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Biochemical Analysis and Characterization of Silver Nanoparticles Synthesized by the Endophytic Fungus *Plectosphaerella oligotrophica* S8A26 Associated with *Anaphalis contorta*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Endophytic fungi associated with medicinal plants produce compounds that have vast biological activities. *Anaphalis contorta* is a highly therapeutic plant that has been used in traditional medicine by various communities. In the present study, the endophytic fungus *Plectosphaerella oligotrophica* S8A26 was isolated from the stem part of *A. contorta* and assessed for its plant growth promotion abilities, and showed positive results for phosphate solubilization and ammonia production. The secondary metabolites were analyzed for the presence of seven biochemicals, which indicate the presence of flavonoids, phenols, saponins, and steroids. The total flavonoid content and total

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phenolic content were recorded to be 25.67 µg of QE/mg of EE and 5.06 µg of GAE/mg of EE, respectively. The isolate was allowed to synthesize silver nanoparticles and characterized using X-ray diffraction, Energy Dispersive X-ray, Particle size distribution, and Zeta potential. XRD analysis has shown the AgNPs are crystalline with an average size of 24.81 nm. EDX study reveals silver as the main component with 48.63%. Particle size distribution indicates that the majority of the particles occur in the size range of 65–70 nm. Zeta potential value of −31.8 mV shows that the AgNPs are stable. Studies like the biological activities of the endophyte as well as AgNPs, secondary metabolite profiling, and the isolation of bioactive compounds would constitute important future research.

Keywords: Endophytic fungi; flavonoids; ammonia; AgNPs; zeta potential; pharmaceuticals.

1. INTRODUCTION

The term ‘endophytic fungi’ may be defined as “those fungi that colonize and live within the intercellular spaces of living plant tissue by forming symbiotic relationships without any harmful effect on the host plant” [1]. These endophytic fungi may inhabit the host plants for all or part of their life cycle, depending on the availability of the host tissue. It has been observed that the diversity of endophytic fungal species is higher in tropical and semitropical plants than in those grown in dry and colder regions [2]. Plants with medicinal properties have been exploited for the isolation of endophytic fungi that have potential biomedicine, bioremediation, and bioprospecting properties. In several studies, endophytic fungi have been reported to protect host plants from the attack of pathogens, pests, insects, and herbivores. The secondary metabolites produced by the fungal endophytes contain various bioactive compounds that not only protect the host plant from external invasion but also have vast applications in industries [3] reported the therapeutic properties of natural compounds obtained from endophytic fungi that are used in the treatment of serious diseases like cancer, autoimmune, neurological, and cardiovascular. Endophytic fungi possess growth-promoting properties that not only solubilize and mobilize essential elements but also protect the host plant from pathogenic invasion. *Anaphalis contorta* (D.Don) Hook.f. is a high-altitude perennial herb belonging to the Asteraceae family that has high ethnomedicinal values and is used in traditional medicine for treating high blood pressure, intestinal disorders, cuts and injuries, skin infections, etc. [4,5]. The essential oils from the leaves of *A. contorta* have been reported to show antibacterial and antifungal properties [6].

The Department of Science and Technology (DST), the National Chemical Laboratory (NCL), and the Department of Biotechnology (DBT) in India have made it a top priority to find new bioactive compounds that can treat acute microbiological diseases, especially ones that are not controlled by antibiotics [7]. Plant pathogens are the most important factor in the reduction in crop yield. Conventional farming practices employ chemical treatment of crop fields, which destroys the natural health of the soil, reduces soil microorganisms, decreases water holding capacity, and conversely increases the health hazard. The biological control of plant pathogens is an environment-friendly approach to managing plant diseases and is an important part of sustainable agriculture. Nanoparticles, owing to their small size, have a higher catalytic capacity due to their larger surface area and are highly demanding in various sectors. Endophytic fungi are suitable for nanoparticle synthesis due to their eco-friendly nature and ability to produce larger secondary metabolites in a short period of time. In several studies, silver nanoparticles have shown potent biological activities. Compared with other metals, silver exhibits higher toxicity to a broad spectrum of microorganisms with minimal side effects on mammalian cells [8]. In the present study, the endophytic fungus *Plectosphaerella oligotrophica* S8A26 was examined for the production of plant growth promotion and the secondary metabolites for the presence of phytochemicals. Further, silver nanoparticles were synthesized using *P. oligotrophica* S8A26 and characterized with XDR, EDX, particle size distribution, and zeta potential.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Fungal Endophyte

