

Isolation, identification and biochemical characterization of endophytic fungi from sunflower *Helianthus annuus* L

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Abstract: Endophytic fungi live inside healthy plant tissues and have become common plant partners. These fungi not only help plants grow and produce pigments, enzymes and bioactive compounds, but they can also harm plant tissues and produce toxins. They are found in various parts of plants, such as stem, shoot, root, leaves and bark. Sunflower (*Helianthus annuus* L.) is a universal host for many species of endophytic fungi, which possess unique medicinal properties. This study aimed to isolate, identify and analyze the biochemical characteristics of endophytic fungi from sunflower. Sunflower (*Helianthus annuus* L.) plant was collected and chopped into smaller pieces and put on potato dextrose agar (PDA) medium for fungal growth. Fungi were separated, cleaned and maintained in a continuous subculture. After morphological and molecular identification sample was subjected to biochemical profiling by FTIR. Results showed the presence of *Rhizopus sp.*, *Cladosporium sp.*, *Penicillium sp.* and *Aspergillus sp.* in sunflower and structural determination by FTIR showed a number of bands and peaks.

Keywords: Endophytic fungi; *Helianthus annuus* L; Identification; Morphology; Phytochemicals

Submitted on 01-01-2025 – Revised on 13-02-2025 – Accepted on 06-08-2025

INTRODUCTION

Endophyte” refers to all microorganisms that colonize the internal tissues of a plant during all or part of their life cycle (Hardoim *et al.*, 2015). Microbial endophytes colonizing deep tissues of plant roots are often considered a subset of rhizosphere microbes because large root exudates are chemoattractants that make the rhizosphere zone a large microbial hot spot that can freely enter plant roots and penetrate from the rhizoplane. (Santoyo *et al.*, 2016).

The potential of endophytic fungi as a new source of bioactive chemicals has been recognized worldwide. Their pharmacological properties have received considerable attention, especially in the fields of antimicrobial, anticancer and antidiabetic agents. Fungi have been found to produce more intestinal permeability than bacterial or archaeal toxins. The complex relationship between the fungi that grow inside and their host plants can make their plants act as medicine. (Adeleke and Babalola, 2021). These endophytes have been found to produce various bioactive compounds that can be used for medicinal purposes. (Chaachouay and Zidane, 2024).

Traditional medical systems have used medicinal plants for centuries to treat a variety of diseases. They contain many phytochemicals that can be used for medicinal purposes. Because medicinal plants are more readily available, effective, economical and less toxic than synthetic drugs, their use in herbal medicine is gaining popularity. However, pharmacological studies have not

been able to accurately determine the potential of medicinal plants due to a lack of knowledge about their uniqueness and efficacy (Ekor, 2014).

Sunflower (*Helianthus annuus* L.) is a member of the Asteraceae family grown commercially around the world for its many nutritional and health benefits. In 2012, they ranked fourth in the world (8% of 186 Mt of oil produced), behind canola (13%), palm (29%) and soybean (22%) (Prolea *et al.*, 2012). Due to phenolic compounds, flavonoids, polyunsaturated fatty acids and vitamins, sunflower (*H. annuus* L.) seeds and shoots offer significant health benefits such as wound healing, cardiovascular health, antibacterial, anti-inflammatory and antihypertensive properties (Fowler, 2006).

According to (Bashir, 2015) sunflower (*Helianthus annuus* L.) is used in ethnomedicine to treat a variety of diseases such as heart, bronchial, laryngeal and lung diseases. Sunflower and its constituents are popular globally due to these significant nutritional, pharmacological and culinary benefits. Due to its many medicinal properties, the seeds, leaves and roots have been used in herbal remedies to treat various diseases. However, little is known about endophytic bacteria associated with sunflower (*H. annuus* L.) (Balogun *et al.*, 2023).

The detection of active chemicals in plants is useful for studying the fungi growing inside them. The beneficial effects of sunflower seeds can be attributed, at least in part, to their antioxidant properties. The frequent use of medicinal plants as fertilizers plays a vital role in the production of bioactive fungal spores (Kumar *et al.*, 2022).

