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Endophytic *Aspergillus foetidus* from the Sikkim Himalayan Region: GC–MS Based Metabolite Profiling and Antimicrobial Assessment

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Aspergillus foetidus strain **PARLF1**, an endophytic fungus derived from the medicinal plant *Astilbe rivularis*, has been identified as having the potential to produce secondary metabolites that are beneficial to both plants and humans. This research focused on the bioactive components present in the endophyte isolate PARLF1 crude extract using gas chromatography–mass spectrometry (GC-MS). The antimicrobial activity of extract was tested and showed potent antibacterial properties against pathogenic microorganisms by an agar well diffusion method. Volatile organic compound (VOCs) were also quantified using a dual culture plate analysis. This work is the first GC MS profiling of *A. foetidus* obtained from *Astilbe rivularis* of Sikkim himalayan region, and demonstrates its prospects for medicinal use as a source of therapeutically important bioactive compounds.

Keywords : Bioactivity, crude extract, GC-MS, metabolites

INTRODUCTION

Antimicrobial resistance is increasingly posing a substantial threat to human health. Many microbes have evolved particular mechanisms that render them resistant to various medicines, including antibiotics. This prompted a search for effective natural sources that can prevent the growth of disease-causing microorganisms. Since medicinal plants are known to be a reservoir of endophytes, the investigation of endophytes from such plants becomes relevant. Many studies have demonstrated the different qualities these endophytes exhibit, including antimicrobial, anti-inflammatory, anticancer, resistance against phytopathogens and production of volatile organic compounds (VOCs) (Sharma *et al*. 2021). Endophytes should be studied for their diverse bioactive properties.

There has been an increase in the investigation of antimicrobials from natural sources, ideally of microbial origin (Majid *et al*. 2024).

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Due to overexploitation and reckless use, research on bioactive compounds obtained from plants has caused certain medicinally significant plant species to become endangered or even extinct. Investigating endophytes, which are found inside some plant species and have over time developed the capacity to generate bioactive substances that are equivalent to those of the host plant, can help tackle this issue (Jinfeng *et al*. 2017).

The raw material from plant sources is required in bulk for the formulation of pharmaceutical products. This demand collides with the idea of conserving such medicinally important plant species. Therefore, emphasis should be laid on an eco-friendly alternative, which is by utilising endophytes for bioactive metabolite production. Medicinal plants are a major source of bioactive compounds and are known to harbour endophytes. Many endophytes have been able to produce compounds such as alkaloids, peptides, phenols, and flavonoids similar to the host plant (Rana *et al.* 2019). These bioactive compounds find their use in agriculture, industries and phamaceutical domain (Pelo *et al*. 2021).

Consequently, it is essential to explore the secondary metabolites of endophytes from medicinal plants using chromatographic techniques (TLC, HPLC), spectroscopic analysis (GC-MS, NMR, FTIR), and a metabolomics approach (LC-MS).

Based on the ethno-medicinal properties of *Astilbe rivularis*, the endophytes were isolated from this plant and the GC-MS analysis was done on the most promising fungal endophyte isolate. The fungal crude extract showed antimicrobial activity against different test pathogens. The GC-MS analysis was performed to identify the presence of several bioactive chemicals from the crude extract of the isolate PARLF1, identified as *Aspergillus foetidus*. This research will aid in the discovery of new bioactive molecules for application in medicine, agriculture, and the pharmaceutical industry.

The objective of this study was to obtain fungal crude extract, evaluate its antimicrobial efficacy against bacteria test pathogens, assess its ability to produce volatile organic compounds (VOCs) with antagonistic activity against phytopathogens and also to characterise the various secondary metabolites obtained from GC-MS analysis. The GC-MS profiling revealed multiple compounds with reported antimicrobial, antioxidant, antitumor, and nematicidal activities.