Anaphalis contorta plants were collected in sterile plastic bags from the Ukhrul district of Manipur (latitude 25° 8' 41.464" N; longitude 94°

27' 38.289'' E; altitude 2062.14 m a.s.l.) during December and processed for isolation within 24 hours of collection. Stem parts were used for the isolation of fungal endophytes and carried out using the method given by [9], with minor modifications. Surface sterilization was performed by passing through 70% ethanol for 3 minutes, 4% sodium hypochlorite for 2 minutes, and 70% ethanol for 30 seconds. Identification was performed using morphological characteristics and confirmed after ITS-rDNA gene sequencing. The isolate was deposited in the National Fungal Culture Collection of India (NFCCI) and the gene sequence in the GenBank of National Center of Biotechnology Information (NCBI).

2.2 Plant Growth Promotion Activities

The isolate, *P. oligotrophica* S8A26, was assessed for qualitative phosphate (PO_4) solubilization, ammonia (NH_4) production, and hydrogen cyanide (HCN) production.

2.3 Phosphate (PO_4) Solubilization

The isolate was inoculated on Pikovskaya's agar medium petriplates with added calcium phosphate and incubated at 28±1°C for 7 days. The appearance of a clear zone around the fungal colony indicates solubilization of inorganic phosphate [10].

2.4 Ammonia (NH_3) Production

The endophyte was inoculated in a peptone water test tube and incubated for 72 hours at 28±1°C. After the incubation period, Nessler's reagent (0.5 ml) was added and observed for colour change to yellow or brown [11].

2.5 Hydrogen Cyanide (HCN) Production

P. oligotrophica S8A26 was inoculated in a test tube containing Bennett agar media. Onto the wall of the test tube, filter paper dissolved in a solution of picric acid and sodium carbonate was attached after air drying and incubated at 28±1°C for 10 days. The colour change of the filter paper from light yellow into brown or red shows HCN production [12].

2.6 Secondary Metabolite Production

P. oligotrophica S8A26 was cultured in Potato dextrose agar (PDA) medium for 7 days, and mycelial plug (0.5 cm in diameter) was cut off and inoculated in Potato dextrose broth (PDB) for 15 days at 28±1°C. The filtrate obtained was extracted with ethyl acetate three times. The crude extract was dried at 40°C and stored at 4°C [13].

2.7 Biochemical Analysis

The crude extract of *P. oligotrophica* S8A26 was screened for alkaloids, flavonoids, terpenoids, tannins, saponins, steroids, and phenols, and further evaluated for the total phenolic content (TPC) and total flavonoid content (TPC) [14,15].

2.7.1 Test for Alkaloids

The extract was dissolved in Hydrochloric acid (HCl) and added with Mayer's reagent. The development of a cream-coloured precipitate indicates the presence of alkaloids.

2.7.2 Test for flavonoids

The crude extract was mixed with a Sodium hydroxide (NaOH) solution, and the colour change from yellow to colourless after the addition of dilute Hydrochloric acid (HCl) shows the presence of flavonoids.

2.7.3 Test for phenols

The endophyte extract was added with ferric chloride ($FeCl_3$), and the observation of a green colour indicates the presence of phenolic compounds.

2.7.4 Test for saponins

The dried crude extract was mixed with water and shaken vigorously. The formation of intense foam suggests the presence of saponins.

2.7.5 Test for steroids

The ethyl acetate extract was mixed with acetic anhydride and then added with concentrated H_2SO_4. The change of colour from violet to blue or green shows the presence of steroids.

2.7.6 Test for tannins

The fungal extract was mixed with an alcoholic $FeCl_3$. The development of a bluish-black colour, that disappears with the addition of dilute H_2SO_4, followed by the formation of a yellowish-brown precipitate, is an indication of tannins.

2.7.7 Test for terpenoids

The crude extract solution was mixed with chloroform and concentrated H_2SO_4. The formation precipitates with a reddish-brown colouration indicates the presence of terpenoids.

2.7.8 Total phenolic content (TPC)

The Folin-Ciocalteu method was employed to assess the TPC in the crude ethyl acetate extract of *P. oligotrophica* S8A26. Different concentrations of the extract were combined with 10% Folin-Ciocalteu solution and $NaHCO_3$ and incubated at 45 °C for 30 minutes. The absorbance was taken at a wavelength of 765 nm, and calibration curves were constructed using different concentrations of gallic acid (100 to 500 µg/mL) as standard.