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Because endophytic fungi form a close relationship with host plants, they can synthesize nutrients to enhance growth, competition and protection against diseases and herbivores. They are known to produce a variety of bioactive compounds including phenols, lactones, quinones, coumarins, phenylpropanoids, terpenoids, steroids and lignans (Burrigoni and Jeon, 2021).

To find new sources of secondary metabolites with practical biological functions, it is necessary to study the endophytic fungi of sunflower (*H. annuus L.*). Endophytic fungi are important for innovation of new antibiotics, chemotherapeutic drugs and agrochemicals. They may be good sources of new biologically active metabolites due to their relatively unknown nature (Jha *et al.*, 2023).

MATERIALS AND METHODS

Instruments and equipment

Dissecting Scissors and Scalpel (Sigma-Aldrich, USA), Laminar Air Flow Cabinet (LAF-90, Esco Lifesciences, Singapore), Autoclave (Sanyo MLS-3781L Japan), Hot Air Oven (DHG-9140A, Memmert GmbH Germany), Compound Microscope (Olympus CX23 Japan), Stereoscopic Microscope (Nikon SMZ-800N Japan), FTIR Spectrophotometer (Shimadzu IRTracer-100 Japan), Mortar and Pestle / Grinder (IKA A11 Basic Analytical Mill Germany) were used throughout the study.

Collection of plants

The selected plant was collected from common flora of Azad Kashmir, Pakistan and identified by botanical experts. The collected plant components were stored in polythene bags under high humidity conditions to maintain their integrity. The plant materials were cut into 5 mm sections and subjected to a sterilization process. This involved sequential immersion in 70% ethanol and 2% sodium hypochlorite for one minute each, as per the method described by (Pishgouii *et al.*, 2023).

The plant materials were air-dried under sterile conditions. To minimize bacterial contamination, plant segments were placed on potato dextrose agar (PDA) plates supplemented with 50 µg/ml of chloramphenicol. The plates were then sealed with parafilm and incubated in a Biological Oxygen Demand (BOD) incubator at 28 ± 2°C for 5-8 days. Fungal colonies emerging from the plant segments were carefully transferred using sterile techniques from hyphal tips onto fresh PDA plates to establish pure fungal cultures. Morphologically distinct fungal strains were selected for further investigation (Shubha and Srinivas, 2017b).

Isolation of endophytic fungi

Following adequate drying, the surface-sterilized plant material was chopped into smaller pieces and putted on

potato dextrose agar (PDA) medium that had been treated with 100 mg/ml of chloramphenicol.

Every plate was kept at 28°C to encourage the formation of endophytes and any microbial growth was constantly checked. Subculturing was carried out after the microbial growth was seen. After being examined for purity, each endophytic culture was moved to a newly prepared PDA plate (Dhankharet *et al.*, 2013). According to (Jayanthi *et al.*, 2011) the new fungus was separated, cleaned and maintained in a continuous subculture.

Maintenance of endophytes

The purified endophytic isolate was accessioned appropriately after being placed individually to PDA slants. All of the endophytes that had been purified were kept at 4°C.

Fungal morphological characterization

Different criteria were used to identify the fungal strains, including the shape of the hyphae or culture, the traits of the spores and the reproductive structures if the features could be seen using lactophenol or lactophenol cotton blue stains (Watanabe, 2010). The Olympus C-5060 Wide Zoom camera was used to shoot the pictures.

Biochemical profiling of isolated fungi

Fourier-transform infrared Spectroscopy

The presence of different types of chemical bonds or functional groups in phytochemicals was determined using a Fourier Transform Infrared Spectrophotometer (FTIR). All hydroethanolic extracts in dry form were used for FTIR analysis. Potassium bromide (KBr) powder was milled with dried extract powder (10 mg) and pressed into pellets to prepare extract pellets for analysis. After making the pellets, an FTIR spectrometer in the frequency range of 4000-400-4,000/cm was used to identify functional groups as representative of a wide range of important phytochemical constituents (Munir *et al.*, 2024).

