Eco-friendly synthesized silver nanoparticles from endophytic fungus Phyllosticta owaniana – KUMBMDBT-32 and evaluation of biomedical properties

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Abstract

The present study focused on biosynthesis of silver nanoparticles from an endophytic fungus isolated from the medicinal plant *Abrus precatorius*. The endophytic fungus was identified as *Phyllosticta owaniana* KUMBMDBT-32 (NCBI Accession number MW007919). The fungus was cultured in submerged fermentation and extracted with ethyl acetate (1:1 V/V). The extract was subjected to GC-MS analysis which have revealed the presence of 42 bioactive compounds of secondary metabolites. Synthesized silver nanoparticles were characterized by Bio-spectrophotometer, FTIR, SEM-EDX, XRD and DLS. The absorption spectra in the Bio-spectrophotometer analysis showed a peak at 400 nm, confirmed the synthesis of silver nanoparticles. The FTIR study revealed peaks corresponding to various functional groups possibly involved, confirming the reducing and capping of silver nanoparticles. The spherical form of silver nanoparticles was revealed through SEM-EDAX studies, the EDAX study showed that silver atoms are present at 3keV. XRD studies revealed the crystalline structure of the silver nanoparticles. DLS study determined size of synthesized silver nanoparticles i.e., 65.81 nm. Antibacterial activity of synthesized silver nanoparticles was tested against pathogenic bacteria. *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhi* and *K. pneumoniae*. Antibiotic enhancing activity of silver nanoparticles was determined by using commercial antibiotics such as Chloramphenicol, Cefpodoxime, Gentamicin, Ampicillin, and Imipenem. Antifungal activity silver nanoparticles was determined against fungal pathogens such as *Candida albicans*, *Aspergillus brassiliensis*, and *Aspergillus flavus*. Synthesized silver nanoparticles exhibited effective antioxidant, measurement of cell viability by MTT assay and in-vitro and in-vivo anti-inflammatory activities. These results certainly determines pharmaceutical and biomedical importance of silver nanoparticles.

1. Introduction

Nanotechnology is a rapidly evolving branch of science concerned with the synthesis and evolution of various nanomaterials (Rahim et al. 2016). Nanoparticles are typically groups of atoms measuring 1–100 nm in size. Metal nanoparticles' properties are understood to be determined by their size, shape, composition, crystallinity, and structure (Guangquan et al. 2012).

In general, physical processes produce little output, but chemical methods cause environmental pollution due to the use of toxic solvents and the generation of harmful by-products. Bio nanotechnology has recently been researched as an alternative to chemical and physical ones. Bio nanotechnology research has shown that it can provide safe, eco-friendly procedures for the synthesis of noble nanomaterials. It has been claimed that nanoparticles can be produced biologically using a variety of biological systems such as yeast, bacteria, fungi, algae, and plants. When compared to other approaches, microbial biosynthesis of nanoparticles by microorganisms is faster, more effective, cheaper, and does not require the sharing of toxic materials. Fungi are more efficient than other naturally available biological resources, making them more ideal for the synthesis of nano-scale metal particles. For example, fungi produce a lot of protein, which contributes to the particle's high productivity and stability (Ajah et al. 2018; Zhao et al. 2017).

Since the dawn of time, scientists have been fascinated by silver. The combination of silver and nanoparticles has grown in popularity around the world because nanoparticles, unlike bulk materials, have distinct physical, chemical, and biological properties that make them suitable for a variety of applications in disciplines such as healthcare, electronics, and agriculture (Zhao et al. 2017).

In this research investigation, the biosynthesis of silver nanoparticles was achieved by utilizing the filtrate of the endophytic fungus *Phyllosticta owaniana* KUMBMDBT-32, which was isolated from the healthy segments of the *Abrus precatorius*. The bio-spectrophotometer, FTIR, SEM-EDX, XRD, and DLS have been used to analyze, the silver nanoparticles that were synthesised. Biomedical importance of the silver nanoparticles was evaluated by checking for antibiotic enhancing and antimicrobial activities against some important human pathogens, antioxidant, anticancer, in-vitro and in-vivo anti-inflammatory activity.

2. Materials And Methods

2.1. Isolation, identification and molecular characterization of endophytic fungus

Abrus precatorius medicinal plant was collected from Chitradurga district, Karnataka, India. Healthy stem was washed thoroughly under running tap water and surface sterilized by immersing it in 70% ethanol for 60 seconds, then sodium hypochlorite for 3 minutes, and finally 75% ethanol for 30 seconds. Sterilized samples were then rinsed three times with sterile distilled water and dried in laminar air flow (Singh et al. 2017). The plant samples were sliced into small pieces of roughly the same size and placed on potato dextrose agar (Himedia Pvt. Ltd. India) supplemented with streptomycin (30 mg/L, Himedia Pvt. Ltd. India) to prevent endophytic bacteria growth, plates were incubated at 28°C for 5 to 7 days. After incubation pure culture of endophytic fungi was obtained with continuous transfer of hyphal tips on new potato dextrose agar medium plates. The Endophytic fungus was identified microscopically characterized by Lactophenol cotton blue mounting technique. Extraction of genomic DNA from isolated fungus was carried out according to the manufacturer's instructions using a Qiagen kit (USA). PCR with the primer pair ITS 1 5'-CCGTAGGTGAACCTGCGG-3' and ITS 4 5'-TCCTCCGCTTATTGATATGC-3' (Devi et al. 2015). Gel extraction kit was used to purify the PCR products. The sequence was evaluated using NCBI, BLASTn, and a homology search for closely related sequences was used to identify fungus. MEGA-Version 7.0.14 was used to do multiple sequence alignment, and a phylogenetic tree was built using the neighbour-joining (NJ) method.

2.2. Extraction and Gas-Chromatography - mass spectroscopy analysis of Secondary Metabolites

The endophytic fungus *Phyllosticta owaniana* was mass cultivated in 1000 ml potato dextrose broth (PDB) for 21 days under static conditions at room temperature. The broth culture was filtered using Whatman No. 1 filter paper and the resulting filtrate was extracted with ethyl acetate as the solvent system (1:1). The extract was concentrated under decreased pressure and evaporated to dryness at 40°C using a rotating vacuum evaporator (Jayaram et al. 2021). The presence of secondary metabolites in the extract was determined using a GC-MS analyser (Shimadzu Qp 2010 plus). The extract's spectrum was compared to a database at the National Institute of Science and Technology (NIST) library.

2.3. Extracellular synthesis and characterization of silver nanoparticles

Under static circumstances, the endophytic fungus *Phyllosticta owaniana* was cultured in Potato Dextrose Broth (PDB) and incubated for 7 to 14 days at 28 ± 2°C. Biomass was filtered using Whatman filter paper no. 1 after incubation, and the filtrate was used to synthesize silver nanoparticles. 1 mM silver nitrate was added to an equal volume (1:1) of fungal filtrate and incubated for 24 hours in the dark at 28 ± 2°C. The synthesis of silver nanoparticles was confirmed by the absorbance spectrum in Bio-spectrophotometer after a 24-hour incubation period. The spectrum was recorded between 200 and 700 nm, the cell filtrate solution with silver nitrate solution had turned from light brown to dark brown, indicating the synthesis of silver nanoparticles. Synthesized silver nanoparticles were purified by centrifuging a solution of silver nanoparticles at 15,000 rpm for 15 min three times with a continuous washing of pellet with sterile distilled water. After drying the final pellet in a hot air oven at 50°C for 24 hours, the pure powder was obtained and silver nanoparticles was characterized by FTIR, SEM-EDX, XRD and DLS. The functional groups presumably involved in the production and stability of silver nanoparticles were investigated using FTIR analysis. The FTIR spectrum (Model Perkin-Elmer) was acquired with a resolution of 2 cm^{-1} in the range 500–4000 cm^{-1}. Scanning Electron Microscopy - Energy Dispersive Analysis of X-ray (SEM-EDAX) was used to investigate the surface shape and elemental composition of synthesized nanoparticles. Rapid diagnostic systems were studied using X-ray diffraction to examine the crystalline structure and cell characteristics. The Malvern Zeta Sizer Nano series compact scattering spectrometer was used to measure the size of the disseminated silver nanoparticles using the dynamic light scattering (DLS) technique (Natala et al. 2016).

