Phytochemical profiling, antioxidant capacity and indole-3-acetic acid production by endophytic fungi and bacteria isolated from Achyranthes aspera L.

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Abstract
Endophytic microorganisms associated with medicinal plants are recognized as promising sources of bioactive secondary metabolites with applications in pharmaceuticals and sustainable agriculture. In this study, endophytic bacteria and fungi were isolated from Achyranthes aspera and evaluated for their phytochemical composition, antioxidant activity, and plant growth–promoting potential. Endophytes were obtained from surface-sterilized tissues and identified using morphological and molecular approaches. Quantitative analysis revealed significant variation in phytochemicals, including phenolics, flavonoids, alkaloids, and tannins. Aspergillus sp. exhibited the highest levels of phenolics (11.21 ± 0.18 mg GAE/mL), flavonoids (3.83 ± 0.31 mg RE/mL), alkaloids (1.42 ± 0.09 mg/mL), and antioxidant activity (2.84 ± 0.12 mg AAE/mL), followed by the black fungal isolate, while bacterial isolates showed comparatively lower activity. All isolates produced indole-3-acetic acid (IAA), with Aspergillus sp. showing the highest production (229.1 ± 12.6 µg/mL). LC–MS analysis confirmed the presence of IAA and kinetin in Aspergillus sp., indicating the production of multiple phytohormones. These findings highlight the superior metabolic capability of fungal endophytes and their potential as sustainable sources of natural antioxidants and plant growth–promoting agents.

Keywords: Achyranthes aspera, endophytic bacteria, endophytic fungi, phytochemicals, antioxidant activity, indole-3-acetic acid, LC–MS

Introduction

Medicinal plants are well recognized as important sources of bioactive secondary metabolites that contribute significantly to the prevention and treatment of various diseases. These metabolites, including alkaloids, phenolics, flavonoids, tannins, and terpenoids, exhibit a wide range of pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, and anticancer activities [1,2]. However, the increasing demand for plant-derived therapeutics, coupled with issues related to overharvesting, seasonal variation, and inconsistent phytochemical yield, has necessitated the exploration of alternative and sustainable sources of these bioactive compounds [3].

Achyranthes aspera L. (family Amaranthaceae), commonly known as prickly chaff flower, is a widely distributed medicinal plant in tropical and subtropical regions. It has been traditionally used for the treatment of inflammatory disorders, respiratory diseases, gastrointestinal disturbances, skin infections, and metabolic ailments [4,5]. Phytochemical investigations of A. aspera have reported the presence of saponins, alkaloids, phenolic compounds, and flavonoids, which are responsible for its diverse therapeutic activities, including antioxidant and anti-inflammatory effects [6,7,8].

In recent years, endophytic microorganisms inhabiting healthy plant tissues without causing apparent harm have gained considerable attention as alternative producers of plant-associated bioactive metabolites. Endophytes are known to synthesize a broad spectrum of secondary metabolites that are structurally and functionally similar to those produced by their host plants, making them promising candidates for pharmaceutical and agricultural applications [1,9]. In addition to bioactive compounds, several endophytes produce plant growth–promoting substances such as indole-3-acetic acid (IAA), siderophores, and cytokinins, which contribute to enhanced plant growth, nutrient acquisition, and stress tolerance [10,11].

Among endophytic microorganisms, fungi represent a highly diverse and metabolically versatile group. Endophytic fungi have been reported to produce antioxidants, antimicrobial agents, anticancer compounds, and phytohormones, highlighting their potential role in bioprospecting and natural product-based drug discovery [1,10]. Similarly, endophytic bacteria, such as Pseudomonas aeruginosa and Bacillus spp., play complementary roles in PGP through IAA production, phosphate solubilization, and endospore formation for host stress tolerance. These bacteria have been isolated from A. aspera and enhance antioxidant activity and growth [12, 13]. Despite the well-documented medicinal importance of A. aspera, systematic investigations focusing on the isolation, molecular characterization, and bioactive potential of its associated endophytic fungi remain limited [9,5].