Statistical analysis

All data are expressed as mean ± standard deviation. One-way ANOVA was included in the statistical analysis. A "P value" of 0.05 was recognized as significant.

RESULTS

In this study, fungal species were isolated from Sunflower (*Helianthus annuus L.*). Plant species were collected and processed to identify endophytic fungi. Four different fungal species were observed under a trinocular microscope. The microscopic and macroscopic features of each fungal species were recorded and identified based on the observed features (<https://mycology.adelaide.edu.au>) (Rodrigues *et al.*, 2007). In this study, fungal species were isolated from Sunflower (*H. annuus L.*). Plant

species were collected and processed to identify endophytic fungi. Four different fungal species were observed under a trinocular microscope (Rodrigues *et al.*, 2007). The microscopic and macroscopic features of each fungal species were recorded and identified based on the observed features Mycology Online. Available at: <https://mycology.adelaide.edu.au> (Accessed 5 May 2023).

Table 1: Endophytic fungal species identified based on macro and micro characteristics of (*Helianthus annuus L*) sunflower.

Fungal isolates	Fungal name
Isolate 1	<i>Rhizopus sp.</i>
Isolate 2	<i>Cladosporium sp.</i>
Isolate 3	<i>Penicillium sp.</i>
Isolate 4	<i>Aspergillus sp.</i>

DISCUSSION

Macroscopic and microscopic images of the isolated fungal species are shown in fig. 1 and 2. Table 1 describes the macroscopic and microscopic features of the isolated fungal species. Four fungal species were isolated from the samples belonging to the families Rhizopodaceae, Davidiaceae, Aspergillaceae and Trichocomaceae. The isolated endophytic fungi were classified as *Rhizopus sp.*, *Cladosporium sp.*, *Penicillium sp.* and *Aspergillus sp.* according to their microscopic and macroscopic features. (Table 1).

Biochemical profiling

According to infrared spectroscopy in table 2, the absorption peaks at range 4,000 to 2,500/cm was associated with the stretching vibration of signal bond formed by hydrogen and other element like O-H, N-H and C-H; peaks at 2500 to 2000/cm explored the absorption of light due to triple bond e.g. C≡C, C≡N; peaks at 2000 to 1500/cm represent the presence of compound with double bonds including C=C, C=O while absorption peaks at 1500 to 650 /cm consisted of many complicated bands and this part of the spectrum is unique to each compound also called fingerprint region. It is rarely used to identify particular functional groups like C-C, C-N, C-O, C-Cl, C-I, S-S and N=O*. According to infrared spectroscopy in fig 3. selected sample extracts FTIR analysis revealed absorption peaks at 3,500-4,000/cm, which meant that there were hydrogen bonds in intramolecular and intermolecular regions. The absorption peak at 2,940/cm was attributed to the asymmetric stretching vibration of C-H. The peak at 2,201/cm might be related to stretching vibration of C-H. The absorption peak at 1,889/cm was attributed to the symmetrically stretching vibration of C=O. The absorption peaks at 1,548/cm might be related to the stretching vibration of C=N and C=C. The peaks at 1400 to 1500/cm (1,455/cm, 1,444/cm and 1,442/cm) might be attributed to strong to medium vibration of C=C and N=O functional groups. The absorption peaks at 1,388/cm and 1,328 were due to the stretching vibration

of C-O. The peaks at 1,097/cm were attributed to the asymmetric stretching vibration of C-O-C (Table 1, Fig. 3a, 3b, 3c and 3d). Isolation, identification and biochemical profiling of endophytic fungi from *Helianthus annuus L.* (sunflower) highlight the diversity and potential utility of fungal endophytes. This study, based on macroscopic and microscopic identification of four different fungal species, *Rhizopus sp.*, *Cladosporium sp.*, *Penicillium sp.* and *Aspergillus sp.* These results are consistent with previous studies showing that endophytic fungi are ubiquitous among plant species and contribute significantly to host health and ecological interactions (Akram *et al.*, 2023).