2.4. Evaluation of antibiotic enhancing activity of synthesized silver nanoparticles

The disc diffusion approach was used to increase the antibacterial activity of produced silver nanoparticles from the endophytic fungus *Phyllosticta owaniana* in combination with some significant commercial antibiotics (Rahi et al. 2014). In this method, 10 microgram (mcg) concentration of standard antibiotic discs [(Chloramphenicol (CHL), Cefpodoxime (CPD), Gentamicin (GEN), Ampicillin (AMP), and Imipenem (IPM), Himedia Laboratories Pvt. Ltd. India)] were impregnated with synthesised silver nanoparticles and placed on Mueller-Hinton agar (MHA) medium plates pre-inoculated with bacterial test pathogens [*Escherichia coli* [MTCC-1599], *Staphylococcus aureus* [MTCC-4734], *Pseudomonas aeruginosa* [MTCC-1934], *Salmonella typhi* [MTCC-734], *and Klebesilla pneumoniae* [MTCC-7028]] and incubated at 37°C for 24 hours. Standard antibiotic discs were used as positive control, while Dimethyl Sulfoxide (DMSO) was used as a negative control. Following incubation, the formation of a zone of inhibition was observed, and the increase in zone area was calculated by calculating the mean surface area of each antibiotic's zone of inhibition (A) and antibiotic + silver nanoparticles' zone of inhibition (B). The fold increase area was determined using the formula (B2-A2)/ A2, where 'A' and 'B' were the antibiotic and antibiotic + silver nanoparticles zone of inhibition, respectively. In the absence of a zone of inhibition, the diameter of the antibiotic disc was believed to be 6 mm. The average standard deviation (SD) was obtained after the measurements were done in triplicate.

2.5. Evaluation of antibacterial activity of synthesized silver nanoparticles

The antibacterial activity of produced silver nanoparticles was tested using a agar well diffusion assay, modified version of the Bauer et al. 1966. Standard bacterial pathogens *Escherichia coli* [MTCC-1599], *Pseudomonas aeruginosa* [MTCC-1934], *Salmonella typhi* [MTCC-734], *Klebesilla pneumoniae* [MTCC-7028] *Staphylococcus aureus* [MTCC-4734] were used to test the antibacterial activity on Mueller-Hinton agar (MHA) medium, 24 hour old inoculated bacteria were uniformly spread and 6 mm wells were made using sterile borer, stock solutions of nanoparticles at different concentrations (10 mg/ml, 05 mg/ml, and 2.5 mg/ml) were added to wells. 1 mg/ml concentration Streptomycin and Ciprofloxacin (HiMedia laboratories Pvt. Ltd. India,) were used as standards and positive controls, Dimethyl Sulfoxide was used as negative control and incubated at 37°C for 24 hours, after incubation zone of inhibition was measured. The average ± standard deviation (SD) was computed after the observations were estimated in triplicates.

2.6. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) assay was determined using micro broth dilution method was performed according to the Clinical and Laboratory Standards Institute (CLSI 2015) Rani et al. 2017. Each well of the micro titre plate added 50 µl of each sterile Mueller Hinton broth. 4mg/ml solution of silver nanoparticles in DMSO (Dimethyl Sulfoxide) and standard antibiotics, the first row of the micro titre plate was treated with streptomycin, ciprofloxacin, and Chloramphenical, followed by serial dilution across. 100 µl of tested bacterial pathogens such as *Escherichia coli* [MTCC-1599], *Staphylococcus aureus* [MTCC-4734], *Pseudomonas aeruginosa* [MTCC-1934], *Salmonella typhi* [MTCC-734], *Klebesilla pneumoniae* [MTCC-7028] inoculums (approximately 1.5×10^8 CFU/ml) were added to each well. A positive control (tested bacterial pathogens) was used, while Mueller Hinton broth (MHB) was used as a negative control. Plates were wrapped in cling film to prevent bacteria dehydration and incubated at 37°C for 24 hours. To avoid errors, experiments were carried out in triplicate. Following incubation, 10 µl of alamar blue dye (resazurin) was added to each well and the plate was incubated in the dark for 3 hours.

2.7. Evaluation of antifungal activity of synthesized silver nanoparticles

The antifungal efficacy of synthesized silver nanoparticles was investigated using the agar well diffusion method (Soni et al. 2014), employing test fungal pathogens such as *Candida albicans* [MTCC-1637], *Aspergillus brassiliensis* [MTCC-1344], and *Aspergillus flavus* [MTCC-9606]. The test fungal culture spore suspension was swab inoculated on sterile (SDA) Sabouraud Dextrose Agar (Himedia Laboratories Pvt. Ltd. India) medium. The wells were made using a sterile borer on Sabouraud Dextrose Agar medium plates and loaded with different concentrations (10 mg/ml and 5 mg/ml), standard antibiotics (Fluconazole, 1 mg/ml) were used as a positive control, and DMSO as negative control was used under aseptic conditions. The formation of a zone of inhibition was noticed after being incubated upright for 2–3 days at 27°C. The average ± standard deviation (SD) was obtained after the measurement was repeated three times.

2.8. Evaluation of *in vitro* antioxidant activity of synthesized silver nanoparticles

2.8.1. DPPH radical scavenging activity

The DPPH radical scavenging assay was used to investigate the free radical scavenging activity of biosynthesized silver nanoparticles in vitro (Rhaman et al. 2015). Various quantities of sample solution (100–500 g/ml) were mixed to 140 microliters of an ethanolic solution containing 0.1mM DPPH. The tubes were incubated at 37°C for 5 minutes in the dark. The absorbance was measured against a blank at 517 nm. L-ascorbic acid is used as a standard. The radical scavenging activity of the endophytic fungus *Phyllosticta owaniana* synthesised silver nanoparticles, given as a percentage of inhibition, was evaluated using the equation below. Each value was presented as a mean standard error.

$$\text{Free radical scavenging activity (\%)} = \left[\frac{\text{absorbance control - absorbance test}}{\text{absorbance control}} \times 100 \right]$$

2.8.2. ABTS radical cation decolorization assay

The ABTS radical cation decolorization experiment, according to Sreeram et al. 2006., was a success. To generate an $ABTS^+$ solution, 7 mM ABTS was combined with 2.45 mM potassium persulfate in water (1:1). This reaction mixture was maintained at room temperature in a container for 16 to 20 hours. An $ABTS^+$ solution was generated with methanol as a diluting agent that had an absorbance of 0.7 at 734 nm. After 30 minutes, different concentrations of synthesized silver nanoparticles of endophytic fungus *Phyllosticta owaniana* (100 to 500 µg/ml) were added to make up to 4ml of ABTS + solution, and the absorbance at 734 nm was measured. The results, which were provided as percentages, were measured using percent scavenging of synthesized silver nanoparticles. The radical cation decolorization assay of the synthesized silver nanoparticles, expressed as a percentage of inhibition, was calculated using the equation below. Each value was presented as a mean standard error.

$$\text{ABTS radical of inhibition (\%)} = \frac{\text{absorbance control - absorbance test}}{\text{absorbance control}} \times 100$$

2.9. Measurement of cell viability by MTT assay

The MTT assay was used to determine the effect of silver nanoparticles synthesised by the endophytic fungus *Phyllosticta owaniana* on the viability of Human Heptaocellular adenocarcinoma cell line (HepG2) and Human renal (Kidney) adenocarcinoma cell line (A498). The cell lines HepG2 and A498 were procured from the National Centre for Cell Science in Pune, INDIA. The cell lines were grown arbitrary and maintained at 37°C in 5% CO2 in DMEM containing 10% foetal calf serum and 1% antibiotic solution containing penicillin and streptomycin until the monolayer was sub-confluent. The MTT reduction assay was analysed spectrophotometrically in accordance with the article (Giridasappa et al. 2021). The water-soluble tetrazolium will be converted by mitochondrial succinate dehydrogenase. When cell lines reached confluency, they were trypsinized, transfected, and transferred at a density of 30 × 103 cells in 100 µL volume per well in a 96 multiwell plate. The reaction plates were incubated in a CO2 incubator at 37°C for 24 hours. A partial monolayer is formed; cancer and fibrocystic cell lines were washed twice with 1×PBS pH 7.4 and replaced with a fresh medium containing various dilutions of prepared silver nanoparticles (12.5–200µg/ mL) and incubated for 24 hours at 37°C. The supernatant was then discarded, and 10 µL of MTT (5 mg/mL) was combined and cultured for 4 hours to allow the cells to absorb the dye. The media was replaced with 100 µl DMSO, and the activity was measured at 570 nm. When compared to the control (without test sample). The percentage of viability was calculated using the following formula.