Therefore, the present study was undertaken to isolate and identify endophytic fungi and endophytic bacteria from Achyranthes aspera and to evaluate their phytochemical composition and bioactive potential [14]. The antioxidant activity and plant growth–promoting properties of the endophytic extracts were assessed through quantitative estimation of phenolics, flavonoids, alkaloids, and indole-3-acetic acid production, with liquid chromatography–

mass spectrometry (LC–MS) employed for metabolite confirmation. This study aims to highlight the pharmaceutical relevance of endophytic fungi and endophytic bacteria associated with A. aspera as sustainable sources of bioactive phytochemicals and phytohormones.

2. Materials and Methods

2.1 Study area and collection of plant material

Healthy and disease-free specimens of Achyranthes aspera L. were collected from Ramgarh district, Jharkhand, India during September–October, corresponding to the peak flowering season. Collected samples were transported to the laboratory in sterile polyethylene bags and processed within 24 h of collection.

2.2 Isolation of endophytic fungi

Leaf and stem tissues were thoroughly washed under running tap water to remove surface contaminants and subjected to surface sterilization following the standard protocol described for endophytic fungi isolation [15, 16]. Briefly, tissues were treated sequentially with 70% ethanol, sodium hypochlorite solution, and sterile distilled water. The sterilized tissues were aseptically cut into small segments (approximately 5 × 5 mm) and placed on Potato Dextrose Agar (PDA) supplemented with chloramphenicol (50 µg/mL) to suppress bacterial growth. Plates were incubated at 25–28°C for up to 14 days. Emerging fungal colonies were repeatedly subcultured to obtain pure isolates. Sterility checks were performed by imprinting sterilized tissues on PDA plates to confirm the effectiveness of surface sterilization.

2.3 Isolation of endophytic bacteria

For bacterial isolation, sterilised leaf and stem tissues were macerated in sterile phosphate-buffered saline (PBS) using a mortar and pestle. Serial dilutions (10^{-1} to 10^{-6}) were prepared and plated on Nutrient Agar (NA). Plates were incubated at 28–30°C for 24–72 h. Distinct colonies were subcultured to obtain pure isolates, including NB3 and NB4 [17]. Sterility was verified by imprinting tissues on NA plates.

2.4 Identification and staining of endophytic bacteria and fungi

Fungal DNA was extracted using the CTAB method [17]. The ITS region was amplified using primers ITS-1F and ITS-4R [18]. Amplicons were sequenced via Sanger method and identified using BLAST. Fungal structures were stained with lactophenol cotton blue for microscopy.

Bacterial isolates were identified via colony morphology, Gram staining, and biochemical tests (catalase, oxidase). Gram staining was performed using crystal violet, iodine, alcohol, and safranin. NB3 appeared as Gram-positive rods with endospores (likely Bacillus sp.), while NB4 showed Gram-negative short rods with green pigment (most probably Pseudomonas aeruginosa) [12,13].

2.5 Production and extraction of fungal metabolites

Pure endophytic fungal isolates were cultured in Potato Dextrose Broth and Czapek–Dox Broth under submerged fermentation conditions at 28°C with shaking at 150 rpm for 7–14 days. After incubation, cultures were filtered to separate mycelial biomass from the culture filtrate. Secondary metabolites were extracted from the filtrate using organic solvents such as ethyl acetate, methanol, and chloroform, following standard extraction procedures [9]. The organic layers were concentrated under reduced pressure using a rotary evaporator and stored at 4°C until further analysis.

2.6 Qualitative phytochemical screening

Preliminary qualitative phytochemical screening of the fungal extracts was carried out to detect the presence of alkaloids, phenolics, flavonoids, tannins, saponins, glycosides, carbohydrates, and proteins using standard chemical tests as described by Harborne and Sofowora [19,3]. The presence of phytochemicals was confirmed based on characteristic colour changes or precipitate formation.

2.8 Quantitative estimation of phytochemicals

2.8.1 Determination of total phenolic content

Total phenolic content (TPC) of the fungal extracts was estimated using the Folin–Ciocalteu method with gallic acid as the standard [20]. Absorbance was measured spectrophotometrically, and the results were expressed as milligrams of gallic acid equivalents (GAE) per millilitre of extract.