Ecological role and diversity of fungal endophytes

Endophytic fungi play an important role in plant ecosystems by improving nutrient acquisition, stress tolerance and pathogen resistance. The diversity of fungal species observed in sunflower (*Helianthus annuus L.*) samples, belonging to the families Rhizopodaceae, Davidiellaceae, Aspergillaceae and Trichocomaceae, suggests that sunflower (*Helianthus annuus L.*) contain a fungal diversity of endophytes with potential for biotechnological and pharmaceutical applications. As reported by Rodrigues *et al.* (2007), the identification of fungal endophytes requires a meticulous approach that includes both macroscopic and microscopic characterization, a method that was applied in this study.

Identification and characterization

The use of three-dimensional microscopy to study the macroscopic and microscopic properties of fungi provides a useful tool. Morphological characteristics such as colony size, color and morphology are important for distinguishing different species. For example, the *Rhizopus sp.* exhibited characteristics typical of the Rhizopodaceae family, including the formation of clusters, whereas *Cladosporium sp.* showed the characteristic black color associated with the Davidiellaceae family. These findings are consistent with the established standards for fungal identification, further supporting the validity of the results (Salvamaniet *al.*, 2014).

Biochemical profiling and potential applications

Endophytic fungi are known to produce a wide variety of compounds with potent antimicrobial, antimicrobial and anticancer activities. The combination of diverse organisms such as *Penicillium sp.* and *Aspergillus sp.* which are well-documented producers of bioactive compounds opens new avenues to explore their potential therapeutic applications. For example, the genus *Penicillium sp.* is a well-known source of penicillin and other bioactive compounds, while *Aspergillus sp.* is popular for its industrial and pharmaceutical applications. These endophytes can be used as promising carriers for drug discovery and agricultural bioproduction. (Usmanet *al.*, 2024).

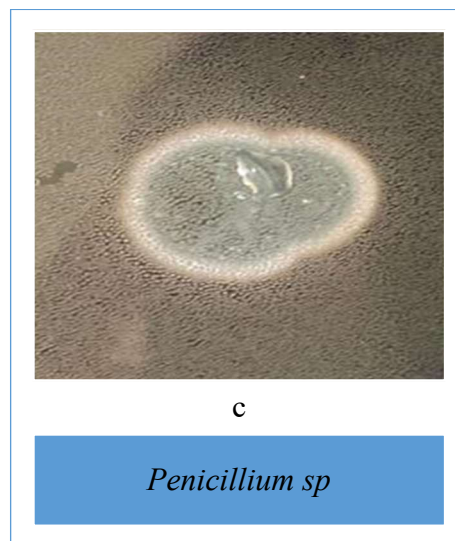
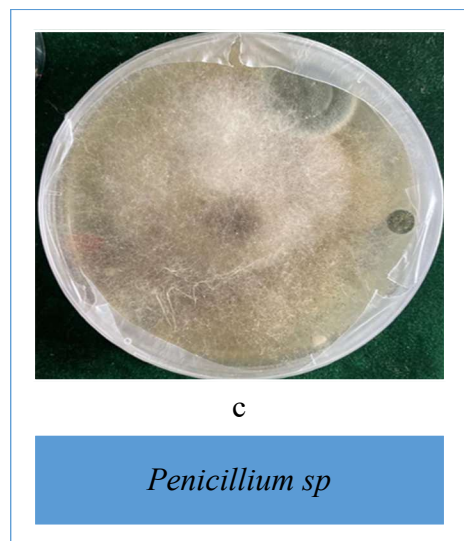
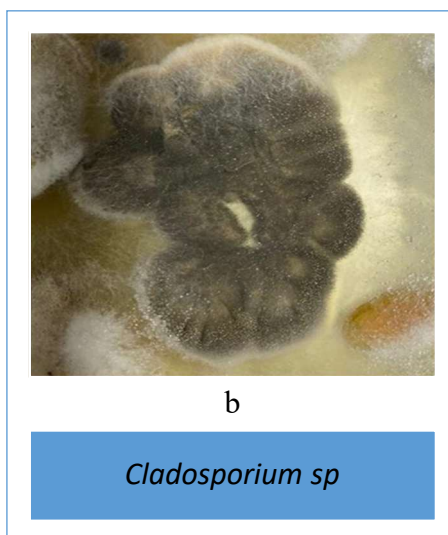
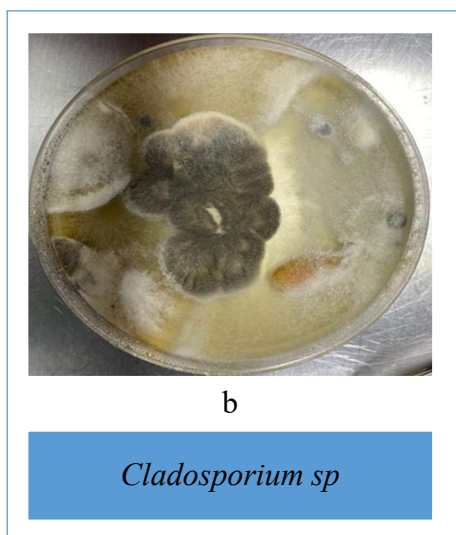