2.7.9 Total flavonoid content (TFC)

The TFC was calculated following the colorimetric technique. The crude extract of *P. oligotrophica* S8A26 was diluted with deionized water and added with sodium nitrite solution. The mixture was incubated at room temperature for 6 minutes. Aluminium chloride solution was added to the mixture and incubated for 5 minutes with added sodium hydroxide solution. The solution was again diluted with distilled water and incubated at 25 ºC for 30 minutes, and the absorbance was recorded at 510 nm. The flavonoid concentration was determined by employing a standard curve (5 to 100 µg/mL) of quercetin.

2.8 Synthesis of Silver Nanoparticles (AgNPs)

P. oligotrophica S8A26 was cultured on PDB for 10 days at 28±1 °C and the fungal hyphae mat was collected after washing in distilled water. Then the mat was suspended in 100 ml of sterilized distilled water for 48 hours at 28±1 °C and filtered. The filtrate was treated with 1 mM silver nitrate solution and incubated for another 24 hours at room temperature in dark conditions for reduction and observation for colour change. The AgNPs solution was centrifuged at 10,000 rpm for 15 min, and the AgNPs pellets obtained were over-dried and stored at 4°C [16].

2.9 Characterization of AgNPs

The biosynthesized silver nanoparticles were characterized by X-ray diffraction (XRD), Energy dispersive X-ray (EDX), Particle size distribution, and Zeta potential.

2.9.1 XRD analysis

X-Ray diffraction (XRD) was employed as a technique to examine the crystalline structure of the silver nanoparticles. The silver nanoparticles were finely ground and homogenized in order to achieve a uniform size and placed on the XRD grid. The study utilised Cu-Kα radiation with a wavelength (λ) of 1.5406 Å, operating at 40 kV and 40 mA. Data collection took place within the 2θ range of 10° to 80°, with a scan speed of 2°/minute [17]. The particle size of the prepared samples was determined by using Scherrer's equation as follows:

$$D = \frac{K\lambda}{\beta 1/2 \cos \Theta}$$

Where, D is average crystalline size, β is line broadening in radians (full width at half maximum of the peak in radians), λ is wavelength of X-ray and Θ is braggs angle, and K is constant (geometric factor = 0.94).

2.9.2 EDX analysis

The compositional analysis of the presence of elemental silver was carried out through Energy dispersive X-ray (EDX) detector. The emission of rays from the nanoparticles in the X-ray detector, which exhibit peaks at specific electron volt values, provides confirmation for the presence of elemental silver [18].

2.9.3 Particle size distribution and zeta potential of silver nanoparticles

The determination of the sizes and stability of the distributed biosynthesized nanoparticles was carried out by the utilization of the Dynamic light scattering (DLS) technique on Zetasizer Nano ZS [19]. The particle size distribution was assessed to identify the size, while the zeta potential was measured to evaluate the stability of the nanoparticles. A diluted solution of nanoparticles was made in deionized water and subjected to sonication at 35°C for a duration of 20 minutes in order to eliminate any agglomeration. A volume of 2000 µL of silver nanoparticles was placed into transparent disposable zeta cells for conducting the dynamic light scattering (DLS) investigation. The data were subjected to analysis through the monitoring process conducted at a temperature of 25ºC and a

scattering angle of 90º. The analysis of particle size distribution involves the examination of variations in light scattering resulting from the Brownian motion of nanoparticles, and the average size was calculated using the given formula:

> Average size = sum (size x frequency) of all particles/sum of all the frequencies

On the other hand, the zeta potential determines the extent of electrostatic repulsion or attraction between nanoparticles present in the suspension and is calculated using the Smoluchowski equation as given below:

> Zeta Potential = (4 × π × Dynamic viscosity of liquid × Ionic mobility)/Relative permittivity of solvent