2.8.2 Determination of total flavonoid content

Total flavonoid content (TFC) was determined using the aluminium chloride colorimetric method described by Chang et al. [21], with rutin as the reference standard. Absorbance was recorded at 510 nm, and flavonoid content was expressed as milligrams of rutin equivalents per milliliter of extract.

2.8.3 Estimation of total alkaloid content

Total alkaloid content was quantified using the bromocresol green (BCG) spectrophotometric method with atropine as the standard [22]. Absorbance was measured at 470 nm, and alkaloid concentration was expressed as milligrams per millilitre of extract.

2.9 Evaluation of antioxidant activity

The antioxidant activity of endophytic fungal extracts was evaluated using the ferric reducing antioxidant power (reducing power) assay [23]. Absorbance was measured at 700 nm, and antioxidant activity was expressed as

milligrams of ascorbic acid equivalents (AAE) per millilitre of extract.

2.10 Indole-3-acetic acid (IAA) production

Endophytic isolates were screened for IAA production by culturing them in Luria–Bertani broth at 28–30°C for 48–72 h under shaking conditions. Cell-free supernatants were obtained by centrifugation and reacted with Salkowski reagent. The development of a pink colour indicated IAA production. Absorbance was measured at 530–550 nm, and IAA concentration was calculated using a standard curve prepared with commercial IAA [24].

2.11 LC–MS analysis

Liquid chromatography–mass spectrometry (LC–MS) analysis was performed to confirm the presence of phytohormones and bioactive metabolites in selected fungal extracts. Chromatographic separation was carried out using a reverse-phase column under gradient elution conditions. Mass spectra were acquired in positive ionisation mode, and compound identification was based on retention time, accurate mass measurement, mass error (ppm), and comparison with spectral libraries [25].

2.12 Statistical analysis

All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation (SD). Data analysis was performed using standard statistical procedures.

3.Results

3.1 Isolation and identification of endophytic fungi and Bacteria

Endophytic microorganisms were successfully isolated from surface-sterilised leaf and stem tissues of Achyranthes aspera. Fungal isolates included Aspergillus sp. and a black fungal isolate, which were identified based on morphological characteristics and ITS sequencing [9]. NB3 appeared as Gram-positive rods with endospore formation, indicating characteristics of Bacillus sp., whereas NB4 showed Gram-negative short rods with green pigmentation, consistent with Pseudomonas aeruginosa. Lactophenol cotton blue staining was performed for morphological identification of fungal endophytes. Microscopic observations revealed characteristic features of Aspergillus flavus, including olive-green conidial heads, globose vesicles, and radiating phialides. In addition, the black fungal isolate was identified as Rhizopus stolonifer based on the presence of well-developed stolons, rhizoids, and large globose sporangia arising opposite the rhizoids. These structural features are consistent with standard taxonomic descriptions of these fungal species and confirm their identity. These isolates exhibited distinct growth patterns and were selected for further phytochemical and biochemical analysis [12,13].

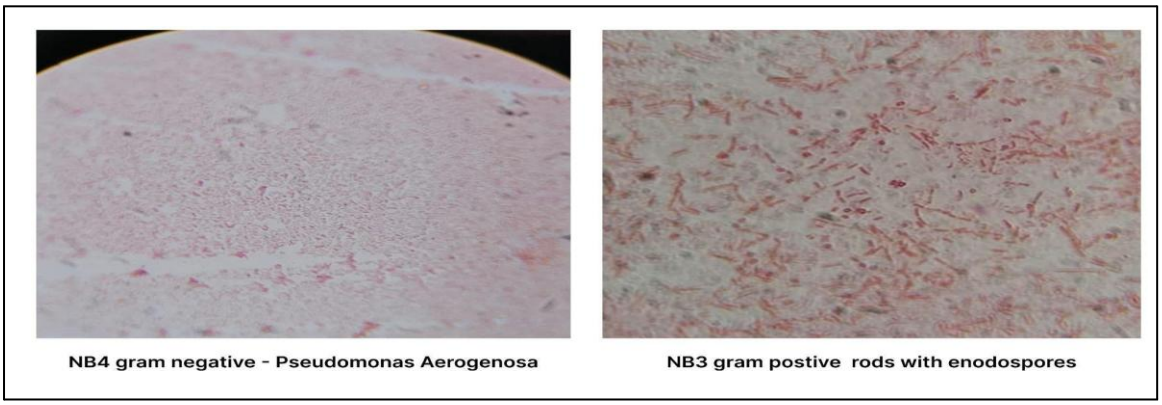


Figure 1. Gram staining of bacterial endophytes (NB3: Gram +ve rods with endospores; NB4: Gram -ve short rods with green pigment)