% of viability = cell treated with samples/untreated control cells × 100.

2.10. Evaluation of in vitro anti-inflammatory activity of synthesized silver nanoparticles

2.10.1. Egg albumin denaturation test.

The egg albumin denaturation test, as previously described, was used to evaluate the in vitro anti-inflammatory activity of the synthesised nanoparticles [Gupta et al., 2015]. The reaction mixture (5mL) contained 5 different concentrations of nanoparticles (10, 50, 100, 150, and 200 µg/ml), 0.2 mL of egg albumin, 2.8 mL of phosphate buffered saline (PBS, pH 6.4), and 2 mL of each. An equivalent volume of distilled water was used as a control. After being incubated at (37°C) for 15 minutes, the mixtures were heated at 70°C for 5 minutes. After cooling, absorbance was measured at 660 nm using a vehicle as a blank. Diclofenac sodium was used as a control drug and was treated similarly to measure absorbance at final concentrations of 10, 50, 100, 150, and 200 µg/ml. The experiment was performed three times. The percentage inhibition of protein denaturation was calculated using the following formula.

% inhibition = $[A_{control} - A_{sample} / A_{control}] \times 100$

Where $A_{control}$ is the absorbance of solution without silver nanoparticles, A_{sample} is the absorbance of solution with sample silver nanoparticles/standard.

2.10.2. Evaluation of in vitro anti-inflammatory activity by BSA denaturation activity.

The protein denaturation experiment was carried out according to the methodology outlined by (Gunathilake et al. 2018). 0.02 mL of extract, 4.78 mL of phosphate buffered saline (PBS, pH 6.4), 0.2 mL of 1% bovine albumin, and 4.78 mL of mixture made up the reaction mixture (5 mL). The mixture was mixed, heated at 70°C for 5 minutes after 15 minutes of incubation in a water bath (37°C). After cooling, the turbidity was measured at 660 nm with a UV/VIS spectrophotometer. Phosphate buffer solution was used as the control. The experiment was carried out three times. Using the following formula, the % inhibition of protein denaturation was determined. The percentage inhibition of protein denaturation was calculated using the following formula.

% inhibition = $[A_{control} - A_{sample} / A_{control}] \times 100$

Where $A_{control}$ is the absorbance of solution without silver nanoparticles, A_{sample} is the absorbance of solution with sample silver nanoparticles/standard.

2.10.3. Membrane Stabilization Test

Human Red Blood Cell (HRBC) Suspension Preparation In order to prevent clotting, 2 mg of disodium EDTA was added to a centrifuge tube containing human blood before it was centrifuged at 1000g for 10 min. The erythrocyte pellet was collected after centrifugation and gently resuspended after three gentle washings with ten volumes of pyrogen-free saline (0.9% NaCl). The pellet was gently resuspended in normal saline to 10.0% (v/v) after the suspension was centrifuged once more at 1000g for 10 min.

2.10.4. Hypotonicity-Induced Hemolysis

Synthesize silver nanoparticles were dissolved in distilled water (hypotonic solution), isotonic phosphate buffer solution, and the necessary concentrations of 100–500 µg/ml in the centrifuge tubes. In the control tubes, 5 ml of distilled water was present. Additionally, as a positive control, Diclofenac sodium was dissolved in isotonic phosphate buffer solution at the necessary concentration (100–500 µg/ml). Each tube received 0.1 ml of erythrocyte suspension, which was gently mixed in. The tubes were then placed in a water bath and heated to 37°C for 30 minutes. The tubes were then centrifuged for 5 minutes at 3000 rpm. Using a UV spectrometer, the absorbance of the supernatant at 540 nm is used to calculate the amount of haemoglobin produced because of haemolysis. Using an assumption, the percentage of haemolysis was estimated. By assuming that all haemolysis produced in the presence of distilled water is 100%, the percentage of haemolysis was calculated. Equation was used to determine the extract's % inhibition of haemolysis (Bagur et al. 2020).

$$\%\text{Inhibition of Haemolysis} = [1- OD_2\text{-}OD_1 / OD_3\text{-}OD_1] \times 100$$

Where, OD_1, OD_2, and OD_3 are the absorbance of the test sample in an isotonic solution, the absorbance of the test sample in a hypotonic solution, and the absorbance of the control sample in a hypotonic solution, respectively.

2.11. In - vivo Anti-inflammatory activity of synthesized nanoparticles

2.11.1. Experimental Mice

Swiss albino female mice weighing 25 to 35 gms were purchased from the Liveon Biolabs Pvt. Ltd., Tumkur, Animal Facility. All animal experiments were conducted in accordance with the regulations set forth by the Committee for the Purpose of Supervision of Experiments on Animals (CPCSEA), India's national regulating organisation for animal experimentation with approval number were consented by the Office of Institutional Animal Ethical Committee (IAEC) (Reg.No:123/PO/C/99/CPCSEA under the rules 5(a) of the “Breeding of experiments on animal (control and supervision rules 1998”)). Unless

otherwise stated, mice were divided into 3 groups of 6, per group, in all animal tests.

2.11.2. Carrageenan-induced paw edema in mice.

Acute inflammation for evaluating anti-inflammatory treatments. Using a modified version of the previously reported procedure, the anti-inflammatory efficacy of produced silver nanoparticles was evaluated using carrageenan-induced paw edema in mice [Ghorbanzadeh et al. 2015; Gupta et al. 2015]. 0.1 ml of 1% carrageenan was subcutaneously injected into the right hind paw of mice to cause inflammation. One hour prior to the delivery of carrageenan, mice were pretreated with intra-peritoneal injections of the usual drug, Diclofenac sodium (10 mg/kg) and synthesized silver nanoparticles (10µg to 150µg/mouse). Two hours after the animals were sacrificed, the carrageenan-induced edema feet were dissected, and paw edema was assessed for histopathology, the dissected paws were weighed, compared to the controls, and stored in 10% formalin. The inhibitory effect was determined by using following formula:

%inhibition of edema = [(Vc-Vt)/Vc] × 100

Where Vc is the edema volume in the control group and Vt is the edema volume in tested group.

3. Results And Discussion

3.1. Isolation and molecular characterization of endophytic fungus

The endophytic fungus was isolated from a stem sample of *Abrus precatorius*. A whitish block endophytic fungus was discovered (Fig. 1, a, b, c and d). The cell morphology was recorded with respect to spore chain and structure of hyphae (Fig.e). The 18s rRNA gene was amplified using the ITS1 primer and ITS4 primer, and the 18s rRNA gene was identified using Sanger's dideoxy Nucleotide sequencing of the amplified ITS region. Blastn analysis revealed considerable multiple sequence alignment and pairwise results revealing 100 percent identity with the sequence of *Phyllosticta owaniana* – KUMBMDBT-32, which was deposited in NCBI GenBank with the accession number MW007919. The obtained sequencing files were utilized to construct a phylogenetic tree using the neighbor-joining (NJ) method utilizing software such as MEGA- Version 7.0.14 (Fig. 2).

