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Antibacterial potential of the endophyte *Phyllosticta fallopiae* from wild tomato (*Solanum pimpinellifolium*)

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Abstract. *Sarma A, Biswas PR, Tayung K. 2025. Antibacterial potential of the endophyte* Phyllosticta fallopiae *from wild tomato* (Solanum pimpinellifolium). *Biodiversitas 26: 4180-4192.* The growing threat of antimicrobial resistance has necessitated the exploration for undiscovered novel antimicrobials from natural sources. *Solanum pimpinellifolium*, a wild relative of cultivated tomato is known for its built-in resistance to a variety of pests and pathogens. Literature studies indicated that such plants are colonized by distinct endophytic microbes that produce secondary metabolites of multiple applications. A total of 205 endophytic fungi were isolated from healthy leaf tissue of *S. pimpinellifolium* and five most dominant isolates were determined for antibacterial activity against eight clinically significant human pathogens using agar well diffusion method. The most potent isolate was identified at molecular level via ITS sequencing. Minimum Inhibitory Concentration (MIC) was determined using broth microdilution, and time-kill kinetics were performed to evaluate bactericidal effect. The extract was further analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) to identify active metabolites. The metabolite obtained from *Phyllosticta fallopiae* SPK5 (GenBank accession no. OR770585) was the most effective and showed the highest antibacterial activity against *Enterococcus faecalis* (25.67±0.58 mm), *Bacillus anthracis* (23.67±2.08 mm) and *Staphylococcus aureus* (21.00±1.00 mm). The extract showed promising Minimum Inhibitory Concentrations (MICs) of 15.62 µg/mL against *Enterococcus faecalis* and reduction in viable cell count in 12 h. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the potent extract revealed presence of seven major compounds including fatty acids, ketones and heterocycles such as Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), octadecanoic acid, and 1-nonadecene, which are reported to have bioactive properties. These findings highlight the potential of wild tomato-associated endophytic fungi as a promising source of novel antimicrobial compounds, with possible applications in pharmaceutical development and management of multidrug-resistant pathogens.

Keywords: Endophytic fungi screening, GC-MS, minimum inhibitory concentration, *Phyllosticta fallopiae*, *Solanum pimpinellifolium*

INTRODUCTION

The rapid increase in Antimicrobial Resistance (AMR) poses a critical global health challenge, undermining the efficacy of existing antibiotics and facilitating the emergence of multidrug-resistant pathogens. This crisis affects both developed and developing nations, with severe implications for healthcare, agriculture, and ecosystems. Recognizing its severity, the World Health Organization (WHO) has listed AMR among the top ten global public health threats, emphasizing the urgent need for novel antimicrobial agents. Natural products, particularly those derived from microorganisms, have historically served as valuable sources of antibiotics and continue to hold promise for overcoming resistance (Newman and Cragg 2020). Endophytic fungi have gained considerable attention in this context for their capacity to produce diverse biologically active secondary metabolites with antimicrobial, anticancer, and anti-inflammatory properties (Wen et al. 2022; Usman et al. 2024). These microorganisms colonize internal plant tissues without causing disease and often enhance host resistance to both biotic and abiotic stresses (Schulz and Boyle 2005). Their metabolites include alkaloids, terpenoids, and polyketides, many of which are structurally unique and chemically diverse (Kharwar et al. 2011; Jha et al. 2023). Given their ecological versatility and widespread occurrence across plant species and habitats, endophytic fungi represent a rich and underexplored reservoir for bioprospecting new antimicrobial leads.

The diversity and biosynthetic capacity of endophytic communities are strongly shaped by ecological characteristics of their host plants. Wild plant species, unlike domesticated crops, are exposed to fluctuating temperatures, drought, nutrient limitations, and intense microbial competition. Such ecological pressures foster the evolution of robust defense strategies, often reflected in the recruitment of chemically diverse and functionally specialized microbial symbionts (Porras-Alfaro and Bayman 2011). *Solanum pimpinellifolium*, the wild ancestor of cultivated tomato, exemplifies this phenomenon. Native to harsh coastal environments of South America, it exhibits remarkable genetic diversity and resilience against pests and pathogens (Razali et al. 2018; Cialli et al. 2024). Unlike domesticated tomato varieties, which have undergone genetic bottlenecks, *S. pimpinellifolium* retains numerous defense-related genes and diverse secondary metabolite pathways that contribute to its resistance (Zhu et al. 2018). Reports indicate that it withstands fungal pathogens such as *Fusarium oxysporum*, *Phytophthora infestans*, and *Botrytis cinerea*, as well as bacterial pathogens including *Xanthomonas* spp. and *Pseudomonas syringae* (Sullenberger et al. 2022; Hassan et al. 2024; Saikia et al. 2025). Leaf extracts have also shown antimicrobial activity against human pathogens such as *Escherichia coli*, *Bacillus cereus*, and *Klebsiella pneumoniae* (Aladesida et al. 2021). These traits suggest that wild tomatoes may harbour a chemically rich and biologically potent endophytic community. Wild tomatoes have been reported to harbour a more diverse and chemically active endophytic community than cultivated varieties, likely due to their adaptation to complex natural environments (Ratnaweera et al. 2015; Mili et al. 2021). Despite extensive studies on endophytes from cultivated tomato and other Solanaceae members (Pelo et al. 2020; Biswas et al. 2023), research on the endophytic microbiota of *S. pimpinellifolium* remains limited. Previous studies from cultivated tomato tissues have reported bioactive fungi including *Fusarium, Penicillium*, and *Colletotrichum* (Eljounaidi et al. 2016; Rashid 2021). However, systematic exploration of endophytes from wild tomato, particularly their antimicrobial metabolites, is scarce. Among potential candidates, *Phyllosticta* species have been occasionally reported as endophytes, yet there is meagre information about the bioactive metabolites of *Phyllosticta fallopiae*. Very few investigations have attempted to identify its bioactive metabolites, and to date, its antimicrobial compounds have not been systematically characterized. This gap is particularly important because understanding species-specific metabolites not only highlights the novelty of the isolate but also provides insight into unique chemistries that may contribute to antimicrobial drug discovery.

In light of these considerations, the present study aimed to isolate and identify endophytic fungi from healthy leaves of *S. pimpinellifolium*. The isolates were screened for antibacterial activity against clinically important bacterial pathogens. Among the isolates, one fungus, identified based on ITS rDNA sequencing as *Phyllosticta* sp., showed strong antibacterial activity. The ethyl acetate extract of this isolate showed potent inhibitory effects against pathogens including *Enterococcus faecalis, Bacillus anthracis*, and *Staphylococcus aureus*. To elucidate the chemical basis of this activity, the bioactive extract was analysed using Gas Chromatography-Mass Spectrometry (GC-MS), which revealed multiple bioactive compounds. These findings highlight the untapped potential of wild tomato-associated fungal endophytes as promising sources of novel antimicrobial agents.

