leaves contain active constituents mainly withanine, somniferine, somnine, somniferinine, withananine, pseudo-withanine tropane, pseudotropine, choline, anaferine, anahydrine, and isopelletierine [18]. Leaf juice encompasses anti-inflammatory, antitumor, antistress, antioxidant, mind-boosting, immune-enhancing, and rejuvenating properties [19]. The roots of *W. somnifera* are employed to treat constipation, rheumatism, loss of memory, and neurodegenerative disorders and people have a tradition of use of this plant as a medicinal agent [20,21].

Since many of the bioactive molecules are present in *W. somnifera* as reported earlier by many authors, in the present investigation we have focused on the isolation of endophytic fungi from *W. somnifera* leaves, preparation of endophytic extracts and its phytochemical analysis, synthesis of AgNPs using endophytic extracts, characterization of AgNPs, antibacterial and antimitotic assay which is not yet reported for the AgNPs.

METHODS

Isolation of endophytic fungi from *W. somnifera*

Fresh leaves of *W. somnifera* were collected from village Dibbur, Tumkur, Karnataka, India, using sterile polythene bags and authenticated by the Department of Botany, Tumkur University, Tumakuru, Karnataka, India. These were incised into 0.5 mm pieces and surface sterilized for 1 min using 0.01% mercuric chloride solution followed by washing

with sterile distilled water and transferred into a potato dextrose agar medium and incubated at 30°C for 7 days. The growth of endophytic fungi and subcultures was maintained in potato dextrose broth.

Preparation of endophytic (*Aspergillus* sp.) extracts

After 7 days of incubation, the fungal mat was harvested and crushed using pestle and mortar and filtered using Whatman filter paper no. 1. The collected filtrate was centrifuged at 10,000 rpm for 15 min, and supernatant was stored for further process.

Synthesis of AgNPs by endophytic *(Aspergillus* sp.) extracts

About 90 ml of aqueous 5 mM silver nitrate (Merck) was added to 10 ml endophytic extracts in the Erlenmeyer flask, followed by the incubation of 24 hrs at room temperature in the dark. The change of color from colorless to dark brown indicated the reduction of metallic silver from silver ions. The sample treated with silver nitrate was centrifuged at 10,000 rpm for 10 minutes using Remi centrifuge. Supernatant was ruled out, and unreacted silver nitrate and endophytic extracts were repeatedly washed thrice with double distilled water. The pure pellet was preserved for future experiments.

Characterization of AgNPs

Preliminary identification of AgNPs was confirmed using ultraviolet (UV)-visible spectrophotometer (Agilent Cary 60) at the wavelength between 300 and 700 nm. The X-ray diffraction (XRD) spectra of AgNPs were recorded by Rigaku – SmartLab X-ray diffractometer with monochromatism Cu-Kα radiation. The diffracted intensities were recorded for 2θ Angles from 10° to 80°. The size and the surface morphology of the synthesized AgNPs were studied by scanning electron microscope (Ultra 55 Model-II, Carl Zeiss SEM machine).

Antibacterial assay

Disc diffusion method was used to evaluate antibacterial activity of the synthesized AgNPs [22-24]. The antibacterial assay was carried out against multidrug-resistant bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Klebsiella* sp. collected from the Department of Microbiology, Shridevi Institute of Medical Sciences and Research Hospital, Tumakuru, Karnataka, India. Taxim was used as a standard antibiotic. 5 mm diameter sterile filter paper discs were impregnated with AgNPs and Taxim (100 µg/discs) and placed on agar plates that had been already inoculated with the microorganisms separately. The zone of inhibition was determined on incubation at 37°C for 24 hrs.