3.2. Extraction and Gas-Chromatography - mass spectroscopy analysis of secondary metabolites

The endophytic fungus *Phyllosticta owaniana* extract was analysed using gas chromatography and mass spectrometry (GC-MS), 42 metabolites were discovered by comparing with the NIST database (Table 1 and Fig. 3). The bioactive compounds were identified based on their retention time, the major compounds peak 18 (RT 7.818 Area % 24.79) is 2-Furancarboxaldehyde, 5-(hydroxymethyl), peak 14 (RT 6.682 Area % 10.33) is 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, peak 19 (RT 8.132 Area % 8.21) is 1,2,3-Propanetriol, monoacetate, peak 10 (RT 5.770 Area % 5.94) is 1,3,5-Triazine-2,4,6-triamine, peak 22 (RT 9.548 Area % 5.05) is Phenol, 2-methoxy-3-(2-propenyl), peak 25 (RT 10.835 Area % 4.83) is 2-Pentanone, 5-(acetyloxy)- $$ 2-Pentanone, 5-hydroxy-, acetate, peak 20 (RT 8.815 Area % 4.63) is Propanal, 2,3-dihydroxy-, (S), peak 8 (RT 5.263 Area % 4.02) is Pentanoic acid, 4-oxo, Many biological parameters were present in the majority of the bioactive secondary metabolites compounds it helps to synthesis of silver nanoparticles.

Table 1
List of identified bioactive compounds of ethyl acetate extract of endophytic fungus *Phyllosticta owaniana*.

Sl. No.	Compound	Molecular Formula	Molecular Weight	Retention Time	Peak	Area %
01	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126 g/mol	7.818	18	24.79
02	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144 g/mol	6.682	14	10.33
03	1,2,3-Propanetriol, monoacetate	$C_5H_{10}O_4$	134 g/mol	8.132	19	8.21
04	1,3,5-Triazine-2,4,6-triamine	$C_3H_6N_6$	126 g/mol	5.770	10	5.94
05	Phenol, 2-methoxy-3-(2-propenyl)-	$C_{10}H_{12}O_2$	164 g/mol	9.548	22	5.05
06	2-Pentanone, 5-(acetyloxy)- $$ 2-Pentanone, 5-hydroxy-, acetate	$C_7H_{12}O_3$	144 g/mol	10.835	25	4.83
07	Propanal, 2,3-dihydroxy-, (S)-	$C_3H_6O_3$	90 g/mol	8.815	20	4.63
08	Pentanoic acid, 4-oxo-	$C_5H_8O_3$	116 g/mol	5.263	8	4.02
09	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162 g/mol	13.707	31	3.77
10	Butanoic acid, 2-ethyl-3-oxo-, methyl ester	$C_7H_{12}O_3$	144 g/mol	3.237	1	3.69
11	(2R,4R)-(-)-Pentanediol	$C_5H_{12}O_2$	104.15g/mol	3.601	2	1.79
12	2-Cyclopenten-1-one, 2-hydroxy-	$C_5H_6O_2$	98.1g/mol	3.745	3	0.88
13	2-Furanmethanol, 5-methyl-	$C_6H_8O_2$	112.13g/mol	4.063	4	0.21
14	2-Furancarboxaldehyde, 5-methyl-	$C_6H_6O_2$	110.11g/mol	4.203	5	0.37
15	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	144.12g/mol	4.397	6	1.93
16	2H-Pyran-2,6(3H)-dione	$C_5H_4O_3$	112.08g/mol	4.593	7	1.11
17	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	$C_6H_8O_3$	128.13g/mol	5.455	9	0.34
18	Cyclopropyl carbinol	C_4H_8O	72.11g/mol	5.939	11	0.09
19	Maltol	$C_6H_6O_3$	126.11g/mol	6.214	12	0.25
20	Butanoic acid, 2-methyl-3-oxo-, ethyl ester	$C_7H_{12}O_3$	144.17g/mol	6.508	13	0.68
21	2,4-Difluoroanisole $$ 2,4-Difluoro-1-methoxybenzene	$C_7H_6F_2O$	144.12g/mol	6.923	15	0.53
22	Methyl 6-oxoheptanoate $$ 5-Acetylvaleric acid, methyl ester	$C_8H_{14}O_3$	158.19g/mol	9.078	21	0.23
23	DL-Proline, 5-oxo-, methyl ester	$C_6H_9NO_3$	143.14g/mol	9.875	23	2.47
24	Cyclohexanecarboxamide, N-tetrahydrofurfuryl	$C_{12}H_{21}NO_2$	211.3g/mol	10.101	24	0.57
25	2,4-Methylene-D-epirhamnitol	$C_7H_{14}O_5$	178.18g/mol	11.202	26	0.19
26	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.5g/mol	11.614	27	0.24
27	2-(3-Methyl-undec-3-enyl)-[1,3]dioxolane	$C_{15}H_{28}O_2$	240.38g/mol	12.200	28	0.18
28	Ethyl N-(o-anisyl)formimidate	$C_{10}H_{13}NO_2$	179.22g/mol	12.576	29	0.18
29	d-Glycero-d-galacto-heptose $$ Heptose	$C_7H_{14}O_7$	210.18g/mol	13.044	30	0.83
30	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242.4g/mol	13.937	32	0.81
31	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42g/mol	14.321	33	0.25
32	2-Amino-6-nitrobenzoic acid, methyl ester	$C_8H_8N_2O_4$	196.16g/mol	15.403	34	0.26
33	3-Methoxy-4-tetrazol-1-yl-phenylamine	$C_8H_9N_5O$	191.19g/mol	15.848	35	0.10
34	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.5g/mol	16.046	36	0.38
35	Sorbitol	$C_6H_{14}O_6$	182.17g/mol	16.596	37	0.81
36	1-(1-Ethyl-2,3-dimethyl-cyclopent-2-enyl)-ethanone	$C_{11}H_{18}O$	166.26g/mol	17.432	38	0.17

Sl. No.	Compound	Molecular Formula	Molecular Weight	Retention Time	Peak	Area %
37	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.5g/mol	17.742	39	0.49
38	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.5g/mol	17.968	40	0.15
39	dl-Phenylalanine, N-benzyl-	$C_{16}H_{17}NO_2$	255.31g/mol	18.373	41	0.16
40	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278.34g/mol	21.539	42	0.88
41	Cholest-5-en-3-ol (3.beta.)-	$C_{30}H_{50}O_2$	442.7g/mol	26.066	43	0.12
42	beta.-Sitosterol	$C_{29}H_{50}O$	414.7g/mol	28.186	44	0.10

3.3. Characterization of synthesized silver nanoparticles

The effectiveness of the endophytic fungus *Phyllosticta owaniana* synthesized silver nanoparticles was assessed visually by observing the change in color of the reaction mixture from light brown to dark brown (Fig. 4a, b) which was induced by the reduction of silver ions to silver nanoparticles. Bio-spectrophotometer was used to evaluate the synthesized silver nanoparticles.

3.3.1. Bio-spectrophotometer

The surface Plasmon resonance (SPR) of endophytic fungus *Phyllosticta owaniana* synthesised silver nanoparticles produced a peak centred near 413 nm indicating the reduction of silver nitrate into silver nanoparticles (Fig. 5). It was also observed that bioreduction of silver ions into silver nanoparticles, which indicates the release of extracellular proteins in the colloidal solution and their possible mechanism in the bioreduction process.

3.3.2. Fourier Transform Infrared Spectroscopy (FTIR)

The interaction of silver ions with the bioactive components contained in the endophytic fungus *Phyllosticta owaniana* fungal extracts that are responsible for the stabilization of silver nanoparticles was investigated using FTIR studies. The FTIR spectra of synthesized silver nanoparticles (Fig. 6) exhibit a peak at 3260 cm-1, indicating the existence of stretching vibrations in the Hydroxy group, H-bonded OH stretch. The Methyl C-H asym. sym. Stretch is responsible for the peaks 2903 cm^{-1} and 2851 cm^{-1}. Primary amine, NH bend group is responsible for the peaks 1614 cm^{-1} and 1524 cm^{-1}. The 1232 cm^{-1} peak is produced by symmetrical aromatic tertiary amine CN stretch. Aromatic C-H in-plane bend correlates to the band 1018 cm^{-1}. The presence of secondary metabolites, compounds, and proteins that are likely involved in the synthesis of silver nanoparticles was clearly revealed by FTIR results, as well as the fact that proteins play an important role in the stabilization of silver nanoparticles by capping, which prevents agglomeration and formulation stabilization. The obtained data was compared to [Nandiyanto et al. 2019], these findings are consistent with previous reports of Netala et al. 2016 worked on the biogenesis of silver nanoparticles utilizing the endophytic fungus *Pestalotiopsis microspora* and evaluation of antioxidant and anticancer capabilities.