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Fungal Endophyte

The morphological identification characteristics of *P. oligotrophica* S8A26 include: pale pink mycelium, spores capsule shape with single septa, growth rate slow and sporulation occuring around 6 – 7 days after incubation. Morphological identification was confirmed by ITS-rDNA gene sequencing, which showed 99.61% of the known stains of *Plectospaerella oligotrophica*. The NFCCI and GenBank accession number obtained were “NFCCI 5222” and “OR357719” (Table 1, Figs. 1 – 2). Endophytic fungi are an essential part of medicinal plants and inhabit a peculiar habitat that is beneficial to both. Tropical and sub-tropical plants harbour diverse fungal endophytes. [20] conducted a study in a tropical forest in Indonesia and isolated 21 endophytic fungi from the roots of *Paraserianthes falcataria*. In a similar study, [21] isolated 31 endophytic fungi from the leaves and fruits of *Tamarindus indica* collected from Malaysia, belonging to 15 genera. Different types of *Plectosphaerella* have been found to live on aquatic plants in southwest China [22]; *Plectosphaerella guizhouensis* and *Plectosphaerella nauculaspora* [23]; *Plectosphaerella oligotrophica* from *Panax bipinnatifidus* [24]; *Plectosphaerella cucumerina* from *Cynanchum auriculatum* [25]; and *Plectosphaerella niemeij* are all types of *Plectosphaerella*. ITS-rDNA sequencing is considered an important technique for molecular identification of endophytic fungi, which gives rapid and reliable results [26].

3.2 Plant Growth Promotion Activities

P. oligotrophica S8A26 solubilize inorganic phosphate and also produce ammonia but unable to generate hydrogen cyanide (Fig. 3). Nitrogen and phosphorus are essential elements for the growth and development of plants, as they are required in crucial metabolic processes such as photosynthesis, cell cycle, and the synthesis of proteins and enzymes [27]. They are supplied in crop fields in the form of chemical fertilizers, which pose environmental and health hazards. The endophytic isolate *P. oligotrophica* S8A26 has the potential to supply nitrogen in the form of ammonia and could solubilized inorganic phosphate that can be easily absorbed by the plant roots. The plant growth promotion activities of endophytic fungi are of great importance as they are environment-friendly, reliable, and easily applicable in the fields. Evaluation of other activities like siderophore production and plant growth hormone production is required to fully understand the growth promotion abilities of *P. oligotrophica* S8A26.

3.3 Secondary Metabolite Production

The crude extract of *P. oligotrophica* S8A26 yields 513 mg (approximately) of dry metabolite from the filtrate of 1000 mL of PDB (Fig. 4). Secondary metabolites produced by fungal endophytes contain biologically active compounds that have vast applications in the fields of agriculture, pharmaceuticals, medicine, and industries.

3.4 Biochemical Analysis

The endophytic fungus *P. oligotrophica* S8A26 produces flavonoids, phenols, saponins, and steroids. The TFC and TPC were observed to be 25.67 µg of quercetin equivalent/ mg of endophyte extract and 5.06 µg of gallic acid equivalent/ mg of endophyte extract, respectively (Table 2). Flavonoid compounds are widely used for applications in nutraceuticals, pharmaceuticals, medicine, textiles, and cosmetics [28]. [29] isolated the anticancer flavonoid chrysin (5,7-dihydroxy flavone) from the endophytic fungi *Alternaria alternata*, *Colletotrichum capsici*, and *C. taiwanense* associated with *Passiflora incarnata*. Plenolic compounds have several health-promoting properties, such as antioxidant, anticancer, and

use in the treatment of cardiovascular and neurodegenerative diseases. They also possess anti-ageing properties, for which they are used in the cosmetic industry [30]. In a recent study, [31] isolated two phenolic compounds, tyrosol and p-hydroxyphenylacetamide, from the endophytic fungus *Coriolopsis rigida* obtained from the medicinal plant *Cochlospermum regium* that showed potent antioxidant activity with an EC_{50} of 0.33 mg/mL and effective allelopathic activity against the seedlings of *Lactuca sativa* and *Raphanus sativus*. Compounds belonging to saponins have been known to show antibacterial, antifungal, antiviral, anti-inflammatory, anticancer, antioxidant, and immunomodulatory effects [32]. [33] extracted eight saponins, viz., Cyclamine saponin, Aspoligonin A, Sarsapogenin, Asparacosin A, Schidigera saponin D, Aspargoside A, Dioscin, and Protodioscin, from the endophytic fungi *Aspergillus terreus*, *A. flavus*, *Penicillium* sp., and *Talaromyces pinophilus* associated with the medicinal plant *Asparagus racemosus* and found to exhibit strong antimicrobial and antioxidant activities. Natural steroids are rich sources of bioactive compounds with antioxidant, anticancer, and antioxidant activities [34]. In an investigation, nine steroids, namely, norcyclocitrinol A, erythro-11α-hydroxyneocyclocitrinol, pesudocyclocitrinol A, neocyclocitrinols A–D, cyclocitrinol, and 24-epicyclocitrinol, were extracted from the secondary metabolites of the endophytic fungus *Penicillium chrysogenum* isolated from *Huperzia serrata*, and all of them have shown anticancer activity [35]. [36] isolated the endophytic fungus *Nigrospora sphaerica* from *Euphorbia hirta*, and its extract has shown TPC and TFC values of 77.74 ± 0.046 mgGAE/g and 230.59 ± 2.0 mgRE/g, respectively. The endophytic fungus *P. oligotrophica* S8A26 in our study might be an important source of bioactive compounds. The isolation and evaluation of the bioactivities of the compounds would be an important aspect for pharmaceutical and industrial applications.