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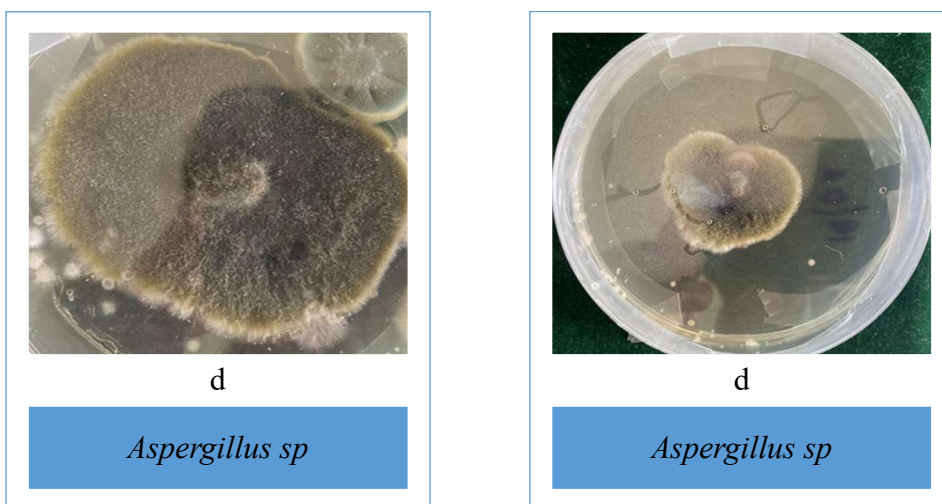


Fig. 1: Colony morphology of endophytic fungi isolated from *Helianthus annuus* L. on PDA medium: (a) *Rhizopus sp.*, (b) *Cladosporium sp.*, (c) *Penicillium sp.*, (d) *Aspergillus sp*

