Discovery of Antibacterial Compounds from Medicinal Plant Endophytes

by

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Abstract:

Antimicrobial resistance is an escalating global health threat that necessitates the discovery of new bioactive compounds. Fungal endophytes, which live asymptomatically within plant tissues, are increasingly recognized as promising sources of novel secondary metabolites with antimicrobial activity. In this study, fungal endophytes were isolated from seven medicinal plant species and screened for antibacterial activity against clinically relevant bacterial pathogens. A total of 264 morphologically distinct fungal endophytes were isolated from surface-sterilized root and leaf tissues. Initial antibacterial screening identified 58 isolates (22%) capable of inhibiting the sensitive *Acinetobacter baumannii* strain AB258. Antibacterial isolates were disproportionately derived from root tissues (74.1%), suggesting that roots represent a particularly rich source of bioactive endophytes. Of these active isolates, 24 demonstrated broader-spectrum activity against additional bacterial strains. Fifteen isolates producing the strongest inhibition zones were selected for crude extract preparation and secondary screening using disc diffusion assays against a bacterial panel including methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Acinetobacter* spp. Several extracts exhibited multi-strain inhibitory activity, with isolates DL16, GB29, and GB2 producing the largest zones of inhibition. Three candidate isolates (GB2, LV6, and DL16) were prioritized for chemical investigation. Thin-layer chromatography revealed distinct metabolite profiles, with GB2 displaying the greatest compound separation. Consequently, GB2 was selected for further purification using flash chromatography, yielding three pure fractions. Minimum inhibitory concentration (MIC) assays of these fractions revealed relatively high MIC values (128–256 $\mu g\ mL^{-1}$) across tested strains, indicating limited antibacterial potency of

individual compounds but suggesting potential synergistic interactions among metabolites. Overall, this study highlights the diversity of fungal endophytes associated with medicinal plants and demonstrates their potential as sources of antibacterial metabolites. These findings support continued exploration of plant-associated fungi for novel antimicrobial compound discovery.

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Introduction:

The rise of antimicrobial resistance has created an urgent demand for new therapeutic compounds, as conventional antibiotics are becoming increasingly ineffective against multidrug-resistant pathogens (Tiwari & Bae, 2022). To address this global health challenge, natural products remain one of the most promising sources of novel antibiotics, since the majority of clinically used antimicrobials are derived from microbial metabolites (Schneider, 2021). Including fungal endophytes, that associate symbiotically with plants without causing harm to their hosts. These fungi represent a potential valuable reservoir of bioactive compounds (Hoque et al., 2022; Tiwari & Bae, 2022).

Endophytic fungi inhabit the internal tissues of roots, stems, and leaves, where they form long-term, mutualistic, commensal, or sometimes latent pathogenic associations with their plant hosts (Higginbotham et al., 2013; Oliva et al., 2021; Zhou et al., 2021). Their ecological success is shaped by a combination of host-specific traits and environmental factors, including soil composition, moisture availability, and climatic conditions (Ayala et al., 2023; Huusko et al., 2017; Oita et al., 2021). Functionally, fungal endophytes can influence host physiology by enhancing nutrient uptake, improving stress tolerance, and producing defensive metabolites that suppress pathogens and herbivores (Molina-Montenegro et al., 2023; Watts et al., 2023).

Fungi produce a vast diversity of secondary metabolites, small molecules that do not contribute directly to growth or reproduction but are critical for mediating ecological interactions such as defense against stressors, competition with microbes, or predator deterrence (Eagan & Keller, 2025). These metabolites span diverse chemical classes, including alkaloids, polyketides, terpenoids, and peptides, and they exhibit a wide range

of biological activities such as antimicrobial, cytotoxic, antiparasitic, and neuroprotective effects (Aly et al., 2010; Jiang et al., 2019; Jia et al., 2024). While fungal secondary metabolism has been extensively studied in certain groups, endophytic fungi remain comparatively underexplored. Traditional fungal isolation methods often favor fast-growing species that thrive in many conditions, which means rare or slow-growing endophytes are frequently overlooked (Aly et al., 2010). As a result, the diversity of endophyte-derived metabolites and their ecological functions remain incompletely characterized, highlighting the need for continued exploration of these organisms for novel bioactive compounds (James et al., 2020).

Following isolation, the range of applications for endophyte-derived secondary metabolites spans industry, agriculture, and medicine. These compounds include volatile aromatics used in perfumery (Monggoot et al., 2017), plant-growth–promoting metabolites such as indolic auxins produced by *Trichoderma* spp. (Contreras-Cornejo et al., 2009), and clinically valuable molecules like ambuic acid with potent antimycotic activity and the cardiotonic glycoside digoxin (Li et al., 2017; Kaul et al., 2013). However, beyond these broader applications, one of the most critical and urgently relevant roles of endophyte-derived metabolites lies in their potential as new antibiotic compounds. For example, *Trichoderma longibrachiatum* SMF2 produces peptaibols such as trichokonins A (TKA), which strongly inhibit the rice pathogen *Xanthomonas oryzae* pv. *oryzae*. TKA disrupts bacterial membrane integrity, causing cell deformation and leakage of intracellular contents (Zhang et al., 2022). Likewise, an *Aspergillus fumigatus* endophyte from *Albizia lucidior* produces compounds active against *Staphylococcus aureus* that target DNA gyrase and topoisomerase IV (Hussein et al., 2022). More broadly,

natural-product antibiotics, including those derived from endophytes, commonly act on conserved bacterial processes such as cell-envelope biosynthesis, protein synthesis, DNA/RNA synthesis, and β-lactamase inhibition, which are major targets in modern antibiotic discovery (Butler et al., 2024).