MATERIALS AND METHODS

Sample collection, isolation and identification of endophytic fungi

Healthy and mature leaves of wild tomatoes (*Solanum pimpinellifolium*) were collected from hilly region of Karbi Anglong District, Assam, India (Voucher no. GUBH20942). The leaf samples were surface-sterilized following the method described by Schulz et al. (1993), with slight modifications. Briefly, the specimen was immersed in 70% ethanol (1 min), 1% sodium hypochlorite (3 min), and sterile distilled water (2×1 min). Leaf fragments (0.5 × 0.5 cm) were aseptically placed in Potato Dextrose Agar (PDA) plates supplemented with streptomycin (100 µg/mL) to inhibit bacterial contamination. Plates were incubated at 28±2°C for 7-14 days under a 12 h light/12 h dark photoperiod and relative humidity of 70% in a controlled incubator. Fungal growth was monitored regularly, and emerging colonies were sub-cultured to obtain pure isolates (Kharwar et al. 2008). Endophytic fungi were tentatively identified based on their morphological traits and microscopic observation of reproductive spores referring fungal identification manuals (Gilman 1971; Barnett and Hunter 1998).

Fermentation and extraction of metabolite

The five most dominant isolates were selected based on their relative abundance. Pure cultures of the selected isolates were cultured in 250 mL Erlenmeyer flasks containing 100 mL of Potato Dextrose Broth (PDB) and incubated at 28°C under static conditions for 7 days, following the protocol of Strobel and Daisy (2003). After the incubation period, the cultures were filtered (pore size 0.20µm) and resulting filtrates were extracted three times with equal amount of Ethyl Acetate (EtOAc). The combined organic layers were concentrated to dryness under reduced pressure with rotary evaporator set at 40°C. The obtained crude extracts were stored at -20°C for further analysis.

Determination for antibacterial activity

The crude fungal extracts were screened for antibacterial activity using the agar well diffusion assay (Balouiri et al. 2016; Prastiyanto et al. 2022; Wary et al. 2022). Eight human test pathogenic bacteria were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India: *Bacillus anthracis*, *Staphylococcus aureus*, *Escherichia coli, Enterococcus faecalis*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Shigella flexneri*. Bacterial suspensions adjusted to 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL) were spread evenly on Nutrient agar plates (Plate size 90 mm). Wells of 7 mm (Depth 4 mm) were bored and filled with 100 µL of fungal extract (20 mg/mL). Streptomycin (10µg/mL) was used as a positive control, whereas 10% Dimethyl Sulfoxide (DMSO) was used as a negative control in all subsequent assays. After the incubation period at 37°C for 24 hours, zone of inhibition was measured in millimetres.

Determination of Minimum Inhibitory Concentration (MICs)

The Minimum Inhibitory Concentrations (MICs) of ethyl acetate extract of the most potent fungal isolate (*Phyllosticta* sp.) were determined against the eight selected human pathogens—using the broth microdilution method in sterile 96-well microtiter plates (Prastiyanto 2021), following the guidelines of the Clinical and Laboratory Standards Institute (CLSI M07-A10, 2020). The extract was initially dissolved in Dimethyl Sulfoxide (DMSO) and subsequently diluted in Nutrient broth to obtain concentrations ranging from 1000 to 7.8 µg/mL. Each well was inoculated with 20 µL of bacterial suspension adjusted to 0.5 McFarland standard (1×10^7 CFU/mL), diluted to reach a final concentration of 1.3×10^6 CFU/mL. After incubation at 37°C for 24 hours, bacterial growth was measured using a microplate reader at 495 nm in all three replicates. The MIC was determined as the minimum concentration of the extract that completely prevented visible microbial growth (Rodríguez-Melcón et al. 2022).

Time kill study

Time-kill assays were performed to evaluate the bactericidal kinetics of the active extract against the most sensitive pathogens (*E. faecalis*, *B. anthracis* and *S. aureus*). The assay was conducted at extract concentrations equivalent to 1×, 2×, and 4× the MIC. Bacterial cultures were exposed to the extract in sterile tubes and incubated at 37°C (n = 3). Aliquots were taken at 0, 2, 4, 8, 12, and 24 hours, serially diluted in Phosphate-Buffered Saline (PBS), and plated on Nutrient agar for CFU enumeration. The bactericidal activity was interpreted based on the reduction in CFU/mL over time. A ≥ 3 $\log_{10}$ reduction in CFU/mL compared to the initial inoculum was considered bactericidal (Smith and Kirby 2018).

Molecular identification and phylogeny

The endophytic fungus that produced the most active extract was identified through molecular analysis of the Internal Transcribed Spacer (ITS) region. Genomic DNA was extracted using a modified CTAB method (Saha and Tayung 2025). The ITS region was amplified using the universal primers ITS1 and ITS4. The resulting PCR amplicons were purified and sequenced using Sanger sequencing method. The generated sequences were subjected to a BLASTn search against the NCBI GenBank database for determining species-level identity. To infer phylogenetic relationships, the ITS sequence of the isolate and closely related sequences retrieved from GenBank were aligned using ClustalW. Neighbour-joining method was used for construction of phylogenetic tree with 1000 bootstrap replications in MEGA X software (Kumar et al. 2018).

Metabolite characterization by GC-MS analysis

The most active extract produced by the endophytic fungus (*Phyllosticta* sp.), which exhibited significant antimicrobial activity, was characterized by Gas Chromatography-Mass Spectrometry (GC-MS) analysis to identify the major compounds present in the secondary metabolites. The analysis was conducted using a GC-MS system (Agilent Technologies 7890B GC system coupled with a 5977A MSD) utilizing HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was programmed initially at 60°C (maintained for 2 min), followed by an increase of 10°C/min up to 280°C and held for 10 minutes. Helium served as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was set at 250°C, and the sample (1 μL) was injected in splitless mode. The MS parameters were as follows: ionization voltage 70 eV, ion source temperature 230°C, and scan range 50-600 m/z. The total run time was 40 minutes. The acquired mass spectra were analysed using the NIST/EPA/NIH Mass Spectral Library (NIST 14). Compounds were identified by comparing retention times and library spectra (SI≥90%) with reference spectra in the library. The identified compounds were categorized according to their chemical class and reported biological activities from previous literature databases (Kim et al. 2016; Barba-Ostria et al. 2022).