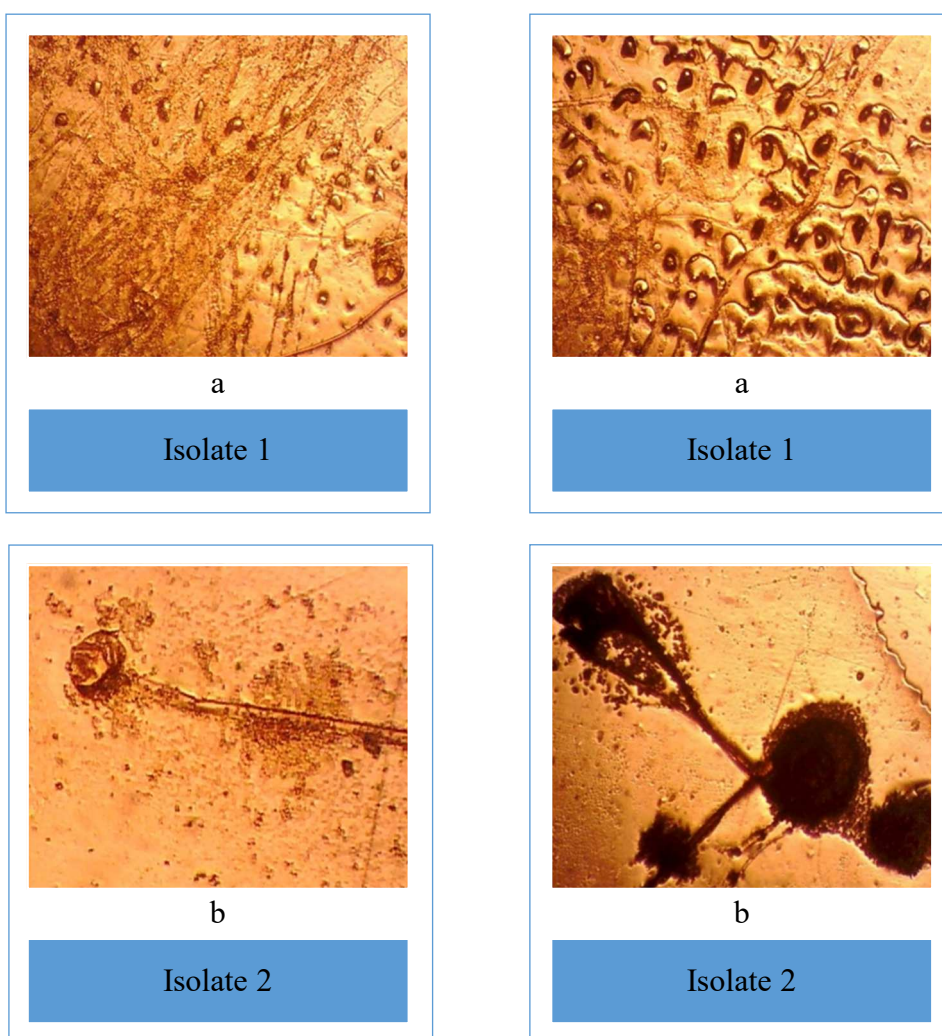


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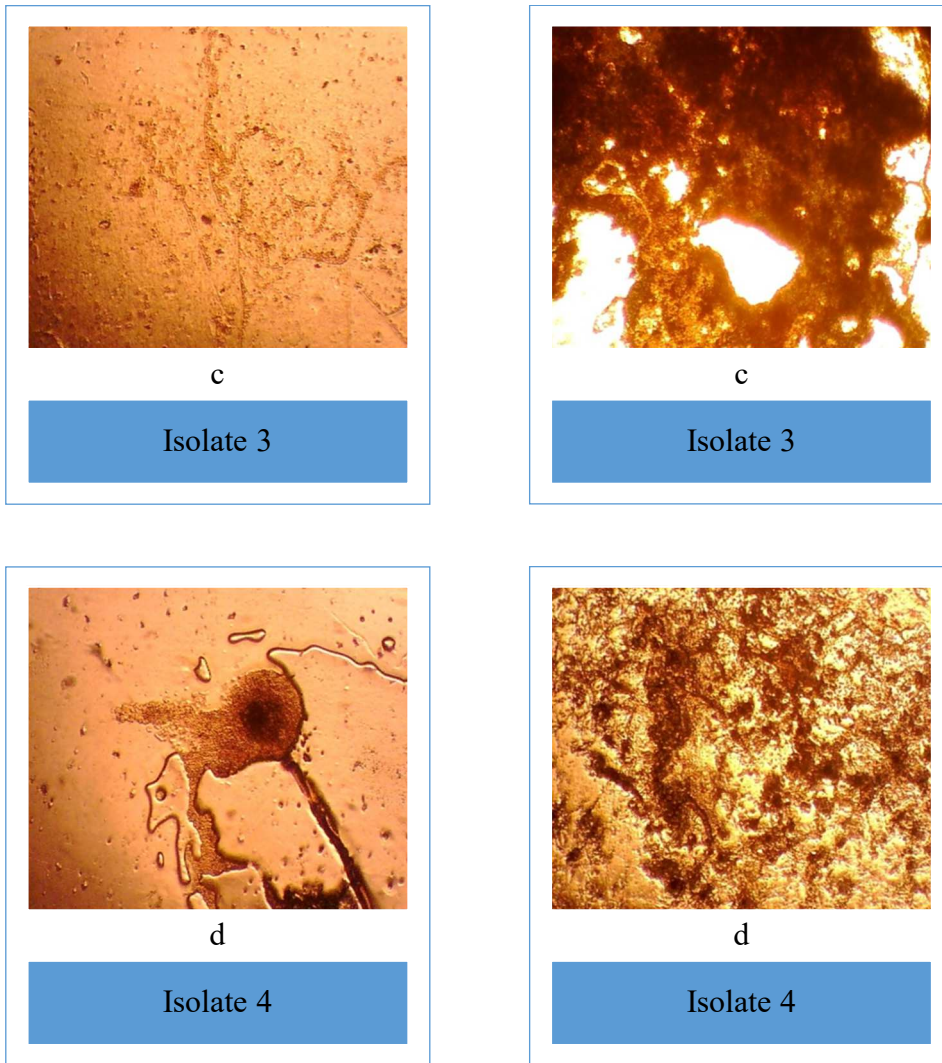
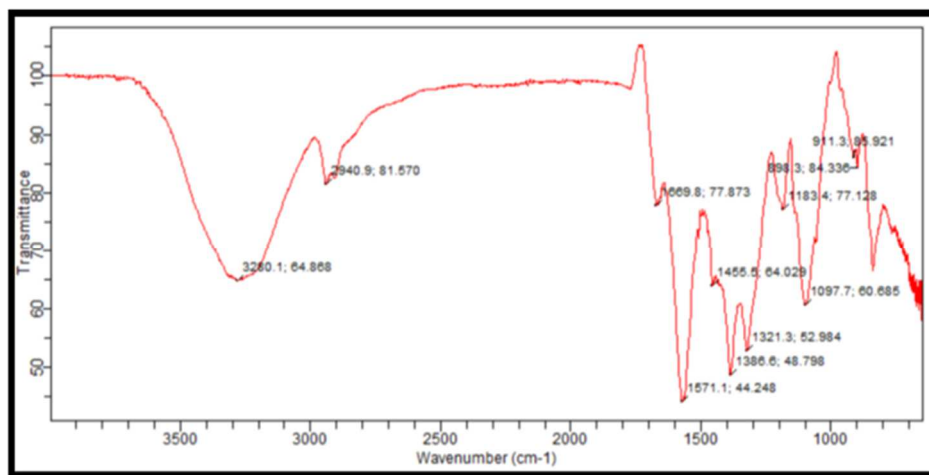


Fig. 2: Microphotographs of isolated endophytic fungi at 10× and 40× magnifications: (a) *Rhizopus sp.*, (b) *Cladosporium sp.*, (c) *Penicillium sp.*, (d) *Aspergillus sp.*



(a)

Table 2: Different functional groups and possible phytochemical constituents identified using FTIR

Wavenumber (cm ⁻¹)/ Possible functional groups	<i>Rhizopus sp.</i>	<i>Cladosporium sp.</i>	<i>Penicillium sp.</i>	<i>Aspergillus sp.</i>	Reference
4,000 to 2,500/cm O-H, N-H and C-H Stretch vibration	3280 2940	3441	2924	2924	(Munir <i>et al.</i> , 2024).
2500 to 2000/cm C=C, C=N Stretch vibration			2178 2119 2089	2201 2178 2119	
2000 to 1500/cm C=C, C=O, C=N Stretch vibration	1889 1571	1681 1548 1504	1997 1975 1755 1681 1515	1755 1696 1681 1515	(Naumann, 2000).
2000 to 1500/cm C=C, C=O, C=N Stretch vibration	1889 1571	1681 1548 1504	1997 1975 1755 1681 1515	1755 1696 1681 1515	(Shubha <i>et al.</i> , 2017b).
2000 to 1500/cm C=C, C=O, C=N Stretch vibration	1889 1571	1681 1548 1504	1997 1975 1755 1681 1515	1755 1696 1681 1515	