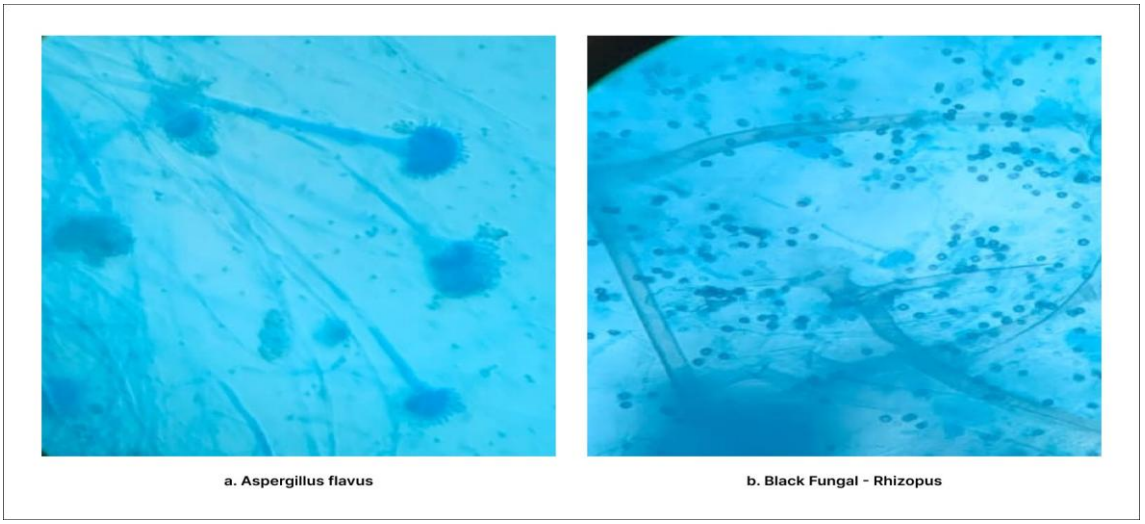


Figure 2. Lactophenol cotton blue staining of endophytic fungal isolates showing morphological characteristics of a. Aspergillus flavus (olive-green conidia and radiate conidial head) and b. Black fungal

- Rhizopus (sporangia, rhizoids, and sporangiophores)

3.2 Qualitative phytochemical screening

Qualitative phytochemical analysis revealed the presence of alkaloids, phenolics, flavonoids, tannins, and saponins in the endophytic fungal extracts, with variation among isolates. The black fungal isolate and Aspergillus sp. exhibited a wider range of detectable phytochemical classes compared to NS3 and NS4 [11,27].

Table 1. Qualitative phytochemical profile of endophytic fungal extracts

Isolate	Alkaloids	Phenolics	Flavonoids	Tannins	Saponins	Overall phytochemical richness
NS3	Absent	Low	Absent	Absent	Absent	Low
NS4	Absent	Low	Low	Low	Absent	Low–Moderate
Black fungal isolate	Present	High	High	High	Present	High
Aspergillus sp.	Present	High	High	Moderate	Present	High

3.3 Quantitative phytochemical analysis

The quantitative estimation of phytochemicals revealed significant variation among the endophytic isolates (Table 2). Among all isolates, Aspergillus sp. exhibited the highest phenolic content (11.21 ± 0.18 mg GAE/mL) [7,26], flavonoid content (3.83 ± 0.31 mg RE/mL) [2,27], and alkaloid content (1.42 ± 0.09 mg/mL) [1,11], indicating strong biosynthetic potential.

The black fungal isolate also demonstrated high phytochemical richness, particularly in tannins (4.10 ± 0.00%) [26] and flavonoids (3.45 ± 0.29 mg RE/mL) [2,27]. In contrast, bacterial isolates NS3 and NS4 showed comparatively lower phytochemical concentrations, although NS4 exhibited moderate flavonoid content (1.52 ± 0.13 mg RE/mL) [2,27].