MATERIALS AND METHODS

Fungal crude extraction

The production of crude extract obtained from a fungal endophyte, *Aspergillus foetidus*, which was isolated from the medicinal plant *Astilbe rivularis*. The fungal isolate was inoculated in potato dextrose broth and incubated for 21 days at 28 ± 2°C. After 21 days of incubation and adequate growth of the isolate, the broth was separated from the fungal mat using Whatman filter paper 4. The fungal mat was dried in the oven. The dry weight of the fungal mat was obtained.

To assess the bioactive metabolites/compounds from cell-free supernatant, the culture filtrate obtained after separation was extracted by mixing equal amounts of suitable organic solvents, i.e., ethyl acetate, in a separating funnel. The filtrate and solvent were shaken repeatedly to ensure that they mixed fully. Then the solution was allowed to settle down for some time until two distinct layers were formed in the separating funnel. The bottom layer was allowed to flow, while the top layer was collected in a round bottom flask before being concentrated in a rotary evaporator at the appropriate temperature and pressure (Muzzamal *et al*. 2012; Wiyakrutta *et al*. 2004).

Volatile activity of the isolate PARLF1

To investigate volatile activity, the following fungal test pathogens were employed. These phytopathogens were acquired from the Indian Agriculture Research Institute's Plant Pathology Division in New Delhi. The dual culture approach was employed to investigate volatile antagonistic activity. Using this technique, the endophyte isolate was cultivated for two days in PDA plates. Agar blocks of the test pathogen were extracted from a culture that was five days old and placed on fresh agar plates. These plates' inoculated bases were removed and put on top of the endophyte-containing Petri plate. After being parafilm-sealed, the plates were incubated for five days at 28 ± 2°C. Additionally, control plates with only test pathogens were maintained (Dennis and Webster, 1971; Sharma *et al*. 2024).

The percentage of inhibition was calculated as follows:
Inhibition % = ((Dc - Dt)/Dc) x 100

(where Dc = diameter of the test pathogens in control plates, Dt = diameter of the test pathogens in test plates).

GC-MS analysis of crude extracts of fungal endophyte

The solvent was allowed to completely dry till the crude extract was obtained. The crude extract was extracted carefully, which was further analysed for its bioactivity using disc diffusion method, chromatographic techniques and bio-autography (Muzzamal *et al*. 2012; Wiyakrutta *et al*. 2004). The crude extract thus obtained was analysed for different metabolites by GC-MS. GC-MS analysis was done on Shimadzu QP-2010

Plus with Thermal Desorption System TD 20 at the Advanced Research Instrumentation Facility, Jawaharlal Nehru University, New Delhi. The major constituents of VOCs can be classified as hydrocarbons, alcohols, ketones, aldehydes, esters, ethers, acids, and other classes of terpenes based on the total integrated peak regions of the GC-MS elution profile. Furthermore, the compounds obtained in the GC-MS analysis were checked for different properties based on several reports.

Data analysis

Each bioactivity test was conducted in triplicate, and the data were recorded accordingly. The values are presented as mean ± standard deviation (SD). Microsoft Excel was used to prepare the calculations and graphs.

Antimicrobial activity of fungal crude extract

The antibacterial activity of crude extracts was evaluated using the agar well diffusion method. *Bacillus cereus* MTCC 430, *Klebsiella pneumonia* MTCC 103, *Staphylococcus aureus* MTCC 3160 and *Streptomyces pneumoniae* MTCC 655 were used to examine the fungal crude extract. In this procedure, a one-day culture of test pathogens was obtained and sprayed onto nutrient agar plates using a sterile loop. A sterile cork borer was used to create two wells. One well contained 30µl of crude extract sample, while the other had 30µl of streptomycin solution as a positive control. Plates were incubated at 32 ±2°C in a BOD incubator-shaker for one day.The inhibition zones were calculated as 10 mm (+++, strong inhibition), 2 to 10 mm (++, moderate inhibition), 1 mm (+, weak inhibition), less than 1mm (no activity) (Lodi *et al*. 2023).