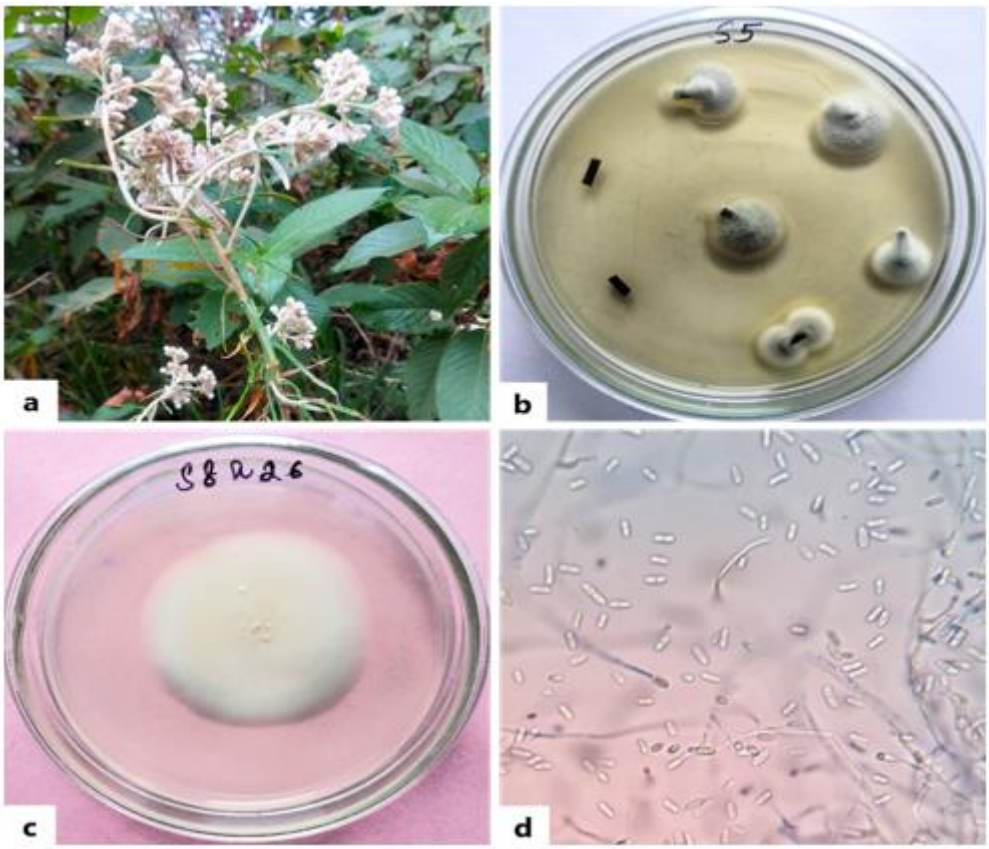

Fig. 1. (a) Anaphalis contorta plant, (b) isolation of fungal endophyte from plant segments, (c) culture morphology of Plectosphaerella oligotrophica S8A26, and (d) micrograph of *P. oligotrophica* S8A26

Table 1. The BLAST analysis report for *P. oligotrophica* S8A26 showing closely related five taxa available in the GenBank database

GeneBank Accession No.	Description	Max score	Query cover	Query coverage (%)	E value	Identity (%)
MT447499	*Plectosphaerella oligotrophica* strain GFRS31	921	921	100	0.0	99.61
MT447492	*Plectosphaerella oligotrophica* strain GFRS24	921	921	100	0.0	99.61
MT221576	*Plectosphaerella* sp. isolate RFE 5	921	921	100	0.0	99.61
MT032654	*Fusarium oxysporum* isolate G549	921	921	100	0.0	99.61
MN522947	*Plectosphaerella cucumerina* clone 2014_1356	921	921	100	0.0	99.61