Tannin content showed notable variation among the isolates, with the highest levels observed in the black fungal isolate (4.10 ± 0.00%), followed by Aspergillus sp. (3.90 ± 0.10%), while NS4 showed low tannin levels and NS3 showed negligible content [26].

Similarly, antioxidant activity varied significantly among the isolates, with Aspergillus sp. exhibiting the highest activity (2.84 ± 0.12 mg AAE/mL), followed by the black fungal isolate (2.28 ± 0.10 mg AAE/mL), whereas NS3 and NS4 showed comparatively lower activity [7,8].

Overall, fungal endophytes demonstrated superior phytochemical production and antioxidant potential compared to bacterial isolates.

Table 2. Quantitative phytochemical composition and antioxidant activity of endophytic isolates.

Isolate	Phenolics (mg GAE/mL)	Flavonoids (mg RE/mL)	Alkaloids (mg/mL)	Tannins (%)	Antioxidant (mg AAE/mL)
NS3	3.57 ± 0.15	0.46 ± 0.05	ND	0.00 ± 0.00	1.24 ± 0.06
NS4	3.32 ± 0.17	1.52 ± 0.13	ND	0.40 ± 0.05	1.64 ± 0.08
Black fungal isolate	9.90 ± 0.17	3.45 ± 0.29	1.18 ± 0.08	4.10 ± 0.00	2.28 ± 0.10
Aspergillus sp.	11.21 ± 0.18	3.83 ± 0.31	1.42 ± 0.09	3.90 ± 0.10	2.84 ± 0.12

Note: Values expressed as mean ± SD (n = 3); ND = Not detected.

3.4 Indole-3-acetic acid (IAA) production

All endophytic isolates demonstrated the ability to produce indole-3-acetic acid (IAA), with significant variation among them (Table 3). Aspergillus sp. exhibited the highest IAA production (229.1 ± 12.6 µg/mL), followed by the black fungal isolate (183.3 ± 8.4 µg/mL).

Bacterial isolates NS4 (96.2 ± 6.1 µg/mL) and NS3 (72.4 ± 5.8 µg/mL) showed comparatively lower IAA production, consistent with their moderate plant growth–promoting potential [9,10].

Table 3. Indole-3-acetic acid (IAA) production by endophytic fungal isolates

Endophytic isolate	IAA production (µg/mL)
NS3	72.4 ± 5.8
NS4	96.2 ± 6.1
Black fungal isolate	183.3 ± 8.4
Aspergillus sp.	229.1 ± 12.6

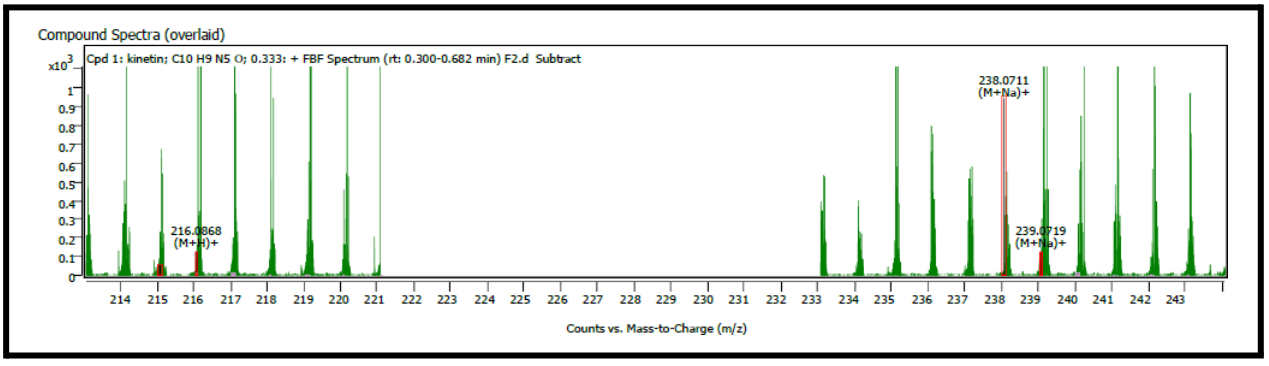
3.5 LC–MS confirmation of phytohormones

LC–MS analysis confirmed the presence of phytohormones in selected endophytic fungal extracts (Table 4). In Aspergillus sp., both indole-3-acetic acid (IAA) and kinetin were identified based on accurate mass measurement, low mass error (<5 ppm), and high match scores (>90).