RESULTS AND DISCUSSION

Dual plate assay

In the dual plate assay, the volatile compounds produced by the endophyte were able to restrict the growth of phytopathogens. The percentage inhibition was observed against *P. infestans* (81.8%) followed by *A. alternata* (74%) and *F. solani* (24%). However, the least inhibition was observed against *S. sclerotiorum* with 21.9% as shown in Figs. 1 and 2.

GC-MS analysis of the fungal endophyte PARLF1

This fungal endophyte, PARLF1, identified as *Aspergillus foetidus*, was selected for GC-MS analysis as this isolate exhibited all plant growth-promoting traits. The area and retention time of some of the compounds, along with different bioactive properties and reports from other sources, have been mentioned in Table 1. The GC-MS profiling of the crude fungal extract revealed the presence of several bioactive metabolites such as 1-Dodecanol, Dodecane, 2-Methyltetracosane, 1-Tetradecanol, Tetradecane, Octadecane, 1-chloro-, Benzophenone, Dotriacontane, n-Hexadecanoic acid, Eicosane, Oleic Acid, Octadecanoic acid, n-Hexadecanoic acid. Among these bioactive metabolites, Benzophenone was the compound that had been reported from *Aspergillus* sp. and had antimicrobial and antioxidant properties. This study is the first report of GC-MS analysis of the crude extract of fungal endophyte *Aspergillus foetidus*. The GC-MS analysis revealed a total of 61 compounds. Across 61 the emission profile of *Aspergillus foetidus* revealed a high content of Alcohols (34 %) followed by that of Esters (22.4 %), derivatives (amide, phosphate, organosilicon, sulfonyl chloride (14.8 %), hydrocarbons (14.6 %) and acids (7.1 %). Minor contributions (<5% each) were observed from ketones, aldehydes, ethers, and triterpenoid sterols.Interestingly 23 compounds were found to have different bioactive functions and properties. The analysis also revealed the presence of the analysed compounds from other plant and endophyte sources. These compounds detected in this analysis, concerning their specific properties, indicate that they are sources of natural compounds. Other reports of GC-MS analysis from *Aspergillus* sp. have revealed the presence of many bioactive compounds.

This GC-MS analysis reveals the presence of top 10 compounds among the 61 compounds in terms of their area percentage is as follows: Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydroxy (9.76%), 1-Hexadecanol

Table. 1 List of compounds obtained from the crude extracts of *Aspergillus foetidus.*