In this study identification and characterization the endophytic fungi from sunflower (*Helianthus annuus L.*) was carried out, further biological and molecular studies are needed to elucidate their metabolites. Furthermore, investigating the interactions between endophytes and their host plants can provide insight into how these fungi contribute to plant growth, stress response and disease resistance. Endophytic fungi associated with *Helianthus annuus L.* has shown great potential of bioactive compounds.

Isolation, identification and biological and structural determination of these fungi indicate that the potent of antimicrobial, anti-inflammatory and anticancer activities. These findings highlight the importance of exploring natural fungi as a useful material for drug development. These compounds may play an important role in combating antibiotic resistance, controlling oxidative stress-related diseases and developing novel cancer therapies. Furthermore, since sunflower is a widely cultivated crop, it is a simple and sustainable way to supply these beneficial fungi.

Future research should focus on the development of immunomodulators, elucidating the mechanism of action of these compounds and conducting ongoing clinical trials to evaluate their therapeutic potential. This study supports the importance of fungi for the development of biomedical science and demonstrates their role as a source of research and drug discovery

CONCLUSION

This study successfully isolated and characterized endophytic fungi from Sunflower (*Helianthus annuus L.*), revealing their potential as important sources of bioactive compounds. The isolated fungal species *Rhizopus sp.*, *Cladosporium sp.*, *Penicillium sp.*, and *Aspergillus sp.* were subjected to Fourier Transform Infrared Spectroscopy (FTIR) analysis to determine the presence of structural and functional groups in their metabolites.

The FTIR spectra exhibited characteristic absorption bands corresponding to various functional groups, including O–H and N–H stretching vibrations (indicative of hydrogen bonding and hydroxyl or amine groups), C–H asymmetric stretching (representing alkanes), and multiple C=O, C=N, and C=C stretching vibrations, which signify the presence of carbonyl, imine, and alkene compounds, respectively. Additionally, peaks related to N=O and C–O–C functional groups were observed, suggesting the presence of nitro and ether linkages associated with complex organic metabolites.

The diversity of functional groups detected indicates that these endophytic fungi possess rich biochemical potential and may produce a wide range of secondary metabolites such as phenolics, flavonoids, terpenoids, and alkaloids compounds known for their antimicrobial, antioxidant, and pharmaceutical properties. These findings highlight the biotechnological significance of endophytic fungi

residing in *Helianthus annuus* L., suggesting that this plant serves as a promising host for isolating novel fungal strains with potent bioactive capabilities.

Future research should focus on purification and structural elucidation of the individual metabolites produced by these endophytes, alongside evaluating their biological activities through antimicrobial, antioxidant, and cytotoxicity assays. Such investigations may contribute to the discovery of new natural products with potential therapeutic applications.

Acknowledgments

The authors would like to express their sincere gratitude to the University of Poonch Rawalakot, Azad Jammu and Kashmir, Pakistan, for providing laboratory facilities and technical assistance throughout this research.

Authors' contributions

Shamaila Kausar and Muhammad Akram contributed equally to the experimental design, data collection and analysis, and participated in the drafting of the manuscript. Fatima Ali, Farheen Ansari and Syed Zeeshan Haider Naqvi were responsible for research supervision, manuscript revision, and final approval. All authors have read and approved the final manuscript.

Funding

There was no funding.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

This study was approved by the Ethics Committee of University of Lahore (Approval number is IMBB/BBBC/22/730)

Conflict of interest

There are no conflicts to declare.

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