Antimitotic assay

To study the antimitotic activity of the synthesized AgNPs, 48 hrs *Allium cepa* root tips treated with synthesized nanoparticles, quercetin (10 mg/ml, 5 mg/ml; quercetin – 1 mg/ml), and distilled water were used. Root tips were fixed with 1:3 aceto-alcohol and squash was prepared by acetocarmine stain and was observed for cellular, nucleolus, and chromosomal abnormalities [25]. The mitotic indices were assigned to each root tip manually by scoring approximately 500 cells under bright field light microscopy of high resolution (×100 oil immersions), and mitotic phases of the cells were observed. Dividing cells include normal prophase, metaphase, anaphase, and telophase. The cells were examined for abnormalities such as chromosomal fragments, vagrant chromosomes, chromosomal bridge, chromosomal gaps, anaphase, multipolar anaphases, and telophases, and sticky chromosomes. The mitotic index was calculated using the formula:

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{total number of cells}} \times 100$$

RESULTS AND DISCUSSION

An endophytic fungus isolated from the leaf of *W. somnifera* was identified as *Aspergillus* sp. based on the colony growth, morphological characteristics and hyphae and was mass cultured for the synthesis of AgNPs and further studies (Fig. 1).

Synthesis of AgNPs

The fungal mat was used for the preparation of endophytic extract. The collected extract was treated with aqueous 5 mM silver nitrate solution [26]. The color change from pale yellow to dark brown indicates the reduction of silver ions (Fig. 2).

Characterization of AgNPs

For preliminary confirmation of AgNPs, UV-visible spectroscopy was used where the strong absorption peak at 400 nm (Fig. 3) confirmed the AgNPs [27] which then further confirmed by XRD and scanning electron microscopy (SEM). XRD results were remarkable in confirmation of synthesized AgNPs with distinct XRD peaks at 2θ values of 38°, 44°, 64°, and 77° lattice planes were observed which indexed the facts of

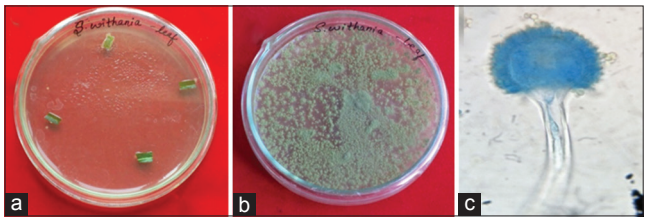

Fig. 1: (a) Inoculation of leaf on potato dextrose agar (PDA) medium. (b) Growth of endophytic fungus on the PDA medium after the incubation period of 7 days. (c) *Aspergillus* spp.

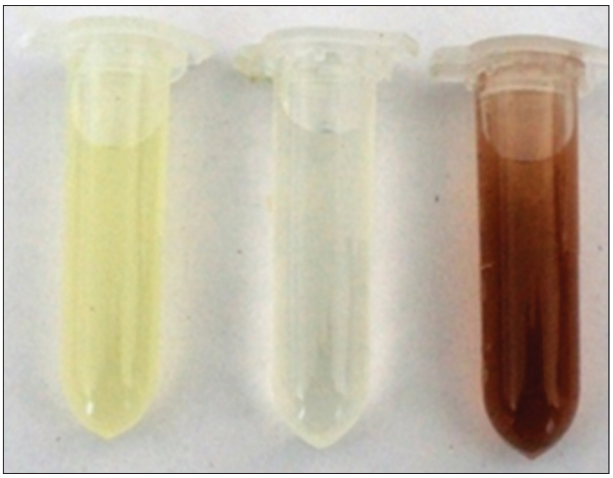

Fig. 2: Synthesis of silver nanoparticles by endophytic fungi (*Aspergillus* spp.) extracts

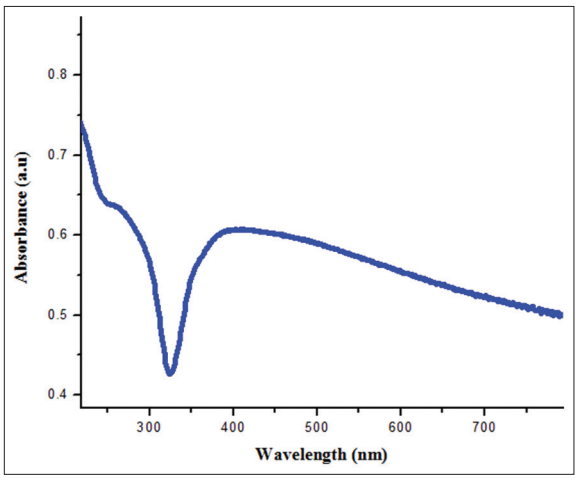

Fig. 3: Ultraviolet-visible spectra of silver nanoparticles synthesized by *Aspergillus extract* of *Withania somnifera*