Name of the compound	Retention time	Peak area %	Nature of compound	Activity
1-DODECANOL	7.891	0.64	Alcohol	Ability to inhibit bacterial growth (de Sousa Ferreira *et a*l., 2023). Antibacterial activity against *Staphylococcus aureus* (Togashi *et al*., 2007).
Dodecane	8.014	0.58	Hydrocarbon	Antibacterial activity (Nandhini *et al*., 2015).
Benzene, 1,3-bis(1,1-dimethylethyl)-	8.767	1.10	Hydrocarbon	Bioactive compounds identified in plant species *Pila virens* (Gayathri *et al*., 2017).
Dodecane, 4,6-dimethyl-	9.089	0.49	Hydrocarbon	Metabolites of the perennial grass *Phragmites australis* (Frahtia *et al*., 2024).
2-Methyltetracosane	9.405	0.22	Hydrocarbon	Used as a drug for dermatological problems, antibacterial and urinary problems (Duke, 2016).
1-TETRADECANOL	10.726	4.60	Alcohol	Antibacterial, anti-inflammatory (Mujeeb *et al*.,2014).
TETRADECANE	10.824	1.59	Hydrocarbon	Antifungal activity against *C. albicans* (Guo *et al*., 2008); Antibacterial activity against gram positive and gram negative bacteria (Ogunlesi M *et al*., 2010).
HEXADECANE, 2,6,10,14-TETRAMETHYL-	11.945	0.29	Hydrocarbon	Metabolites of the perennial grass *Phragmites australis* (Frahtia *et al*., 2024).
2,4-Di-tert-butylphenol	12.338	1.28	Alcohol	Found as a metabolite in aqueous extract of *Azanza garckeana* seeds (Momodu *et al*., 2022).
Octadecane, 1-chloro-	12.886	0.43	Other	Antibacterial activity (Boussaada *et al.,* 2008).
1-Hexadecanol	13.249	8.48	Alcohol	Metabolite obtained from actinomycetes AIA6 isolates (Kumari *et al*., 2018).
Benzophenone	13.920	**0.62**	Ketone	Metabolite obtained from *Aspergillus* sp. Antimicrobial and antioxidant property (Liu *et al*., 2020).
METHYL (3-OXO-2-PENTYLCYCLOPENTYL)ACETAT	14.013	0.90	Ester	Reported from *Aspergillus flavus* (Singh *et al*., 2023).
8-Pentadecanone	14.192	1.11	Ketone	Metabolite obtained from actinomycetes AIA6 isolates (Kumari *et al*., 2018).
1-Nonadecene	15.502	**7.61**	Hydrocarbon	Reported from *Aspergillus flavus* (Singh *et a*l., 2023).
Heneicosane	15.565	0.62	Hydrocarbon	Reported from two potent endophytes *Enterobacter cloacae* and *Achromobacter xylosoxidans* (Adhikari *et al*., 2023).
Cyclo(L-prolyl-L-valine)	16.085	1.26	Other	Bioactive compound isolated from *Fusarium* sp. extract isolated from *C. officinalis* (A. Asghari *et al*., 2023).
Dotriacontane	16.698	0.48	Hydrocarbon	Reported from *Aspergillus* sp. (Nacef *et al*., 2023).
n-Hexadecanoic acid	17.347	3.60	Acid	Antioxidant, Hypocholesterolemic, Nematicide, Pesticide, Lubricant, Antiandrogenic, Hemolytic, 5-alpha reductase inhibitor, antipsychotic (Sermakkani and Thangapandian V, 2012; Vijisaral and Subramanian, 2014).
Eicosane	17.595	0.42	Hydrocarbon	Antifungal compound reported from *Streptomyces* sp. KF15(Bhat *et al*., 2024).
Oleic Acid	18.997	0.82	Acid	Anti inflammatory, anti androgenic, anticancer, preservative and hypocholesterol properties from a medicinal plant *Pitchavari* (Sreekumar *et al*.,2014).
Octadecanoic acid	19.208	2.07	Acid	Antifungal, Antitumour, Antibacterial (Sermakkani M, Thangapandian , 2012; Akpuaka *et al*., 2013; Vijisaral and Subramanian , 2014).
1-Hexacosanol	21.107	1.41	Alcohol	Detected in pulp oil of *Cassia fistula* with antifungal properties (Irshad *et al*., 2013).