Statistical analysis

Each experiment was conducted in triplicate, and results were presented as mean values with Standard Deviation (SD). Statistical significance was determined at a threshold of $p<0.05$. Statistical differences among treatments were evaluated using One-Way ANOVA followed by Tukey's post hoc test, performed in SPSS version 25 (IBM Corp., Armonk, NY). Graphs and heatmaps were generated using GraphPad Prism version 9.0 and OriginPro 2022, respectively.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

A total of 205 endophytic fungi were isolated from 400 surface-sterilized leaf fragments of *S. pimpinellifolium*. The isolation process involved careful sterilization to eliminate epiphytic contaminants, ensuring that only true endophytes were cultured. Colonies began to emerge within 3-7 days of incubation. The relatively high isolation frequency suggests a strong and stable symbiotic relationship between the host plant and its resident fungal endophytes. The isolates exhibited diverse morphological characteristics, which were used as the basis for preliminary identification. Both colonial morphology on growth media and microscopic features such as spore structure, hyphal organization and reproductive structures were examined to classify the fungi. Surface sterilization controls (plating of the final rinse and leaf imprinting) yielded no fungal growth, confirming the endophytic nature of the isolates. Based on these macroscopic and microscopic observations the isolates were grouped into five distinct fungal genera namely *Colletotrichum* sp., *Phomopsis* sp., *Aspergillus* sp., *Phyllosticta* sp. and *Penicillium* sp. Among these, *Colletotrichum* was the most frequently isolated genus, accounting for 30.2% of the total isolates, followed by *Aspergillus* (26.3%), *Phyllosticta* (19.5%), *Penicillium* (13.7%), and *Phomopsis* (10.2%). The genus-level distribution is summarized in Figure 1. Endophytic diversity in *S. pimpinellifolium* reflects its adaptation to harsh environments and co-evolution with diverse microbes. This diversity provides a reservoir of unique bioactive metabolites with potential antimicrobial applications. It also highlights the impact of domestication bottlenecks, as wild tomato harbors richer endophytic communities than cultivated varieties. A recent study by Gogoi et al. (2024) also reported the presence of *Aspergillus* sp. as endophyte in *S. pimpinellifolium*, supporting our findings. However, the other genera identified in our study, such as *Colletotrichum*, *Phomopsis*, *Phyllosticta* and *Penicillium*, were not reported in their work, indicating possible regional or ecological variations in endophytic communities. This variation is consistent with previous reports demonstrating that host genotype, geographical location, and environmental conditions significantly influence endophytic fungal diversity (He et al. 2023; Zheng et al. 2025). Importantly, the endophytic lifestyle plays a vital role in the functional traits of these fungi. Endophytes inhabit internal plant tissues asymptomatically and are believed to contribute to the defense mechanism of the host by producing bioactive secondary metabolites that deter pathogens, herbivores, or competing microbes. These compounds, often structurally unique, are shaped by co-evolution with the host plant and reflect adaptive responses to shared environmental stressors (Elhamouly et al. 2022; Anjali et al. 2023). Thus, endophytic origin enhances the likelihood of discovering novel metabolites with ecological and therapeutic significance. Notably, several of the genera identified in this study have previously been reported as prolific producers of bioactive secondary metabolites. For instance, species of *Colletotrichum* and *Phyllosticta* are known to produce antimicrobial polyketides and non-ribosomal peptides (Moraga et al. 2019). *Penicillium* and *Aspergillus* species, which are common in endophytic niches, have long been recognized for their ability to synthesize a wide spectrum of pharmacologically relevant compounds, including alkaloids, terpenoids, and diketopiperazines (Nicoletti and Vinale 2018). These findings suggest that the endophytic community of *S. pimpinellifolium* could serve as a valuable reservoir for novel antimicrobial agents.

While a few studies have explored endophytic bacteria from *S. pimpinellifolium* (Basumatary et al. 2021; Saikia et al. 2025), research on its fungal endophytes remains limited. To our knowledge, this is among the few studies providing a genus-level characterization of endophytic fungi from this wild tomato species, highlighting its untapped potential for drug discovery and microbial ecology. Further molecular identification and functional screening are warranted to fully elucidate the ecological roles and biotechnological relevance of these endophytes.

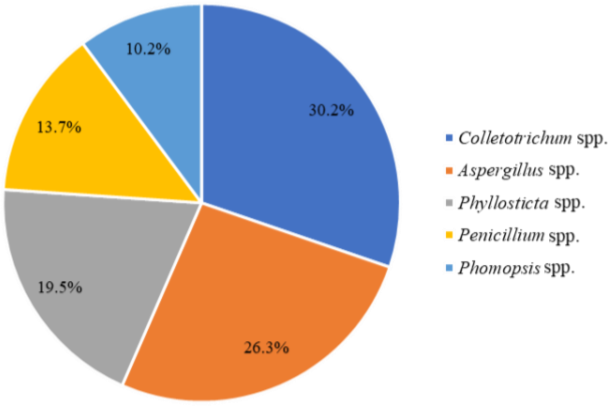

Figure 1. The percentage distribution of endophytic fungal genera isolated from *Solanum pimpinellifolium* leaf tissues

Antibacterial activity of the fungal extracts

The ethyl acetate extracts of five endophytic fungi *Colletotrichum* sp., *Phomopsis* sp., *Aspergillus* sp., *Phyllosticta* sp., and *Penicillium* sp. were determined for their antimicrobial activity against eight pathogenic bacterial strains using the agar well diffusion assay. The results of antibacterial activity of the fungal extracts against the test pathogens are presented in Table 1 and Figure 2. Among the tested endophytes, *Phyllosticta* sp. exhibited the broadest and highest antimicrobial activity, with significant inhibition zones recorded against all test organisms, with zone diameters >20 mm, which falls under the "strong activity" category as per CLSI M02-A12 guidelines. The *Phyllosticta* extract produced large zones for several strains (e.g., *E. faecalis, B. anthracis*), whereas streptomycin remained superior for multiple organisms, including *K. pneumoniae, P. aeruginosa, S. enterica,* and *E. coli* (Table 1). 10% DMSO, which was used as a negative control showed no antibacterial activity. *Phomopsis* sp. also demonstrated notable antimicrobial activity, particularly against *S. aureus* (19.33±0.58 mm), *Shigella flexneri* (18.67±0.58 mm), and *E. faecalis* (16.00±2.00 mm). *Aspergillus* sp. was effective against *E. coli* (19.67±0.58 mm) and *S. aureus* (18.67±1.15 mm), while *Penicillium* sp. displayed strong activity against *Pseudomonas aeruginosa* (22.33±1.15 mm) and *E. faecalis* (19.00±1.00 mm). The result indicated that extract of *Phyllosticta* sp. showed the highest antibacterial activity against the test pathogens and was considered as active extract; therefore, it was selected for further investigation.