3.3.3. Scanning Electron Microscopy – Energy Dispersive Analysis of X-ray (SEM)

Scanning electron microscopy was used to study the synthesized silver nanoparticles. The SEM results revealed surface morphology and shape features with high-density silver nanoparticles synthesised by the endophytic fungus *Phyllosticta owaniana*. Synthesized silver nanoparticles were homogenusly assembled in spherical form when observed under SEM (Fig. 7). Similar to our study Popli et al. 2018 worked on endophyte fungus *Cladosporium* sp., and mediated synthesis of silver nanoparticles exhibiting *in - vitro* antioxidants.

3.3.4. Energy Dispersive Analysis of X-ray (EDAX)

The presence of silver as the predominant constituent element is suggested by the energy dispersive a spectrum of the synthesised nanoparticles endophytic fungus *Phyllosticta owaniana*. Due to surface Plasmon resonance, metallic silver nanoparticles commonly show a significant signal peak at 3 keV. As seen in the Fig. 8, substantial signals from silver atoms were observed, but weaker signals from carbon and oxygen atoms are also recorded. These observations are consistent with a previous study of Popli et al. 2018 on endophyte fungi, *Cladosporium* sp., and mediated synthesis of silver nanoparticles with antioxidant activity in- vitro.

3.3.5. X-ray diffraction (XRD)

XRD analysis was used to determine the crystalline nature of the synthesised silver nanoparticles from the endophytic fungus *Phyllosticta owaniana*. The diffraction peaks at 38.23^0, 44.51^0, 64.60^0, 77.49^0, and 81.67^0 correspond to the planes (111), (200), (220), (311), and (222) appearing at 2Ø angles (Fig. 9). All of the peaks are from the standard JCPDS (Joint committee on powder diffraction standards, file No 87–0719) data and correspond to the face-centered cubic (FCC) lattice phase of silver. The participation of silver nitrate, which was employed for the creation of silver nanoparticles, results in additional intense peaks at 2Ø angles of silver nanoparticles. The synthesis of nanoparticles is indicated by the broadening of Bragg's peaks. The XRD patterns imply that the bioorganic phase crystallizes on the surface of silver nanoparticles. The findings of our study corroborate those of Netala et al. 2016; Popli et al. 2018, who have worked on endophytic fungi mediated synthesis of silver nanoparticles.

3.3.6. Particle size analysis by dynamic light scattering (DLS)

The hydrodynamic radii or sizes of the synthesized silver nanoparticles were calculated using particle size analysis. The average size of the synthesized silver nanoparticles of endophytic fungus was 65.81 nm, as per dynamic light scattering. The solitary peak found that the synthesized silver nanoparticles are of

good quality (Fig. 10). In the same way as our research Ramalingmam et al. 2015; Netala et al. 2016 ; Singh et al. 2017 worked on synthesized silver nanoparticles from endophytic fungi.

3.4. Antibiotic enhancing activity of synthesized silver nanoparticles

After incubation for 24 h, growth inhibition was observed around antibiotic discs impregnated with silver nanoparticles. The results obtained on the ability of silver nanoparticles in combination with antibiotics revealed overall increase in fold area of zone of inhibition which indicated the increase in antibacterial potential of all the antibiotics against the bacterial strains as shown in Table 2 and Fig. 11 to 13 The strong antibiotics Gentamicin 8.06 mm and Ampicillin 5.36 mm combined with silver nanoparticles against *Escherichia coli* (gram negative) *Staphylococcus aureus* (gram positive) formed maximum zone of inhibition. The result of the antibiotic enhancing activity was consisted with earlier report of Rahi et al. 2014 who have worked on silver nanoparticles biosynthesized by endophytic fungus *Penicillium* sp to enhance antibiotic activity.

3.5. Antibacterial activity of synthesized silver nanoparticles

Antibacterial activity of synthesized silver nanoparticles was tested at various concentrations using agar well diffusion method. After incubation zone of inhibition was observed, the result obtained revealed that the silver nanoparticles were extremely effective in inhibiting the growth more or less of entire test bacterial strains (Fig. 14 to 16). However, the maximum inhibitory activity of silver nanoparticles was found against *Pseudomonas aeruginosa* (gram negative bacteria) 15.1 ± 0.17 mm as illustrated in Table 3. The result of antibacterial activity of synthesized silver nanoparticles of endophytic fungus *Phyllosticta owaniana* was consisted with many earlier reports (Netala et al. 2016; Rani et al. 2017) who have worked on green synthesis of silver nanoparticles from endophytic fungi and its antibacterial studies.

Table. 2.1. Zone of inhibition values of antibiotic enhancing activity of endophytic fungus *Phyllosticta owaniana* synthesized silver nanoparticles.

Test pathogenic Bacteria	Measurement of zone of inhibition in diameter (mm)											
	Standard Antibiotic disc (10mcg) and Silver nanoparticles (Mg/mL)											
	Chloramphenicol			Cefpodoxime			Gentamicin			Ampicillin		
	Ab (A)	Ab+AgNP(B)	$(B^2 - A^2) / A^2$	Ab (A)	Ab+AgNP(B)	$(B^2 - A^2) / A^2$	Ab (A)	Ab+AgNP(B)	$(B^2 - A^2) / A^2$	Ab (A)	Ab+AgNP(B)	$(B^2 - A^2) / A^2$
E. coli	16.03 ± 0.05	18.06 ± 0.11	0.26	13.03 ± 0.05	19.03 ± 0.05	1.24	6 ± 00	18.06 ± 0.11	8.06	6 ± 00	11.1 ± 0.17	2.42
S. aureus	13.06 ± 0.11	18.1 ± 0.17	1.02	16.06 ± 0.11	17.1 ± 0.17	0.12	15.06 ± 0.11	17.03 ± 0.05	0.27	6 ± 00	15.13 ± 0.23	5.36
P. aeruginosa	11.06 ± 0.11	14.03 ± 0.05	0.6	14.06 ± 0.11	22.16 ± 0.28	1.47	17.1 ± 0.17	20.1 ± 0.17	0.37	6 ± 00	11.1 ± 0.17	2.42
S. typhi	17.13 ± 0.23	16.13 ± 0.23	0.11	21.06 ± 0.11	24.03 ± 0.05	0.29	17.03 ± 0.05	18.03 ± 0.05	0.12	6 ± 00	10.1 ± 0.17	1.82
K. pneumoniae	15.06 ± 0.11	16 ± 00	0.13	14.06 ± 0.11	20.06 ± 0.11	1.03	15.06 ± 0.11	16.06 ± 0.11	0.13	6 ± 00	11.13 ± 0.23	2.44

Table 3
Zone of inhibition values of antibacterial activity of silver nanoparticles at different concentrations synthesized by using *Phyllosticta owaniana* against bacterial pathogens.

Test pathogenic Bacteria	Measurement of zone of inhibition in diameter (mm)					
	Standard Antibiotics			Silver nanoparticles (mg/ml)		
	Streptomycin	Ciprofloxacin	Chloramphenicol	10	05	2.5
E. coli	20.1 ± 0.17	29.13 ± 0.23	24.06 ± 0.11	12.1 ± 0.17	11.03 ± 0.05	10.03 ± 0.05
S. aureus	10.16 ± 0.23	35.93 ± 0.11	28.03 ± 0.05	12.06 ± 0.11	11.06 ± 0.11	10.1 ± 0.17
P. aeruginosa	23.06 ± 0.09	25.96 ± 0.11	25.1 ± 0.17	15.1 ± 0.17	13.13 ± 0.23	12.13 ± 0.23
S. typhi	21.16 ± 0.28	27.93 ± 0.11	25.13 ± 0.23	14.03 ± 0.05	12.06 ± 0.11	11.16 ± 0.28
K. pneumoniae	23.16 ± 0.28	35.03 ± 0.05	29.1 ± 0.17	12.13 ± 0.23	11.13 ± 0.23	10.06 ± 0.11

Table 4
MIC values of silver nanoparticles synthesized by endophytic fungus Phyllosticta owaniana against bacterial pathogens.