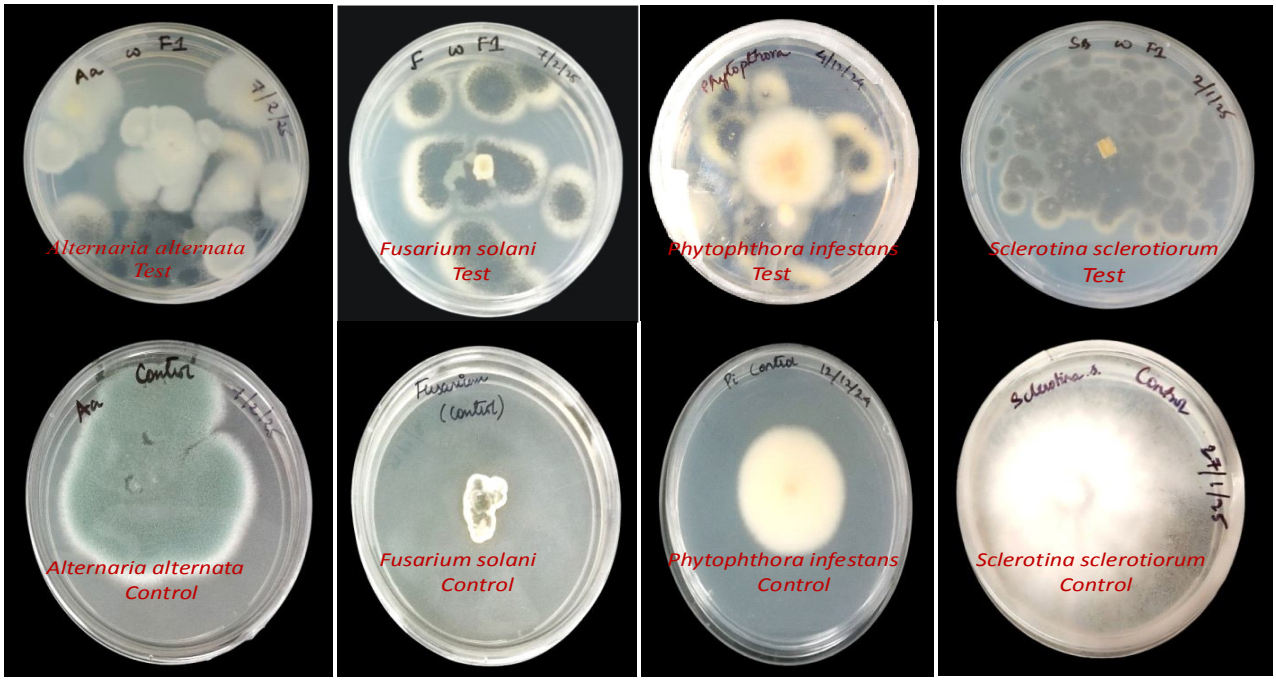


Fig. 1: Images of volatile activity of isolates against test pathogens. The first row depicts the test pathogen and endophyte isolate volatile activity and the second row depicts the control activity of the test pathogen.

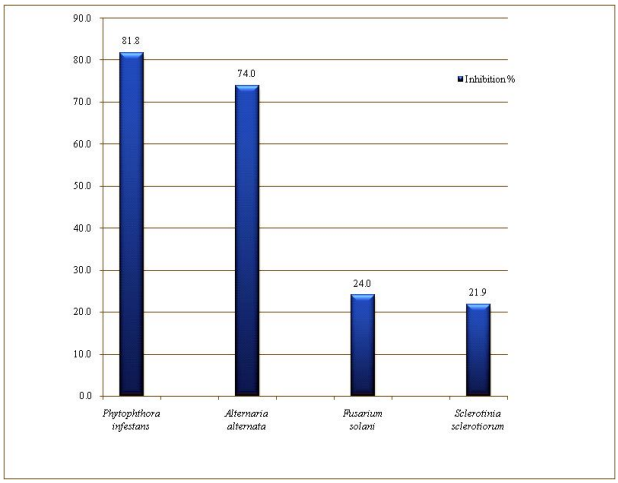
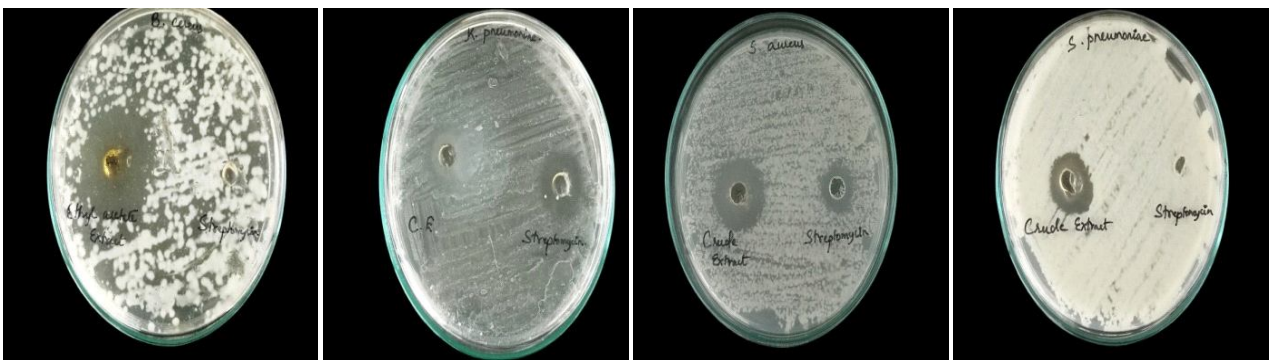