The inhibitory activity of ethyl acetate extract of *Phyllosticta* sp. against a broad range of pathogens, including both Gram-positive and Gram-negative bacteria have also been observed in previous studies. In the present investigation, the maximum inhibition was observed against *E. faecalis* and *S. aureus*, indicating the potential of the extract against opportunistic and drug-resistant pathogens (Wikee et al. 2013; Yang et al. 2017). It was also observed in our present study that metabolites obtained from *Phyllosticta* sp. showed greater activity against Gram-positive bacteria which corroborate with the findings of Chukeatirote et al. (2015). Although, several species of *Phyllosticta* have previously been reported to possess antimicrobial activity, the studies on *P. fallopiae* are limited. To our knowledge, Taher et al. (2023) remains the only significant study to date that has documented the antimicrobial efficacy of *P. fallopiae*, particularly against diabetic wound-associated pathogens like *Bacillus subtilis*, *Shigella boydii*, and *Candida albicans*. The limited exploration of this species presents a valuable opportunity for novel bioactive compound discovery, especially in the context of rising antimicrobial resistance. The ability of *Phyllosticta* sp. to inhibit multiple bacterial strains suggests that it may produce a broad-spectrum antimicrobial compound or a synergistic mix of metabolites. This is particularly relevant in the current scenario, where multidrug-resistant bacteria pose a critical challenge to healthcare systems worldwide. Given that endophytic fungi can exhibit strain- and host-specific metabolite profiles, the unique association of *P. fallopiae* with *S. pimpinellifolium*, a wild tomato species with strong natural resistance traits may contribute to the enhanced bioactivity observed in this study. Additionally, comparable studies on endophytes from other wild Solanaceous plants offer important context for interpreting our findings. Endophytic fungi isolated from *S. linnaeanum*, *S. sodomaeum, S. bonariense* and cultivated tomato (*S. lycopersicum*) have also demonstrated presence of endophytes with notable antimicrobial and antifungal activities (Aydi-Ben-Abdallah et al. 2020; Marzouk et al. 2021). Similarly, bacterial endophytes from *S. lycopersicum* were shown to produce volatile and non-volatile compounds with strong antibacterial activity against both Gram-positive and Gram-negative strains (Zhou et al. 2021). Similar studies have also been observed in other members of Solanaceae family, such as *Trichoderma asperellum* and *Fusarium solani* from *Capsicum annuum* (chilli), which were reported to inhibit the growth of *Ralstonia solanacearum*, highlighting the therapeutic promise of Solanaceae-associated endophytes in both clinical and agricultural contexts (Irawati et al. 2020). This study not only adds to the existing knowledge of *Phyllosticta* as a genus with antimicrobial potential but also emphasizes the importance of exploring wild Solanaceous hosts for uncovering metabolically versatile endophytes.

Minimum Inhibitory Concentration (MICs) of active extract

The MIC of the ethyl acetate extract of *Phyllosticta* sp. was determined against the eight clinically relevant bacterial pathogens through broth microdilution method. The extract showed effective inhibitory activity at varying concentrations depending on the organism (Table 2). The lowest MIC value (15.62 µg/mL) was recorded against *E. faecalis*, indicating high sensitivity to the fungal extract. *Bacillus anthracis* also showed notable susceptibility, with growth inhibition observed at 31.25 µg/mL. Moderate inhibitory activity (MIC: 62.5 µg/mL) was found against *S. aureus, S. flexneri, K. pneumoniae* and *E. coli*. On the other hand, *P. aeruginosa* and *S. enterica* exhibited comparatively lower sensitivity, with higher MIC values of 125 µg/mL, possibly due to their robust outer membrane structures and intrinsic resistance mechanisms.

These findings suggest that the *Phyllosticta* sp. extract possesses broad-spectrum antibacterial activity, with particularly strong effects against Gram-positive bacteria such as *E. faecalis* and *B. anthracis*. The MIC values suggested a strong bacteriostatic activity, showing similar results with Santos et al. (2017), where all concentrations of *P. fallopiae* extract were effective against *E. faecalis* (ATCC 29212) with the highest inhibition ratio of 96.2±3.7%. These observations highlight the enhanced effectiveness of our isolate against *E. faecalis*. This consistency further supports the hypothesis that *P. fallopiae* produces stable, diffusible metabolites with high efficacy against certain opportunistic and drug-resistant pathogens. In our study, the MIC against *E. faecalis* was even lower than that reported in previous work, possibly due to strain-level differences, variation in growth conditions, or the influence of the host plant species (*S. pimpinellifolium*) on metabolite profile of the endophyte.

Table 1. Diameter of inhibition zones (in mm) of endophytic fungal extract against selected test pathogens

Test bacteria	**Endophytic extract**					
	***Colletotrichum* sp.**	***Phomopsis* sp.**	***Aspergillus* sp.**	***Phyllosticta* sp.**	***Penicillium* sp.**	**Streptomycin (Positive control)**
Staphylococcus aureus	15.67 ± 0.58^{d}	19.33 ± 0.58^{b}	18.67 ± 1.15^{c}	**21.00 ± 1.00^{a}**	18.33 ± 0.58^{c}	16.00 ± 1.00^{d}
Bacillus anthracis	13.67 ± 1.53^{e}	15.00 ± 1.00^{d}	13.33 ± 1.15^{e}	**23.67 ± 2.08^{a}**	17.00 ± 1.00^{c}	18.33 ± 0.58^{b}
Pseudomonas aeruginosa	16.33 ± 0.58^{e}	15.67 ± 0.58^{e}	15.33 ± 0.58^{e}	18.33 ± 0.58^{d}	**22.33 ± 1.15^{b}**	12.67 ± 0.58^{a}
Shigella flexneri	17.00 ± 1.00^{c}	18.67 ± 0.58^{b}	17.67 ± 0.58^{c}	**19.33 ± 1.15^{a}**	18.67 ± 0.58^{b}	17.00 ± 1.00^{c}
Klebsiella pneumoniae	13.67 ± 0.58^{d}	13.33 ± 1.52^{d}	15.67 ± 0.58^{c}	**18.33 ± 0.58^{b}**	17.00 ± 1.00^{bc}	13.00 ± 1.00^{a}
Salmonella enterica	**16.67 ± 1.53^{bc}**	16.00 ± 1.00^{c}	15.67 ± 1.15^{c}	16.33 ± 0.58^{bc}	14.67 ± 0.58^{d}	18.67 ± 0.58^{a}
Enterococcus faecalis	12.33 ± 0.58^{e}	16.00 ± 2.00^{cd}	16.00 ± 1.00^{cd}	**25.67 ± 0.58^{b}**	19.00 ± 1.00^{c}	16.00 ± 1.00^{a}
Escherichia coli	18.67 ± 0.58^{b}	17.00 ± 1.00^{c}	19.67 ± 0.58^{b}	**20.00 ± 1.00^{b}**	17.67 ± 0.58^{c}	12.67 ± 1.15^{a}