Test pathogenic Bacteria	STM mg/ml	CPFX mg/ml	CHL mg/ml	AgNPs mg/ml
E. coli	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml	1 mg/ml
S. aureus	1.25 mg/ml	0.03125 mg/ml	0.25 mg/ml	1 mg/ml
P. aeruginosa	1.25 mg/ml	0.03125 mg/ml	0.25 mg/ml	1 mg/ml
S. typhi	1.25 mg/ml	0.0625 mg/ml	1.25 mg/ml	1 mg/ml
K. pneumoniae	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml	1 mg/ml

Table 5
Zone of inhibition values of antifungal activity of silver nanoparticles synthesized by endophytic fungus Phyllosticta owaniana

Test pathogenic Fungi	Measurement of zone of inhibition in diameter (mm)		
	Standard Antibiotics	Silver nanoparticles (mg/ml)	
	Fluconazole	10	5
Candida albicans	17.13 ± 0.23	12.16 ± 0.28	10.06 ± 0.11
Aspergillus brassiliensis	16.06 ± 0.11	11.13 ± 0.23	10.16 ± 0.28
Aspergillus flavus	20.1 ± 0.17	11.1 ± 0.17	10.1 ± 0.17

Table 6
% inhibition of synthesized silver nanoparticles of *Phyllosticta owaniana* and Diclofenac against egg albumin denaturation

Concentration µg/ml	Diclofenac µg/ml	Silver nanoparticles µg/ml
10	44.12 ± 0.79	13.71 ± 0.96
50	66.86 ± 1.18	32.70 ± 2.39
100	77.90 ± 0.68	44.72 ± 0.36
150	85.20 ± 0.59	67.08 ± 1.26
200	90.53 ± 0.00	72.15 ± 1.26
250	94.87 ± 0.34	79.74 ± 1.26
IC_{50} µg/ml	65.17	87.94

Table 7
% inhibition of synthesized silver nanoparticles of *Phyllosticta owaniana* and Diclofenac against egg albumin denaturation

Concentration µg/ml	Diclofenac µg/ml	Silver nanoparticles µg/ml
10	76.19 ± 2.38	7.14 ± 2.38
50	82.53 ± 1.37	28.57 ± 2.38
100	87.30 ± 1.37	44.44 ± 3.63
150	92.06 ± 1.37	57.93 ± 2.74
200	95.23 ± 0.00	76.19 ± 2.38
250	96.82 ± 1.37	88.88 ± 2.74
IC_{50} µg/ml	81.71%	99.67%

Table.8. % membrane stabilization values of hypotonicity-induced hemolysis at different concentrations of synthesized silver nanoparticles by endophytic fungus *Phyllosticta owaniana*

Concentration µg/ml	Diclofenac µg/ml	Silver nanoparticles µg/ml
100	60.91 ± 0.79	43.87 ± 1.12
200	65.28 ± 0.39	49.26 ± 0.73
300	68.73 ± 0.39	53.92 ± 0.84
400	72.64 ± 0.39	57.35 ± 0.73
500	76.55 ± 0.68	63.23 ± 0.73

Table.9. % inhibition values of carrageenan induced paw edema at different concentrations of synthesized silver nanoparticles by endophytic fungus *Phyllosticta owaniana*

Percentage of edema ration					
Control	Standard Drug	Silver nanoparticles concentration µg/ml			
Carrageenan	Diclofenac sodium	10 µg/ml	50 µg/ml	100 µg/ml	150 µg/ml
88.25 ± 0.06	11.34 ±1.68	4.66 ±0.63	9.69 ±0.32	12.80 ±0.53	17.66 ±0.72

3.6. Minimum inhibitory concentration (MIC)

Fungus *Phyllosticta owaniana*, an endophyte a minimal inhibitory concentration was shown by synthesised silver nanoparticles against Gram positive and Gram-negative bacteria. The current findings demonstrated that the minimum inhibitory concentration was effective against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Klebsiella pneumoniae* (Fig. 17. a, b, c, and d Table.4). The outcomes showed that *Phyllosticta owaniana*-produced silver nanoparticles have good minimum inhibitory concentration activity. Similar results were obtained by Rani et al. 2017, who studied the minimal inhibitory concentration activity of endophytic fungi *Aspergillus terreus* using green produced silver nanoparticles.

3.7. Antifungal activity of synthesized silver nanoparticles

After incubation for 2–3 days at 27 ± 2 °C, antifungal activity of endophytic fungus *Phyllosticta owaniana* synthesized silver nanoparticles against most pathogenic *Candida albicans*, *Aspergillus brassiliensis* and *Aspergillus flavus* formed the zone of inhibition values of 12.16 ± 0.28 mm, 11.13 ± 0.23 mm and 11.1 ± 0.17 mm respectively (Fig. 18 to 20 and Table.5). The result revealed that the growth inhibitory activity of synthesized silver nanoparticles against fungal pathogens can be expanded further by expanding the concentration of synthesized silver nanoparticles. Our result of antifungal activity of synthesized silver nanoparticles is confirmatory with the earlier findings (Netala et al. 2016). The antimicrobial activity of silver nanoparticles is the sum of distinct mechanisms. Silver nanoparticles form strong interaction with respiratory chain enzymes which causes uncoupling of electron transport chain (ETC) and oxidative phosphorylation (OP) as a result cell death occurs. The excellent antimicrobial activity of the silver nanoparticles allows them to be used in ingredients of topical ointments to prevent bacterial and fungal infections against open and burn wounds, silver nanoparticles embedded water purifier equipment's, preparation of injection molded plastic products, and employed in coating-based applications, including air ducts and counter tops. The important advantages of silver nanoparticles-based antimicrobial agents are their biocompatibility, health and environmental safety, and their excellent stability.

3.8. *In vitro* antioxidant activity of synthesized silver nanoparticles

3.8.1. DPPH radical scavenging activity

We studied the DPPH radical scavenging characteristics of *Phyllosticta owaniana* synthesised silver nanoparticles by measuring changes in the absorbance of the 1,1-diphenyl-2-picrylhydrazyl radical at 517 nm to understand more about its antioxidative mechanisms. *Phyllosticta owaniana* synthesised silver nanoparticles scavenged the DPPH radicals in a dose-dependent manner (Fig. 21). In terms of radical scavenging activity, the *Phyllosticta owaniana* synthesized silver nanoparticles showed radical scavenging action with an IC_{50} of 65.45 µg/ml, whereas Ascorbic acid had an IC_{50} of 18.53 µg/ml. Each value is represented as mean ± SD of three independent experiments. At $P < 0.05$, values with different superscripts are statistically distinct. Similar to our report of Netala et al. 2016 worked on antioxidant activity of silver nanoparticles produced by endophytic fungus *Pestalotiopsis microspora*, and Akther et al. 2019 worked on biosynthesis of silver nanoparticles synthesized endophytic fungus *Botryosphaeria rhodina* and its DPPH radical scavenging activity.

3.8.2. ABTS radical cation decolorization assay

The ABTS radical was scavenged by *Phyllosticta owaniana* synthesised silver nanoparticles in a concentration-dependent manner (200–1000 µg/mL). Silver nanoparticles *Phyllosticta owaniana* exhibited increased activity as a radical scavenger (Fig. 22). The results show the scavenging activity of ABTS in terms of percent inhibition. The results indicated that the IC_{50} for *Phyllosticta owaniana* synthesized silver nanoparticles was 6.94 µg/ml, g/ml at a concentration of 1000 µg/mL, whereas Ascorbic acid had an IC_{50} of 11.37 µg/ml. Each value is represented as the mean ± SD of three independent experiments. At $P < 0.05$, values with different superscripts are statistically distinct. These result are consisted with earlier report Nallappan et al. 2021 worked on green biosynthesis of silver nanoparticles using leaf extract of *Luffa acutangula*.