Fig 2: Inhibition of phytopathogens by the production of volatile compounds from fungal endophyte *Aspergillus foetidus*



(a) (b) (c) (d)

Fig 3 : Antimicrobial activity of fungal crude extract PARLF1 against (a) *Bacillus cereus (b) Klebsiella pneumoniae (c) Staphylococcus aureus (d) Streptomyces pneumoniae* by agar well diffusion method;Control: 30 µl Streptomycin solution.

(8.48%), 1-Nonadecene (7.61%), 3-PHENOXYPHENOL (6.07%), Furan-2-carboxamide, N-ethyl-N-(3-methylphenyl)- (4.99%), 1-Heptacosanol (4.94%), 1-Tetradecanol (4.6%), Phenol, 3,5-Dimethoxy-, Acetate (4.27%), Behenic alcohol (3.68%), n-Hexadecanoic acid (3.6%). In the GC-MS study, 1-Nonadecene had the highest retention time, 15.502, followed by 1-Hexadecanol (retention time 13.249) and 1-Tetradecanol (retention time 10.726). Benzophenone, an endophyte metabolite isolated from *Aspergillus* sp., was found to have antibacterial and antioxidant properties (Liu *et al*. 2020). Another compound, 1-Nonadecene, has been reported from *Aspergillus flavus* (Singh *et al*. 2023) as well as from two endophytes, *Enterobacter cloacae* and *Achromobacter xylosoxidans* (Adhikari *et al*. 2023).

Crude antimicrobial activity

The crude extracts of the fungal isolate were tested for antimicrobial activity against various bacterial test pathogens, including *Bacillus cereus* MTCC 430, *Klebsiella pneumoniae* MTCC 103, *Staphylococcus aureus* MTCC 3160, and *Streptomyces pneumoniae* MTCC 655, with streptomycin serving as the negative control. The crude extracts demonstrated a discrete zone of clearance. The ethyl acetate extract isolate had the largest obvious zone of inhibition against *B. cereus* (20.7±6.0mm), *Klebsiella pneumoniae* (19.3±7.6mm), *Staphylococcus aureus* (20.7±10.1mm), and *Streptomyces pneumoniae* (17.3±9.5mm) (Fig.3). The emergence of distinct zones indicated the crude extract's antibacterial activity. The inhibition zones were more than 10 mm (+++, strong inhibition) (Lodi *et al*. 2023).

The crude extracts may exhibit high or low antimicrobial activity, and the specific metabolite responsible for this property can be confirmed further by targeting and analysing chemicals. These compounds, which can be fatty acids, fatty acid esters, or aliphatic chains, concentrate in the lipid bilayer of the cell membrane and mitochondria. They disrupt the integrity of the cell structure of microbes, making it more fluid and increasing cell permeability. This allows the contents of the cell to leak out, causing cell death (Solorzano-Santos and Mirana-Novels, 2012; Momodu *et al*. 2022).

The presence of these compounds, as well as their known bioactivity implicates these specific components of the crude extracts of *Aspergillus foetidus* as potential antibacterial compounds. Hence the mentioned endophytic fungus has great antimicrobial potential against the tested pathogens, thus providing an important source for the novel antimicrobial agents and also contains a variety of biologically active secondary metabolites.

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DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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