In contrast, another isolate showed the presence of kinetin only. The observed retention times and mass spectral data are consistent with previously reported LC–MS studies [29,30], showing in figure 1 confirming the reliability of metabolite identification.

Table 4. LC–MS identification of phytohormones in endophytic fungal extracts

Sample code	Identified compound	Molecular formula	RT (min)	Theoretical m/z	Observed m/z	Mass error (ppm)	Match score
B4 (*Aspergillus* sp.)	Indole-3-acetic acid (IAA)	$C_{10}H_9NO_2$	0.410	175.0633	175.0638	2.68	91.47
B4 (*Aspergillus* sp.)	Kinetin	$C_{10}H_9N_5O$	0.410	215.0807	215.0814	3.29	90.14
F2	Kinetin	$C_{10}H_9N_5O$	0.333	215.0807	215.0815	3.58	79.48



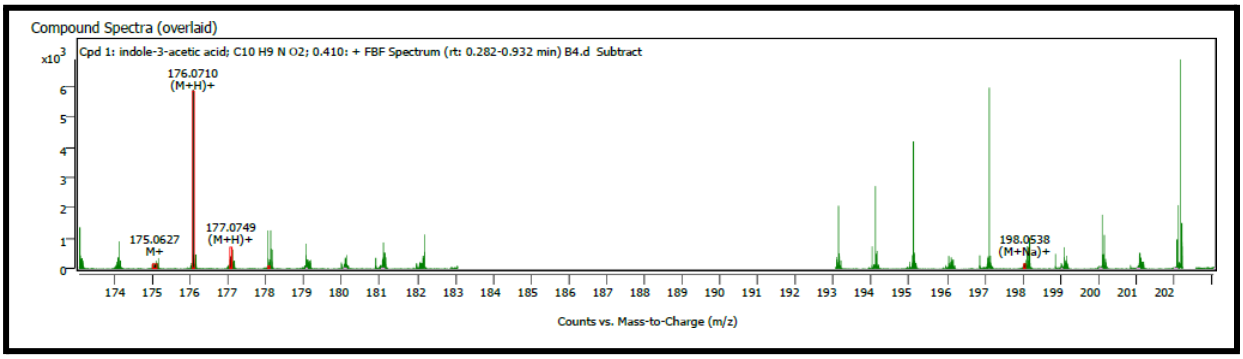


Figure 3. LC–MS chromatograms and mass spectra of endophytic fungal extracts showing detection of indole-3-acetic acid (IAA) and kinetin in Aspergillus sp. (sample B4), and detection of kinetin in sample F2, confirmed by accurate mass measurement and low mass.

4. Discussion

Endophytic microorganisms associated with medicinal plants are increasingly recognised as valuable sources of bioactive secondary metabolites with pharmaceutical and agricultural relevance. In the present study, endophytic bacteria and fungi isolated from Achyranthes aspera

demonstrated the ability to produce diverse phytochemicals, including phenolics, flavonoids, alkaloids, tannins, and phytohormones. Similar findings have been reported in recent studies highlighting the metabolic versatility of endophytes and their capacity to synthesize plant-associated bioactive compounds [9,11]. These observations support the ecological role of endophytes in enhancing host plant defense and adaptation through co-evolutionary interactions and metabolite exchange.

A key outcome of this study is the integrated evaluation of phytochemical composition and antioxidant activity (Table 2), which enables direct comparison across isolates. Marked isolate-specific variation was observed, with fungal isolates such as Aspergillus sp. and the black fungal isolate consistently exhibiting higher levels of phenolics, flavonoids, tannins, alkaloids, and antioxidant activity compared to bacterial isolates NS3 and NS4. Such variability has been widely documented and is attributed to genetic diversity, host–microbe interactions, and ecological adaptation within plant tissues [10,11].