3.9. Measurement of cell viability by MTT assay

HepG2 and A498 cell lines were treated with the silver nanoparticles produced by the endophytic fungus *Phyllosticta owaniana* (Fig. 23 to 24). In cancer cells treated with silver nanoparticles, dose-dependent cytotoxicity was seen; as the concentration of silver nanoparticles increases, so does the cell cytotoxicity considerably. From HepG2 cell lines, the percentage of cell viability based on the IC_{50} value was measured over 24 hours at varied concentrations in 12.5 µg/mL (94.72%), 25 µg/mL(88.08%), 50 µg/mL(76.78%), and 100 µg/mL(55.84%), 200 µg/mL(30.67%), and also A498 cell lines 12.5 µg/mL (98.35%), 25 µg/mL(88.86), 50 µg/mL(82.66), and 100 µg/mL(66.64%), 200 µg/mL(34.53%). The study demonstrated that, when the sample was utilised at a concentration of 200 g/mL, cytotoxicity was found at values of 12.5–200 g/mL (Figs. 25 and 26). These findings are consistent with an earlier paper (Govindappa et al. 2020) that examined the percentage of cell survival of silver nanoparticles produced by endophytic fungi *Cladosporium perangustum*.

3.10. In vitro anti-inflammatory activity of synthesized silver nanoparticles

3.10.1. Egg Albumin Denaturation test.

The production of autoantigens in rheumatoid arthritis has been shown to promote *in vivo* protein denaturation, which is one of the well-known causes of inflammation (Vane and Botting, 1995). In this study, we investigated the possible anti-inflammatory properties of silver nanoparticles from the endophytic fungus *Phyllosticta owaniana* against heat-induced denaturation of egg albumin *in vitro*. At concentrations ranging from 10 to 250 µg/mL, synthesised silver nanoparticles yielded values for the mean suppression of protein denaturation, respectively (Table. 6 and Fig. 27). Like our investigation, the *in vitro* anti-inflammatory activity of egg albumin denaturation activity was used to determine the synthesised silver nanoparticles of *Xylocarpus granatum* extracts (Das et al 2019).

3.10.2. BSA Denaturation Activity

The ability of the endophytic fungus *Phyllosticta owaniana* to synthesise silver nanoparticles was to inhibit protein denaturation in a concentration-dependent manner. Figure 28 illustrates the inhibitory effect of synthesised silver nanoparticles on protein denaturation at various concentrations (10–250 µg/ml). These synthesised silver nanoparticles showed an inhibition percentage of protein denaturation in the range of 7.14 ± 2.38 to 88.88 ± 2.74 at concentrations of 10–250 µg/ml (Table.7). The lowest levels of inhibition were seen in endophytic fungus *Phyllosticta owaniana* synthesised silver nanoparticles. Similar studies by Govindappa et al. 2016 investigated the effects of produced silver nanoparticles on endophytic fungus *Penicillium sp* utilising an in-vitro BSA Denaturation assay to measure their anti-inflammatory activities.

3.10.3. Membrane stabilization test by hypotonicity-induced hemolysis

One of the most popular methods for evaluating the anti-inflammatory efficacy in vitro is Human Red Blood Cells (HRBC) approach, membrane stabilisation. The stabilisation of the erythrocyte or RBC membrane, which is like the lysosomal membrane, suggests that the test sample may also stabilise lysosomal membranes. The results indicated that the endophytic fungus *Phyllosticta owaniana* created silver nanoparticles have strong anti-inflammatory activity at different concentrations, % stabilisation of the synthesised silver nanoparticles, and the common drug Diclofenac sodium, as shown in (Fig. 29 Table.8). Similar findings were obtained by Bagur et al. in 2020 when they tested the membrane stability of synthesised silver nanoparticles of the endophytic fungus *Exserohilum rostrata* using hypotonicity-induced hemolysis.

3.11. In vivo anti-inflammatory activity of synthesized silver nanoparticles

Carrageenan causes paw edema in mice. In our studies, subcutaneous carrageenan injection caused the mouse paws to swell up as a result of edema, indicating a recent flare-up of paw inflammation depicts the thickness of the mice's paws following treatment with synthesized silver nanoparticles produced from the endophytic fungus *Phyllosticta owaniana* and diclofenac sodium. synthesized silver nanoparticles and

diclofenac sodium both displayed suppression when compared to mice given diclofenac at 1 hours (Fig. 30 to 32. Table.9). Photographs were taken of the paws of various mouse groups, including normal control mice and mice with carrageenan-induced paw edoema who had been given diclofenac or synthetic silver nanoparticles. These pictures made it crystal evident how significantly less paw thickness was caused by diclofenac sodium. The *in vivo* anti-inflammatory activity of leaf extract produced silver nanoparticle solutions was assessed using carrageenan-induced hind paw edema in wistar albino rats, and these results are consistent with past reports (Rafie et al. 2014; Helmy et al.2020).

3.11.1. Histopathological analysis

The histopathology examination of Carrageenan induced paw inflammation in Swiss Albino female mice (Fig. 33), After euthanasia, the legs were collected in 10% buffered Formalin solution were preserved before preparing the histological sections using the paraffin method. All sections in ascending grades of ethanol were dehydrated, cleared in xylene then embedded in paraffin wax. Transverse sections (4–5 µm, thickness) were mounted on glass slides and stained with hematoxylin stains. All sections were examined for the evaluation of inflammatory responses (Fig. 34).

4. Conclusion

The present research proposes simple, environmental friendly and cost-effective, biological methods method for extracellular biosynthesis of silver nanoparticles using endophytic fungus, *Phyllosticta owaniana* isolated from the stem sample of *Abrus precatorius* medicinal plant. Fungal culture filtrate mediated the successful synthesis of spherical-form silver nanoparticles, uniform distribution, dynamic light scattering evidenced the average size was found to be 65.81 nm. The silver nanoparticles synthesized very stable and biocompatible. The biosynthesized silver nanoparticles exhibited effective antibiotic enhancing, antimicrobial, antioxidant, anticancer and anti-inflammatory activities. Further, this study suggests that the silver nanoparticles can be used as a potential agent in pharmaceutical and biomedical, importance.

Declarations

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Author contributions

MD-Analysis of research work in laboratory and manuscript writing, TB- designee and correction of research work, AS , SHV-manuscript correction and editing, NG- phylogenetic tree analysis, MGT, NS guide for the experimentation of anti-inflammatory activity, manuscript correction.

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References

1. Ajah H A, Hassan A S and Aja H A (2018) Extracellular Biosynthesis of Silver Nanoparticles using *Fusarium Graminearum* and their Antimicrobial Activity, Journal of Global Pharma Technology, 683-689, ISSN: 0975 -8542.
2. Akther T, Mathipi V, Kumar N S , Davoodbasha M and Srinivasan H (2019) Fungal-mediated synthesis of pharmaceutically active silver nanoparticles and anticancer property against A549 cells through apoptosis, Environmental Science and Pollution Research, 13649–13657, https://doi.org/10.1007/s11356-019-04718-w.
3. Bagur H, Poojari C C, Melappa G, Rangappa N R, Chandrasekhar, Somu P (2020) Biogenically synthesized silver nanoparticles using endophyte rungal extract of *Ocimum tenuiflorum* and evaluation of biomedical properties, Journal of Cluster Science, 31:1241–1255,https://doi.org/10.1007/s10876-019-01731-4.(
4. Bauer AW, Kirby WMM, Sherris JC, Turck M (1966) Antibiotic Susceptibility Testing by a Standardized Single Disk Method. American Journal of Clinical Pathology 45:493–496. Https://doi.org/10.1093/ajcp/45.4_ts.493.