Note: *Data are mean of three replicates (Mean±SD, n = 3). Different superscript letters within the same row indicate statistically significant differences between treatments ($p<0.05$) as determined by One-Way ANOVA. Negative control (10% DMSO) indicates no activity

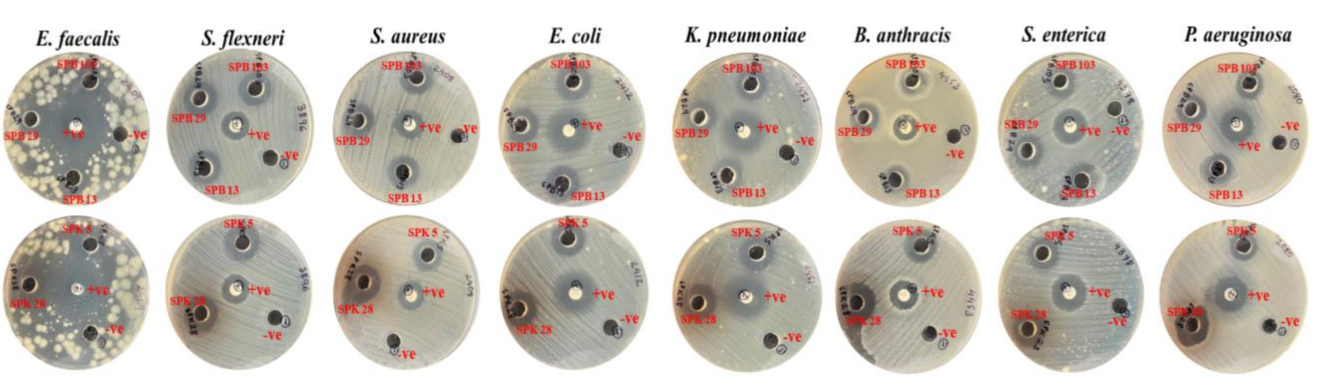

Figure 2. Agar well diffusion assay showing antimicrobial activity of endophytic fungal extracts against bacterial pathogens

Table 2. Minimum Inhibitory Concentration (MIC) of *Phyllosticta* sp. SPK5 organic extract

Test organisms	MIC (µg/mL)	
	***Phyllosticta* sp. extract**	**Reference drug (Streptomycin)**
Gram-positive bacteria		
Staphylococcus aureus	62.5	15.62
Bacillus anthracis	31.25	7.81
Enterococcus faecalis	15.62	3.91
Gram-negative bacteria		
Pseudomonas aeruginosa	125	15.62
Shigella flexneri	62.5	15.62
Klebsiella pneumoniae	62.5	7.81
Salmonella enterica	125	15.62
Escherichia coli	62.5	7.81

Note: *MIC values are mean values of three replicates

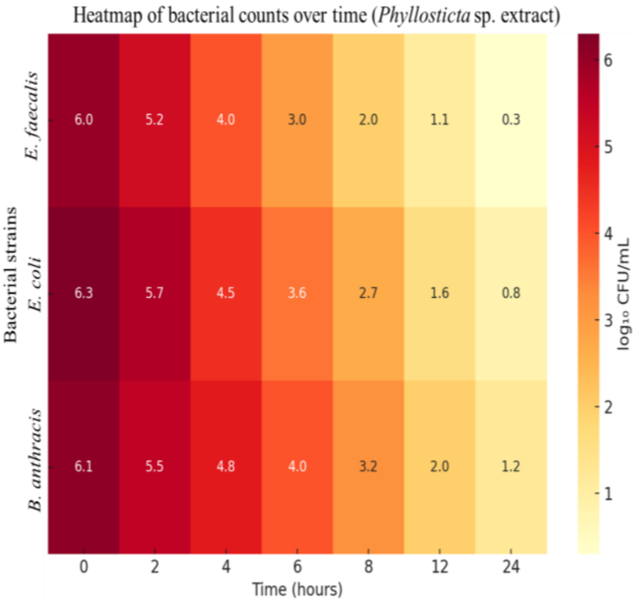

Figure 3. Heatmap representing time-dependent reduction in bacterial counts ($\log_{10}$ CFU/mL) after treatment with *Phyllosticta* sp. extract. Values are mean of triplicate experiments

Time-kill kinetics of the active extract

The time-kill kinetic study demonstrated a progressive and time-dependent bactericidal effect of *Phyllosticta* sp. extract against *E. faecalis*, *E. coli* and *B. anthracis* over a 24-hour incubation period. A heatmap was generated to visualize the changes in viable bacterial counts ($\log_{10}$ CFU/mL) across time points (Figure 3).

A progressive reduction in bacterial load was observed in all three strains, with colour intensities shifting from darker to lighter tones, indicating a decline in cell viability. Among the tested organisms, *E. faecalis* exhibited the most rapid and pronounced killing, with viable counts decreasing from 6.0 to 0.3 $\log_{10}$ CFU/mL by 24 hours. *E. coli* showed a steady decline from 6.3 to 0.8 $\log_{10}$ CFU/mL, while *B. anthracis* showed a comparatively slower rate of killing, with final counts reaching 1.2 $\log_{10}$ CFU/mL at the same time point. The heatmap visualization effectively highlights the time-dependent and organism-specific bactericidal effect of the extract. All three strains exhibited a ≥3 $\log_{10}$ CFU/mL reduction by 24 hours, confirming the strong antimicrobial potential of the extract. Notably, the extract was particularly effective against *E. faecalis*, evidenced by the sharper gradient of reduction in the heatmap. A time-dependent reduction in viable cell count was observed, with near-complete killing of *E. faecalis* within 12 hours and significant reductions in *E. coli* and *B. anthracis* within 24 hours. These findings highlight the ability of the extract not only to inhibit bacterial growth but also to cause irreversible damage to microbial cells over time.