The strong antioxidant activity observed in Aspergillus sp. and the black fungal isolate correlates well with their elevated phenolic and flavonoid contents, as presented in Table 2. Phenolic compounds are well known for their redox properties, enabling them to act as reducing agents and free-radical scavengers. Similar correlations between phenolic concentration and antioxidant activity have been reported in previous studies [7,8]. In contrast, bacterial isolates exhibited comparatively lower antioxidant activity, which may be associated with reduced phenolic biosynthesis, although their role in plant growth promotion remains significant.

Selective production of alkaloids and tannins further highlights the metabolic diversity among the isolates. Alkaloids are pharmacologically important due to their antimicrobial, anti-inflammatory, and anticancer properties, while tannins contribute to antioxidant and protective functions. The restricted occurrence of these compounds in fungal isolates suggests strain-dependent biosynthetic pathways, as reported in earlier studies [1,11]. Fungal dominance in these metabolite classes may reflect the presence of specialised gene clusters involved in secondary metabolism.

All endophytic isolates demonstrated the ability to produce indole-3-acetic acid (IAA), although significantly higher levels were observed in fungal isolates, particularly Aspergillus sp. This supports the role of endophytes as plant growth–promoting agents. Similar findings have been reported for endophytic microorganisms isolated from medicinal plants, where IAA production enhances root development and nutrient uptake [9,10]. Bacterial isolates such as Pseudomonas aeruginosa and Bacillus sp. showed moderate IAA production, consistent with their known mechanisms, including tryptophan-dependent auxin biosynthesis and stress tolerance through endospore formation [12,13].

LC–MS analysis provided confirmatory evidence for phytohormone production, with Aspergillus sp. producing both indole-3-acetic acid and kinetin. This validates the colorimetric IAA assay and demonstrates the ability of a single endophytic isolate to synthesise multiple phytohormones. The coexistence of auxins and cytokinins suggests a complex regulatory role in plant–microbe interactions, as supported by previous LC–MS-based studies [28,29,30]. Although LC–MS analysis was not performed for bacterial isolates, their IAA production indicates potential for similar metabolite synthesis, warranting further investigation.

Overall, the findings of this study demonstrate that endophytic microorganisms associated with Achyranthes aspera, particularly Aspergillus sp., possess significant potential as sustainable sources of bioactive metabolites. The integration of phytochemical and antioxidant data strengthens the interpretation of their functional capabilities and highlights their applicability in pharmaceutical development and sustainable agriculture. Future studies focusing on compound isolation, bioactivity-guided fractionation, and molecular characterisation will further elucidate their biotechnological potential and enable the development of effective microbial consortia for

enhanced efficacy.

5. Conclusion

The present study demonstrates that endophytic bacteria and fungi isolated from Achyranthes aspera are capable of producing a diverse range of bioactive secondary metabolites, including phenolics, flavonoids, alkaloids, tannins, and phytohormones. The integrated analysis of phytochemical composition and antioxidant activity revealed significant isolate-specific variation, with fungal endophytes, particularly Aspergillus sp., exhibiting superior biosynthetic potential.

The strong correlation between elevated phenolic and flavonoid contents and enhanced antioxidant activity highlights the functional significance of these metabolites in free-radical scavenging and redox balance. In addition, the ability of all isolates to produce indole-3-acetic acid confirms their role as plant growth–promoting endophytes, with fungal isolates showing comparatively higher production levels.

LC–MS-based confirmation of indole-3-acetic acid and kinetin in Aspergillus sp. further substantiates the metabolic versatility of endophytic fungi and validates the biochemical assays employed in this study. The coexistence of multiple phytohormones within a single isolate suggests a complex regulatory role in plant–microbe interactions and enhanced applicability in sustainable agriculture.

Overall, the findings establish endophytic microorganisms associated with Achyranthes aspera as promising and sustainable sources of bioactive compounds with potential applications in pharmaceutical development and agricultural bioformulations. Future research focusing on compound isolation, structural characterisation, and bioactivity-guided studies is warranted to fully exploit their therapeutic and biotechnological potential.

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