Fig. 2. Sequence allignment report for P. oligotrophica S8A26 with the closest genetic neighbour strain Plectosphaerella oligotrophica strain GFRS31 (MT447499) in the NCBI GenBank database

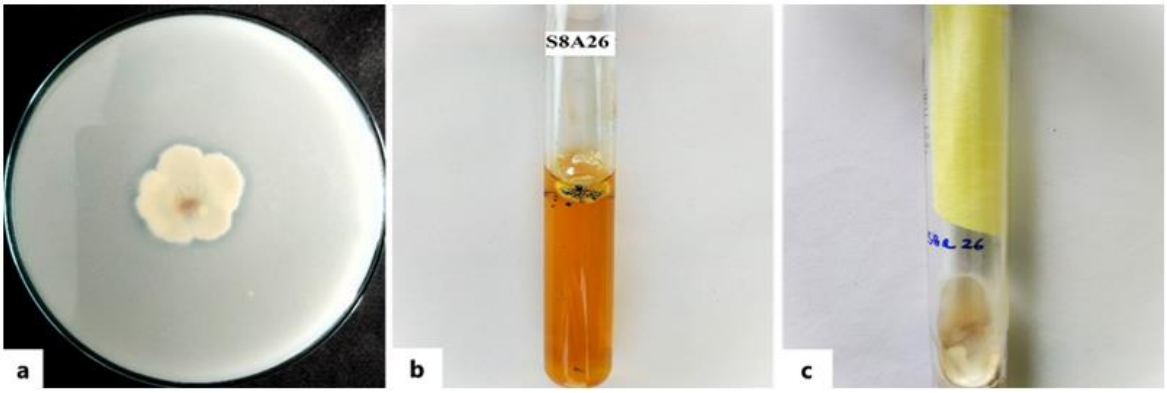

Fig. 3. Plant growth promotion activities of P. oligotrophica S8A26 showing positive results for (a) phosphate solubilisation and (b) ammonia production, and negative result for (c) hydrogen cyanide production

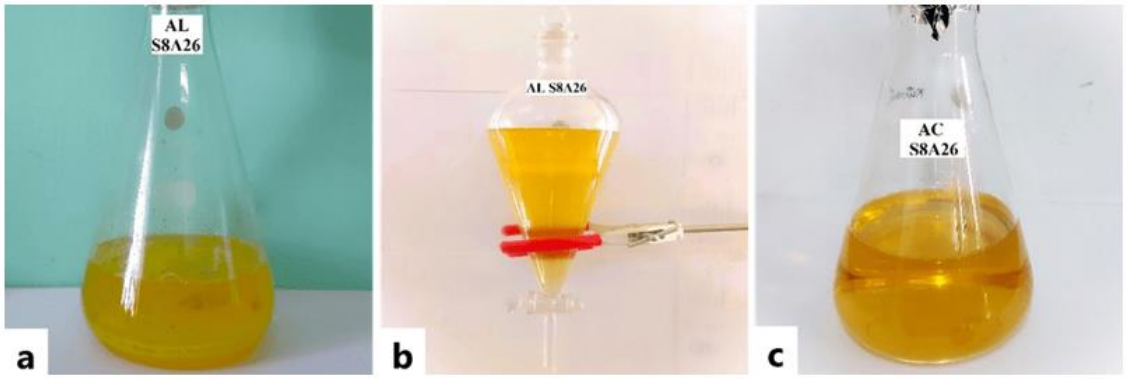

Fig. 4. Secondary metabolite extraction of *P. oligotrophica* S8A26, (a) culture in potato dextrose broth, (b) extraction with ethyl acetate using separating funnel, (c) crude ethyl acetate extract before drying

Table 1. Biochemical analysis of the secondary metabolite produce by *P. oligotrophica* S8A26

Endophyte	Biochemical analysis								
	Qualitative							Quantitative	
	AL	FL	PH	SA	ST	TA	TE	TFC (µg of QE/ mg of EE)	TPC (µg of GAE/ mg of EE)
Plectosphaerella oligotrophica S8A26	-	+	+	+	+	-	-	25.67±0.08	5.06±0.19

AL-Alkaloids, FL-Flavonoids, PH-Phenolics, SA-Saponins, ST-Steroids, TA- Tannins, TE- Terpenoids, QE-Quercetin equivalent, GAE- Gallic acid equivalent, EE- Endophyte extract '+' indicates presence, '-' indicates absence