The pronounced sensitivity of *E. faecalis* to the extract is consistent with previous studies. For instance, Santos et al. (2017) reported that metabolites derived from *P. fallopiae* achieved over 96% inhibition of *E. faecalis* at low concentrations, supporting the idea that this species possess potent anti-enterococcal metabolites. Similarly, Eshboev et al. (2024) demonstrated that secondary metabolites isolated from endophytic fungi exerted time-dependent killing of *E. faecalis* through membrane disruption and leakage of intracellular contents, which may explain the rapid killing observed in our study. While *E. coli* also showed a steady reduction in viable counts (from 6.3 to 0.8 $\log_{10}$ CFU/mL), its response was slower, potentially due to the presence of an outer membrane that limits the permeability of hydrophobic compounds, a well-established resistance mechanism among Gram-negative bacteria (Delcour 2009; Band and Weiss 2015). Nonetheless, a ≥3 log reduction indicates that the extract contains bioactive molecules capable of breaching or circumventing these defences, possibly by targeting membrane-associated proteins or interfering with metabolic pathways (Hashem et al. 2023). *Bacillus anthracis*, though Gram-positive, showed a comparatively slower rate of killing (6.1 to 1.2 $\log_{10}$ CFU/mL in 24 hours), which might reflect strain-specific resistance factors or lower permeability to the bioactive compounds. Previous reports indicate that *B. anthracis* forms a more resilient cell envelope under stress conditions, which can affect antimicrobial efficacy (Chateau et al. 2020). However, the eventual ≥3 $\log_{10}$ reduction confirms that the extract is still effective over prolonged exposure. The heatmap visualization clearly captured these time-based dynamics, with *E. faecalis* showing the most intense reduction gradient, further supporting its high susceptibility. The observed kinetics suggest that the extract exerts not just inhibitory effects but causes irreversible damage, likely by disrupting membrane integrity or interfering with cellular respiration and protein synthesis—mechanisms frequently attributed to fungal-derived secondary metabolites such as diketopiperazines, polyketides, and alkaloids (Conrado et al. 2022).

Molecular identification and phylogenetic analysis of potent isolate

For molecular identification, extraction of genomic DNA was performed from actively growing mycelia of *Phyllosticta* sp. SPK5, and the Internal Transcribed Spacer (ITS) region was amplified using ITS1 and ITS4 primers (Figure S1). The amplified sequences were subjected to BLASTn analysis, revealing high similarity (≥99%) with existing sequences in the NCBI GenBank database. The isolate showed 99.38%

sequence identity with *P. fallopiae* (GenBank Accession no. OR770585) (Figure S2). A phylogenetic tree was constructed using the neighbour-joining method taking 1000 bootstrap replicates, which confirmed the placement of *Phyllosticta* sp. SPK5 within the *Phyllosticta* clade, closely clustering with reference sequences (Figure 4). This molecular evidence corroborated the morphological identification, confirming the identity of the endophyte.

There are several instances where *P. fallopiae* has been reported as an endophyte in earlier studies. In the studies of Chen et al. (2024), *P. fallopiae* was isolated as a foliar endophyte from *Dendrobium nobile*, exhibiting a symbiotic relationship without visible disease symptoms. Members of the *Phyllosticta* genus have also been widely recognized for their dual lifestyles, ranging from endophytes to pathogens, depending on host context and environmental conditions (Jayawardena et al. 2019a). However, endophytic species of *Phyllosticta* are increasingly gaining attention for their bioactive secondary metabolites with pharmaceutical potential. Previous studies have also highlighted the antimicrobial properties of *Phyllosticta* species. Taher et al. (2023) reported strong antibacterial and antifungal activity from an endophytic *P. fallopiae* isolated from medicinal plants. Similarly, Xu et al. (2021) described the isolation of novel polyketide compounds from *P. capitalensis*, showing broad-spectrum antimicrobial activity against human and plant pathogens. Furthermore, metabolomic profiling of endophytic *Phyllosticta* species revealed the presence of bioactive phenolic acids, fatty acids, and polyketides, suggesting their utility as natural antimicrobial agents (Cadamuro et al. 2021). Given its high genetic similarity with *P. fallopiae* and its close clustering with other bioactive strains in the phylogenetic analysis, *Phyllosticta* sp. SPK5 holds promise as a potent antimicrobial producer. Its endophytic nature, reinforced by both molecular and morphological evidence, aligns with an expanding body of research emphasizing the ecological versatility and biotechnological relevance of *Phyllosticta* spp. in sustainable agriculture and natural product discovery.

Characterization of the potent extract using GC-MS

The GC-MS analysis of the ethyl acetate extract of *Phyllosticta* sp. led to the identification of several major bioactive compounds based on their retention times and mass spectral data (Figure 5, Table S1). A total of seven distinct compounds were detected, many of which have been previously reported for their potent antimicrobial and antioxidant properties (Table 3). Among the identified metabolites, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (RT: 11.539 min) and 5-hydroxymethylfurfural (RT: 13.461 min) are well-documented antimicrobial and anti-quorum sensing agents (Kaur et al. 2018; Vijayakumar and Ramanathan 2018; Chen et al. 2021). Structurally related phenolic compounds have also been identified in *Phyllosticta* sp. isolated from one of the important medicinal plants *Guazuma tomentosa*, having antioxidant properties (Elfiati et al. 2022). Notably, pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (RT: 24.724 min), a diketopiperazine, exhibited potential antibacterial, antifungal and cytotoxic activities, and has been widely reported in secondary metabolite profiles of endophytic fungi (Kiran et al. 2018).

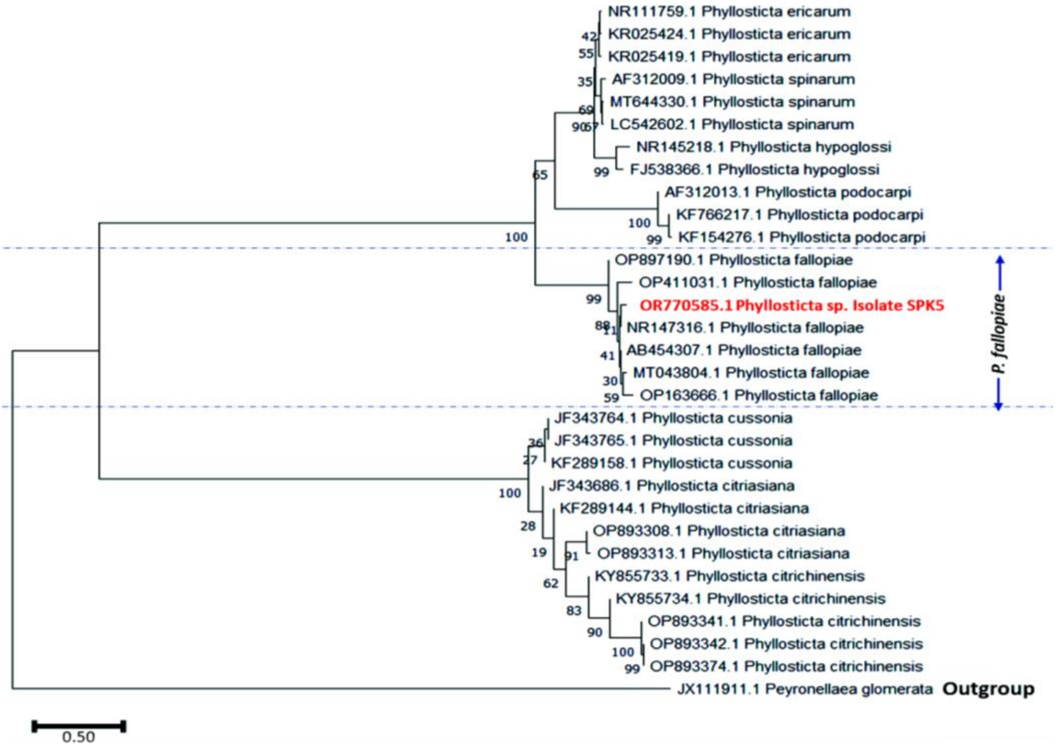

Figure 4. Neighbour-Joining phylogenetic tree based on ITS sequences showing the placement of *Phyllosticta* sp. SPK5 (in red). Bootstrap values (1000 replicates) are indicated at nodes