Table 1: Antibacterial activity of silver nanoparticles synthesized by *Aspergillus* spp. extracts of *W. somnifera*

S.No.	Zone of inhibition of test organisms (mm)				
	Test organisms	Distilled water	Taxim	Silver nanoparticles	Endophyte extracts
1	*E. coli*	0	18±1.4[a]	25±1.0[a]	0
2	*P. aeruginosa*	0	16±1.2[b]	22±1.5[a]	0
3	*S. aureus*	0	15±1.6[b]	24±1.3[a]	0
4	*Klebsiella sp.*	0	17±0.9[b]	20±1.7[b]	0

[a,b]Values are denoted type 1 and 2 errors. Values were expressed as the means of three replicates±SD. Concentration of sample was 100 µg/ml. SD: Standard deviation, *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus, W. somnifera: Withania somnifera*

Table 2: Antimitotic activity of silver nanoparticles

S.No.	Sample	Concentration (mg/ml)	Mitotic index
1	Distilled water	-	96.3±1.1[a]
2	AgNPs	10.0	22.8±1.4[b]
3	AgNPs	5.0	26.9±0.9[b]
4	Quercetin (standard)	1.0	15.4±1.5[a]

[a,b]Values are denoted type 1 and 2 errors. Values were expressed as the means of three replicates±SD. Concentration of synthesized nanoparticles 10 mg/ml, 5 mg/ml, quercetin – 1 mg/ml and distilled water. SD: Standard deviation, AgNP: Silver nanoparticles

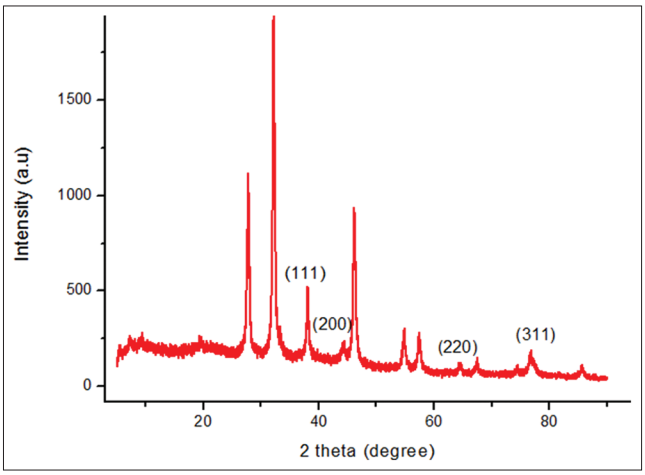

Fig. 4: X-ray diffraction pattern of silver nanoparticles

silver (111), (200), (220), and (311), respectively (Fig. 4) suggesting the biphasic nature of synthesized AgNPs [28], this provides strong evidence in favor of UV-visible spectra. The data were compared with the JCPDS file No. 4-783. The potential peak at 38° stipulated a high degree of crystallinity and which have been already outlined by other authors [29].

SEM revealed the average particle size of 40 nm (Fig. 5) which was calculated using Debye-Scherrer equation.

Antibacterial assay

A study of the antibacterial activities indicated that AgNPs synthesized by *Aspergillus* sp. displayed a greater zone of inhibition when compared with antibiotic Taxim and endophytic extracts (Table 1). A significant antibacterial activity of AgNPs synthesized by *W. somnifera* was reported against Gram-negative bacteria *P. aeruginosa*, *Klebsiella* species, and *E. coli* followed by Gram-positive bacteria *S. aureus* [30]. Many authors were already reported that many plants based AgNPs showed antibacterial activity against human pathogens [31]. Arsia reported that the *Clausena anisata* based AgNPs have shown a maximum zone of inhibition against *P. aeruginosa* followed by *S. aureus* and *E. coli* at 150 µg/ml [32]. AgNPs synthesized by *Carica papaya,* and *Simarouba glauca* exhibited significant antibacterial effect against *E. coli*, *P. aeruginosa*, and *S. aureus* [33,34]. However, our synthesized