3.5 Synthesis and Characterization of Silver Nanoparticles

The endophytic fungus *P. oligotrophica* S8A26 synthesizes AgNPs which was shown by the colour change of the filtrate from light yellow to dark brown due to the reduction of silver nitrate by the secondary metabolite produce. Biosynthesized silver nanoparticles have potential bioactivities with minimal side effects. The size of the nanoparticles is an essential criteria for determining their effectiveness; a smaller size increases bioactivities due to a greater surface area and a smaller volume. Several isolates of endopytic fungi have been known to synthesized AgNPs with various

activities, viz., *Exserohilum rostrate* from *Ocimum tenuiflorum* showed antibacterial, antioxidant, and anti-inflammatory activities [37]; *Penicillium cinnamopurpureum* from *Curculigo orchioides* showed antibacterial activity [38]; *Penicillium radiatolobatum* from *Quercus rubera* showed antibacterial and cytotoxic activities [39]; *Cladosporium perangustum* from *Dendrophthoe falcata* showed antioxidant and anticancer activities [19]; and *Colletotrichum gloeosporioides* from *Berberis aristata* showed antibacterial and antimalarial activities [40].

Table 3. XRD analysis of silver nanoparticles synthesized by *P. oligotrophica* (AS8A26) and calculation of particle size using Scherrer's equation

Position (2Θ)	Height (counts)	FWHM (2Θ)	d-spacing (Aº)	Relative Intensity (%)	AgNPs size (nm)	Average (nm)
27.6890	27.14	0.7872	3.22180	46.03	22.69	
32.1568	58.97	0.3936	2.78364	100.00	39.63	
46.3114	44.68	0.2952	1.96052	75.78	38.89	
54.7421	14.31	0.9840	1.67686	24.26	10.33	24.81
57.5218	14.55	0.7872	1.60226	24.68	12.50	

Table 4. EDX analysis of synthesized silver nanoparticles by *P. oligotrophica* S8A26 displaying the quantitative elemental composition of silver, oxygen, zinc and silicon

Element Line	Weight (%)	Weight Error (%)	Atom (%)
O K	49.72	± 0.83	86.50
Si K	0.45	± 0.07	0.45
Si L	---	---	---
Zn K	1.19	± 0.55	0.51
Zn L	---	---	---
Ag L	48.63	± 0.92	12.55
Ag M	---	---	---
Total	100.00		100.00

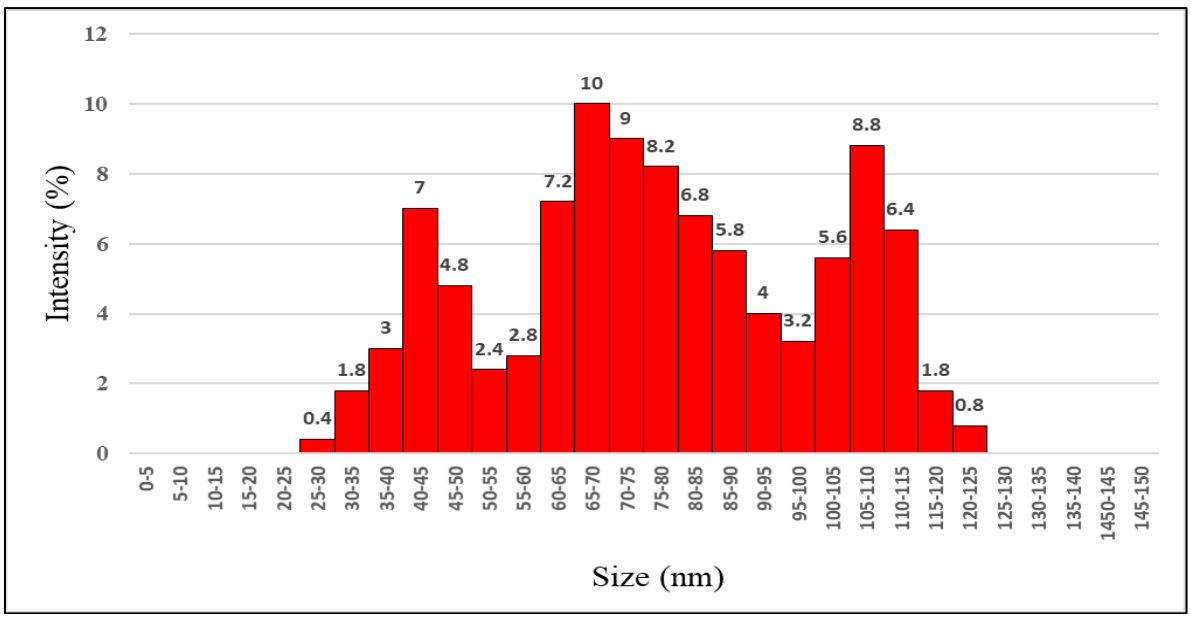

Fig. 5. Dynamic light scattering measurements for particle size distribution of the biosynthesized AgNPs using *P. oligotrophica* S8A26