These cyclic dipeptides are considered pharmacologically privileged scaffolds due to their ability to interact with bacterial membranes and DNA, and are emerging leads in antibiotic and anticancer drug discovery pipelines. These compounds exhibit planar heterocyclic structures with electron-rich nitrogen atoms, features often associated with DNA intercalation and membrane-disruptive properties as predicted by QSAR models. Additionally, free fatty acid derivatives such as palmitic acid (RT: 28.658 min), and stearic acid (RT: 32.313 min) were also detected, all of which are associated with antimicrobial and anti-inflammatory activities (Siddiqui et al. 2017; Ganesan et al. 2024). They are long-chain fatty acid molecules with amphiphilic properties that enable them to disrupt microbial membranes, a mechanism widely supported in QSAR literature (Azmi et al. 2016). Their antimicrobial and anti-inflammatory activities have led to investigations into their application in topical formulations, emulsifiers, and nano-carriers for drug delivery. The detection of 1-nonadecene and n-tetracosanol 1, characterized by long hydrophobic chains, were reported as antimicrobial and anti-inflammatory agents, further validates the therapeutic relevance of the extract (Balachandar et al. 2018; Shalini et al. 2023; Jayaraman and Ramasamy 2024). These molecules are considered natural alternatives to synthetic preservatives and therapeutic excipients due to their bioactive and biodegradable nature. Studies of Taher et al. (2024) documented a wide range of bioactive molecules such as alkaloids, flavonoids, phenolics, quinines, steroids, and terpenoids in *Phyllosticta* spp. associated with a broad range of biological activities, including antimicrobial, anticancer, antioxidant, and herbicidal properties. While this metabolic profiling highlights the chemical diversity and therapeutic potential of *Phyllosticta* sp. isolated from *S. pimpinellifolium*, the complex nature of this bioactive extract necessitates further work. Future studies can be focused on bioassay-guided fractionation and purification to isolate individual active principal compounds. Additionally, Structure-Activity Relationship (SAR) or QSAR analyses will be essential to elucidate the specific mechanisms of action of major compounds. Several *Phyllosticta* species have previously been studied for their metabolomic profiles, revealing the presence of diverse antimicrobial compounds. Notably, *P. capitalensis* has been reported to produce polyketide-derived metabolites such as xenofuranone B, regiolone, and 3,4-dihydroxybenzoic acid (Xu et al. 2021). In addition, endophytic *P. capitalensis* isolated from *Tibouchina granulosa* was found to synthesize a range of bioactive metabolites including ureides (allantoate), coumarins (isofraxidin), N-(3-Oxobutyl)-tyrosine and N-acetylphenylalanine, as well as fatty acids such as linoleic acid and its derivatives, chaulmoogric acid and even muscle relaxant compound metaxalone (Golias et al. 2019). Similarly, *Phyllosticta cirsii* has been shown to produce antimicrobial compounds such as Phyllostoxin and Phyllostin (Evidente et al. 2008). These findings reinforce the pharmaceutical relevance of the metabolite spectrum of the genus *Phyllosticta* and its capacity to produce bioactive natural products with pharmaceutical relevance. In this context, our study highlights *Phyllosticta* sp., an endophyte from *S. pimpinellifolium*, as promising source of antimicrobial agents. The integrative results from antimicrobial screening, MIC determination, time-kill assays and chemical profiling indicate that its bioactivity is likely driven by a consortium of potent secondary metabolites, thereby supporting its potential for further pharmacological evaluation and drug development.

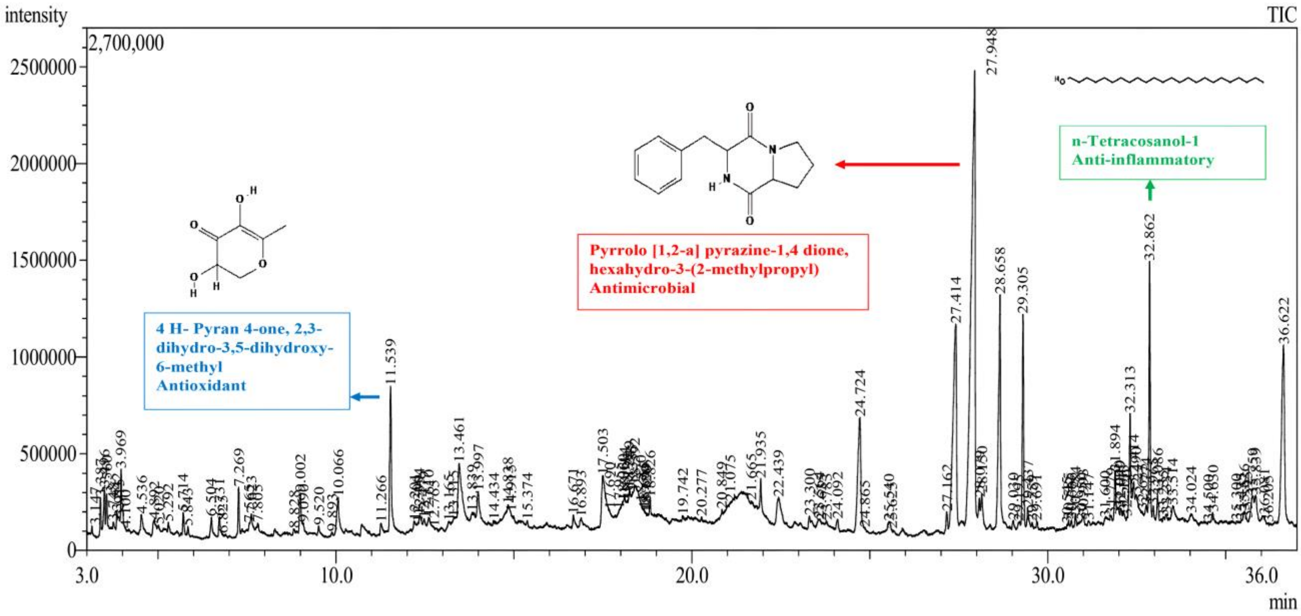

Figure 5. GC-MS chromatogram of ethyl acetate extract from *Phyllosticta* sp. SPK5. Different colours indicate compounds associated with different bioactivities