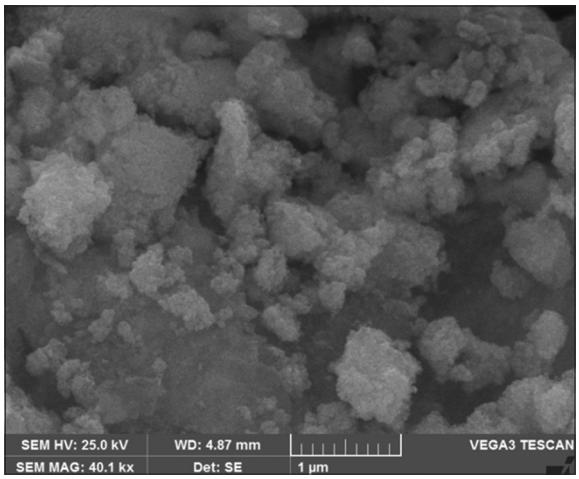

Fig. 5: Scanning electron microscopy images of silver nanoparticle

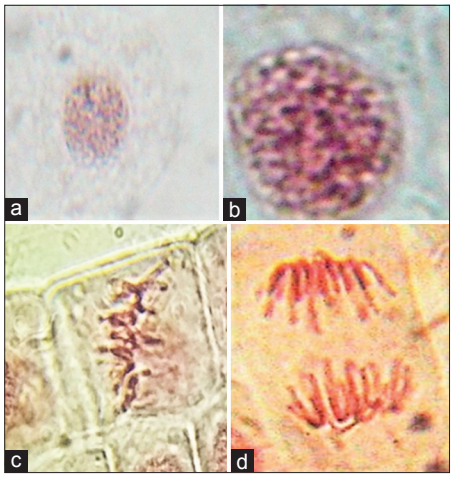

Fig. 6: Normal mitotic phases of *Allium cepa.* (a) Interphase, (b) prophase, (c) metaphase, and (d) anaphase

AgNP has shown a maximum zone of inhibition against *E. coli* followed by *S. aureus*, *P. aeruginosa,* and *Klebsiella* sp. at 100 µg/ml only.

Antimitotic assay

Outcomes of an assay conducted for AgNPs, quercetin and distilled water in different stages of the cell cycle are presented in Figs. 6 and 7 and Table 2. The meristem division of the *A. cepa* is similar to the cancer cell division, and hence we have selected the same for evaluation of anticancer activity of AgNPs [35]. The results of the present study further have opened a path for the development of novel therapy for cancer.

CONCLUSION

In this study, AgNPs were synthesized from the endophyte *Aspergillus* sp. of the leaf of *W. somnifera.* The synthesized nanoparticles

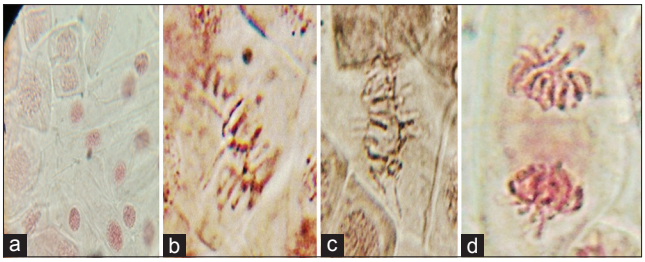

Fig. 7: Mitotic abnormalities of *Allium cepa.* (a) Mega cells and cell shrinkage, (b) chromosomal clumping at metaphase, (c) vagrant chromosome at anaphase, and (d) chromosomal clumping at telophase

with the average size of 40 nm, showed potentially consistent results in the *in vitro* studies such as antibacterial and antimitotic assays. Furthermore, the current method is eco-friendly and suitable for industrial scale production of biologically potent AgNPs.

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