5. Das S K, Behera S, Patra J K , Thatoi H (2019) Green synthesis of sliver nanoparticles using *Avicennia officinalis* and *Xylocarpus granatum* extracts and In vitro evaluation of antioxidant, antidiabetic and anti-inflammatory activities, Journal of Cluster Science, 30:1103–1113, https://doi.org/10.1007/s10876-019-01571-2.
6. Devi L S and Joshi S R (2015) Ultrastructures of silver nanoparticles biosynthesized using endophytic fungi, Journal of Microscopy and Ultrastructure, International Journal of Nanomaterials and Biostructures 29–37, http://dx.doi.org/10.1016/j.jmau.2014.10.004.
7. Ghorbanzadeh B, Mansouri M, Hemmati A, Naghizadeh B, Mard S and Rezaie A (2015) A study of the mechanisms underlying the ant-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. Indian journal pharmacology 47, 292. https://doi.org/10.4103/0253-7613.157127.
8. Giridasappa A, Ismail SM, Rangappa D (2021) Antioxidant, antiproliferative and antihemolytic properties of phytofabricated silver nanoparticles using *Simarouba glauca* and *Celastrus paniculatus* extracts. Appl Nanosci 11:2561–2576. Https://doi.org/10.1007/s13204-021-02084-z.
9. Govindappa M, Farheen H, Chandrappa C P, Channabasava, Rai R V and Raghavendra V B (2016) Mycosynthesis of silver nanoparticles using extract of endophytic fungi, *Penicillium* species of *Glycosmis mauritiana*, and its antioxidant, antimicrobial, anti-inflammatory and tyrokinase inhibitory activity, Advances in Natural Sciences: Nanoscience and Nanotechnology, 1-9, doi:10.1088/2043-6262/7/3/035014.
10. Govindappa M, Lavanya M and Aishwarya P (2020) Synthesis and characterization of endophytic fungi, *Cladosporium perangustum* mediated silver nanoparticles and their antioxidant, anticancer and nano-toxicological Study. Bionanosci 10:928–941. Https://doi.org/10.1007/s12668-020-00719-z.
11. Guangquan Li , Dan He , Qian Y 1, Guan B, Gao S, Cui Y1, Yokoyama K and Wang L (2012) Fungus-Mediated Green Synthesis of Silver Nanoparticles Using *Aspergillus terreus*, International Journal of Molecular Sciences, 466-476, doi:10.3390/ijms13010466.
12. Gunathilake K, Ranaweera K and Rupasinghe H, (2018) In Vitro Anti-Inflammatory Properties of Selected Green Leafy Vegetables. Biomedicines 6, 107. https://doi.org/10.3390/biomedicines6040107.
13. Gupta A K , Parasar D, Sagar A, Choudhary V, Chopra B S, Garg R., Ashish and Khatri N, (2015) Analgesic and Anti-Inflammatory Properties of Gelsolin in Acetic Acid Induced Writhing, Tail Immersion and Carrageenan Induced Paw Edema in Mice. PLoS ONE 10, e0135558. https://doi.org/10.1371/journal.pone.0135558.
14. Helmy A, Shazly M E, Seleem A, Abdelmohsen U, Salem M A, Samir A, Rabeh M, Elshamy A and Singab A N B (2020) The synergistic effect of biosynthesized silver nanoparticles from a combined extract of parsley, corn silk, and gum arabic: in vivo antioxidant, anti-inflammatory and antimicrobial activities, Materials research express, 1-16, https://doi.org/10.1088/2053-1591/ab6e2d.
15. Jayaram H, Marigowda V and Thara Saraswathi K J (2021) Secondary Metabolite Production and Terpenoid Biosynthesis in Endophytic Fungi *Cladosporium cladosporioides* Isolated from Wild *Cymbopogon martinii* (Roxb.) Wats, Microbiology research, 812–828, https://doi.org/10.3390/microbiolres12040059.
16. Nallappan D , Fauzi A N, Krishna B S , Kumar B P , Reddy A V K , Syed T , Reddy C S, Yaacob N S , and Rao P V (2021) Green biosynthesis, antioxidant, antibacterial, and anticancer activities of silver nanoparticles of *Luffa acutangula* leaf extract, BioMed Research International, 1-28, https://doi.org/10.1155/2021/5125681.
17. Nandiyanto ABD, Oktiani R, Ragadhita R (2019) How to Read and Interpret FTIR Spectroscope of Organic Material. Indonesian J Sci Technol 4:97. Https://doi.org/10.17509/ijost.v4i1.15806.
18. Netala V R , Bethu S M, Pushpalatha B, Baki V B, Aishwarya S, Rao V and Tartte V (2016) Biogenesis of silver nanoparticles using endophytic fungus *Pestalotiopsis microspora* and evaluation of their antioxidant and anticancer activities, International Journal of Nanomedicine, 5683–5696, http://dx.doi.org/10.2147/IJN.S112857.
19. Netala V R , Kotakadi V S, Bobbu P, Gaddam S A, Tartt V (2016) Endophytic fungal isolate mediated biosynthesis of silver nanoparticles and their free radical scavenging activity and antimicrobial studies, 3 Biotech, 6:132, DOI 10.1007/s13205-016-0433-7.
20. Popli D, Anil V, Akshatha B. Subramanyam, Namratha M N, Ranjitha V. R, Saroja N. Rao, Rai R V and Govindappa M (2018) Endophyte fungi, *Cladosporium* species-mediated synthesis of silver nanoparticles possessing *in vitro* antioxidant, anti-diabetic and anti-Alzheimer activity, artificial cells, Nano medicine, and biotechnology , S676–S683, https://doi.org/10.1080/21691401.2018.1434188.
21. Rafie H M E and Hamed M A A (2014) Antioxidant and anti-inflammatory activities of silver nanoparticles biosynthesized from aqueous leaves extracts of four *Terminalia* species, Advances in Natural Sciences: Nanoscience and Nanotechnology, 1-11, doi:10.1088/2043-6262/5/3/035008.
22. Rahi, D.K., & Parmar, A.S (2014) Mycosynthesis of silver nanoparticles by an endophytic *Penicillium* species of Aloe vera root, evaluation of their antibacterial and antibiotic enhancing activity, 46-51, ISSN 2277-3851.
23. Rahim A K, Mahmoud S Y and Ali A M (2016) Extracellular biosynthesis of silver nanoparticles using *Rhizopus stolonifer*, Saudi Journal of Biological Sciences, 01-08, http://dx.doi.org/10.1016/j.sjbs.2016.02.025.
24. Rahman MM, Islam MB, Biswas M, Khurshid Alam AHM (2015) In vitro antioxidant and free radical scavenging activity of different parts of Tabebuia pallida growing in Bangladesh. BMC Research Notes 8:. https://doi.org/10.1186/s13104-015-1618-6.
25. Ramalingmam P, Muthukrishnan S, Thangaraj P (2015) Biosynthesis of Silver Nanoparticles Using an Endophytic Fungus,*Curvularialunata* and Its Antimicrobial Potential, Journal of Nanoscience and Nanoengineering, 241-247, http://www.aiscience.org/journal/jnn.
26. Rani R, Sharma D, Chaturvedi M, Jp Y (2017) Green Synthesis, Characterization and Antibacterial Activity of Silver Nanoparticles of Endophytic Fungi *Aspergillus terreus*. Journal of Nanomedicine and Nanotechnology, 01-08, https://doi.org/10.4172/2157-7439.1000457.
27. Seeram NP, Henning SM, Niu Y, et al (2006) Catechin and caffeine content of green tea dietary supplements and correlation with antioxidant capacity. Journal of Agricultural and Food Chemistry 54:1599–1603. https://doi.org/10.1021/jf052857r.
28. Singh T, Jyoti K, Patnaik A, Singh A, Chauhan R, Chandel S S (2017) Biosynthesis, characterization and antibacterial activity of silver nanoparticles using an endophytic fungal supernatant of *Raphanus sativus*, Journal of Genetic Engineering and Biotechnology, 31–39,

http://dx.doi.org/10.1016/j.jgeb.2017.04.005.
29. Soni N, Prakash S (2015) Antimicrobial and mosquitocidal activity of microbial synthesized silver nanoparticles. Parasitol Res 114:1023–1030. Https://doi.org/10.1007/s00436-014-4268-z.
30. Vane JR and Botting R.M (1995) new insights into the mode of action of anti-inflammatory drugs. Inflamm Res 44, 1–10. https://doi.org/10.1007/BF01630479.
31. Zhao X, Zhou L, Riaz Rajoka M S, Yan L, Jiang C, Shao D, Zhu J, Shi J, Huang Q, Yang H and Jin M (2017) Fungal silver nanoparticles: synthesis, application and challenges, Critical reviews in biotechnology, 1-17, https://doi.org/10.1080/07388551.2017.1414141.

Figures

Figures 1 to 34 are available in the Supplementary Files section.

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