Table 3. Major compounds identified from organic extract of *Phyllosticta* sp. SPK5

Retention time	Putative bioactive metabolites	Chemical class	Bioactivity	References
11.539	4H- Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	Pyrone (Lactone)	Antioxidant, anti-inflammatory	Chen et al. (2021)
13.461	5-Hydroxymethylfurfural	Furan derivative (Aldehyde)	Antibacterial, anti-quorum sensing	Kaur et al. (2018; Vijayakumar and Ramanathan (2018)
27.948	*Pyrrolo*[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	Diketopiperazine (Cyclic dipeptide)	Antibacterial, cytotoxic, antifungal	Kiran et al. (2018)
28.658	*n*-Hexadecanoic acid (Palmitic acid)	Saturated fatty acid	Antimicrobial, antioxidant	Ganesan et al. (2024)
29.305	1-Nonadecene	Alkene (Long-chain hydrocarbon)	Antimicrobial, antifungal	Balachandar et al. (2018)
32.313	Octadecanoic acid (Stearic acid)	Saturated fatty acid	Antimicrobial, antioxidant	Jubie et al. (2012)
32.862	*n*-Tetracosanol-1	Long-chain alcohol (Fatty alcohol)	Antibacterial, antifungal, anti-inflammatory	Shalini et al. (2023)

In conclusion, the present study identifies *P. fallopiae* SPK5, isolated from leaves of wild tomato (*S. pimpinellifolium*), as the most potent endophytic fungus with strong antimicrobial potential. The ethyl acetate extract of this isolate demonstrated broad-spectrum activity, notably against *E. faecalis*, *B. anthracis*, and *S. aureus*, with low MIC values and confirmed bactericidal effects in time-kill assays. GC-MS analysis revealed a diverse profile of major bioactive compounds, including saturated fatty acids (palmitic acid and stearic acid), long-chain alcohols (n-tetracosanol-1), unsaturated hydrocarbons (1-nonadecene), aromatic aldehydes and furans (5-hydroxymethylfurfural), and cyclic dipeptides such as the diketopiperazine pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-all known for their antimicrobial, antioxidant, or cytotoxic properties. These findings underscore the potential of *P. fallopiae* SPK5 as a source of structurally diverse natural products with relevance in the pharmaceutical sector for the development of alternative antibiotics, especially against multi-drug-resistant pathogens.

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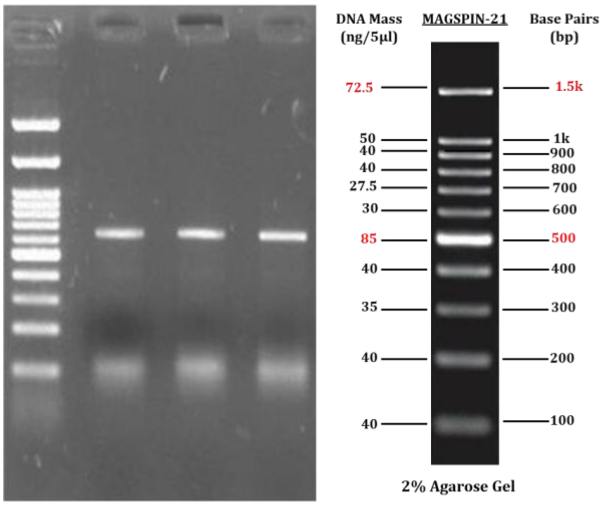

Supplementary file A. Agarose gel electrophoresis image showing PCR amplification of the ITS region from endophytic fungal isolate (*Phyllosticta fallopiae*)


GenBank

Phyllosticta fallopiae isolate SPK 5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GenBank: OR770585.1

FASTA Graphics

Go to:

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KEYWORDS    .
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REFERENCE   1  (bases 1 to 647)
  AUTHORS   Sarma,A. and Tayung,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-NOV-2023) Botany, Gauhati University, Gopinath
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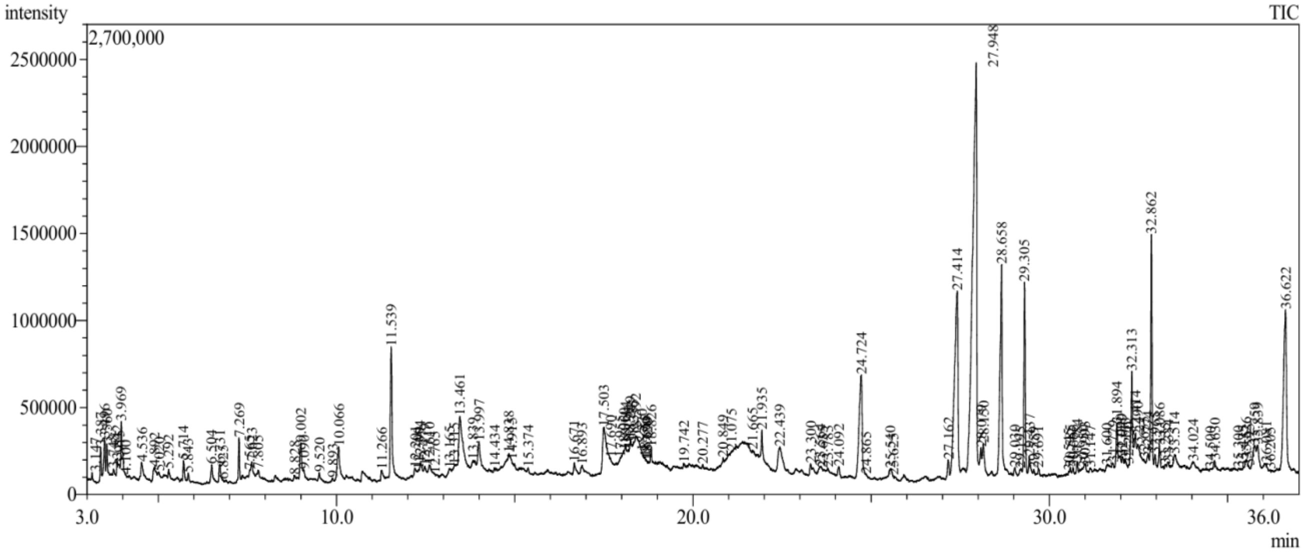
Supplementary file B. NCBI GenBank record of *Phyllosticta fallopiae* SPK 5



Supplementary file C. GC-MS chromatogram of *Phyllosticta fallopiae* SPK5 (Raw file)