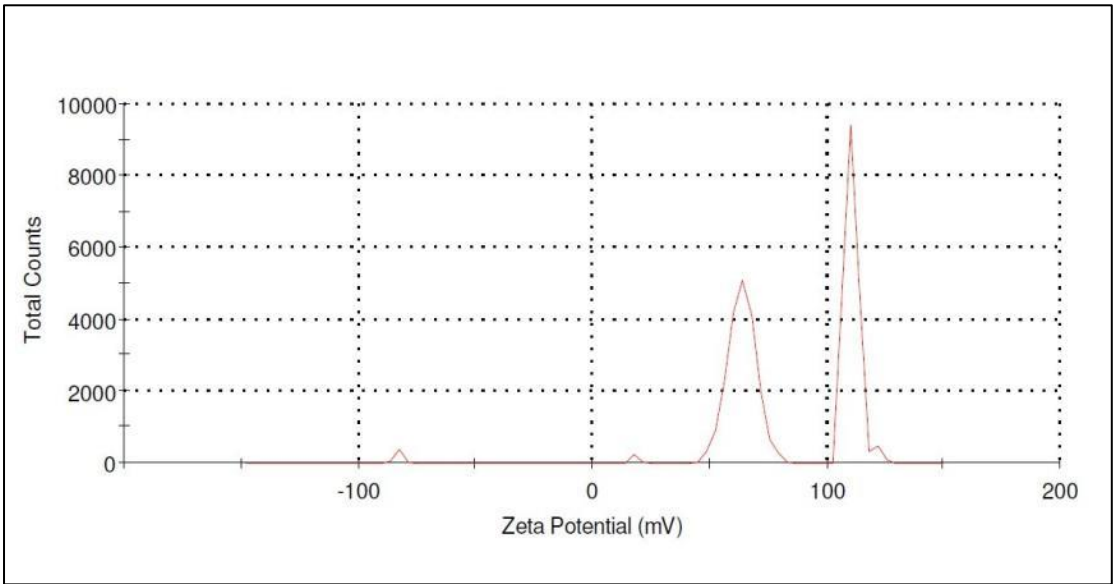

Fig. 6. Zeta potential measurements of synthesized nanoparticles using *P. oligotrophica* S8A26

3.5.1 XRD analysis

54.7421° and 57.5218° which shows the nanoparticles are crystalline. The peaks can be assigned to the planes (122), (111), (200), (220), and (311) facet of silver crystal, respectively. The average size of the synthesized nanoparticles was found to be 24.81 nm (Table 3).

3.5.2 EDX analysis

EDX analysis have shown the presence of silver as the main component. The elemental analysis of the synthesized silver nanoparticles showed the highest proportion of oxygen (49.72%), followed by silver (48.63%), zinc (1.19%) and silicon (0.45%) (Table 4).

3.5.3 Particle size distribution and Zeta potential of silver nanoparticles

Dynamic light scattering measurements for particle size distribution have shown the average size of the AgNPs to be 49.9 nm. The Particle size with the highest intensity (%) occurred in the 65–70 nm range (Fig. 5). The Zeta potential value for the AgNPs was found to be −31.8 mV (Fig. 6).

4. CONCLUSION

From the above results, it can be concluded that the endophyte *P. oligotrophica* S8A26 produces various compounds that might have bioactive properties. Being natural compounds, they posed negligible negative effects on human health. In the last few decades, the approach of environment-friendly agricultural practices like organic farming and integrated farming techniques has increased, and *P. oligotrophica* S8A26, due to its plant growth-promoting abilities, has become an important area of research. Synthesis of nanoparticles using endophytic fungi is encouraged as it is eco-friendly, cost-effective, requires less time, and is highly effective. The isolate *P. oligotrophica* S8A26 synthesizes AgNPs of small size, and further assessment of antimicrobial, antioxidant, anticancer, and anti-inflammatory activities is much needed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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