The functional diversity of fungal metabolites has led to increased interest in endophytes from medicinal plants, which are often enriched in fungi capable of producing biologically active compounds. Medicinal hosts are rich in bioactive phytochemicals which can provide a selective environment that favors fungi capable of producing metabolites with therapeutic properties (Toppo et al., 2024). As a result, the phytochemical environment of medicinal plants may shape the metabolic potential of their endophytes, increasing the likelihood of isolating fungal strains that produce bioactive metabolites (Egamberdieva et al., 2017). Bioprospecting studies support this trend, with medicinal hosts consistently yielding a higher proportion of endophytic fungi that exhibit antimicrobial, anticancer, and other therapeutically relevant activities compared to non-medicinal plants (Hashem et al., 2023; El-Zehery et al., 2024). These patterns suggest that medicinal plants serve as reservoirs for unique fungal diversity that shape endophyte metabolic potential, increasing the likelihood of discovering novel bioactive metabolites.

Despite substantial interest in fungal secondary metabolism, the majority of fungal biodiversity remains uncharacterized. Of the estimated 2.2–3.8 million fungal species on Earth, only ~120,000 have been formally described, and fewer than 5,000 have been identified as endophytes (Hawksworth & Lücking, 2017; James et al., 2020). This gap between known and undiscovered fungal diversity reveals how much of endophyte biology and metabolism is still unexplored. Consequently, bioprospecting endophytic

fungi, particularly those associated with medicinal plants, remains a highly promising strategy for discovering novel and therapeutically relevant antibacterial metabolites that may help address the growing challenge of antimicrobial resistance.

This project aims to discover and characterize antibacterial secondary metabolites from fungal endophytes associated with medicinal plant species. Specifically, I will:

1. isolate fungal endophytes from surface-sterilized tissues of seven plant species and screen for antibacterial activity
2. produce crude extracts from the top antibacterial producing isolates, followed by chromatographic fractionation and chemical characterization of the most active extracts/compounds

Objective 1: Fungal endophytes will be isolated from seven medicinal plants, which will then be screened for antibacterial activity using agar plug diffusion assays. Morphological characterization will be performed for all isolates, and DNA-based identification (ITS sequencing) will be conducted for bioactive strains. We hypothesize that endophytes from diverse plant hosts will yield a subset of isolates with measurable antibacterial activity, some of which may represent under characterized or novel taxa.

Objective 2: The fungal isolates demonstrating the highest antibacterial activity will be fermented in liquid media, and crude extracts will be obtained via solvent extraction. Crude extracts will first be analyzed using LC–MS to characterize their metabolite profiles. Extracts showing the highest bioactivity will then be fractionated using flash chromatography, and the resulting fractions will be evaluated using minimum inhibitory concentration (MIC) assays to identify antibacterial components. We

hypothesize that chemical profiling and fractionation of the most active extracts will reveal chemically diverse secondary metabolites, some of which may represent previously uncharacterized antibacterial compounds

Materials and Methods:

1. Endophyte isolation and antibacterial activity screening

1.1 Host species collection

Endophytic fungi were isolated from seven medicinal plant species (Table 1). These plants were chosen primarily because they are less widely studied as hosts of endophytes, providing an opportunity to discover novel fungal taxa and metabolites. They were also selected because they are locally abundant at the collection sites and have a documented medicinal history, supporting their relevance as reservoirs of bioactive endophytes. To maximize the recovery of diverse endophytic taxa from each host, tissues were sampled from both the rhizosphere (roots) and phyllosphere (leaves). All plant species, except *Arctium lappa*, were collected from a residential garden (49.94393° N, 97.05918° W) characterized by heavy clay soil and natural wood mulch in Winnipeg, Manitoba, Canada. *Arctium lappa* was collected from a naturalized public area within Bunn's Creek Park (49.94910° N, 97.05965° W) in Winnipeg, Manitoba, characterized by clay and organically enriched loamy soil. Whole plants were collected between early and late May 2025 and stored in paper envelopes at 4 °C for a maximum of 48 h prior to processing. Five biological replicates were collected for each plant species, except *Viburnum trilobum*, for which one replicate was collected.

Table 1. Medicinal plant species selected for endophyte isolation, including traditional medicinal uses.

ID	Scientific name	Medicinal Use	Reference
Dandelion (DL)	*Taraxacum officinale*	Exhibits anti-inflammatory, antioxidant, antimicrobial, and anticancer activities	(Di Napoli & Zucchetti, 2021).
Creeping bluebell (CB)	*Campanula rapunculoides*	Alleviate inflammation and abdominal discomfort	(Vasfilova et al., 2020)
Canadian ginger (CG)	*Asarum canadense*	Poultice applied to infected wounds; analgesic effects	(Cavallito & Bailey, 1946)
Lily of the valley (LV)	*Convallaria majalis*	Demonstrates anticancer, anti-inflammatory, and cardioprotective properties	(Witkowska et al., 2024)
Sarsaparilla (SS)	*Aralia nudicaulis*	Poultice applied to wounds and infections; possesses anticancer and antioxidant activity	(Wang et al., 2006)
Greater burdock (GB)	*Arctium lappa*	Treat infection and wounds; diuretic; reduce fever	(Yosri et al., 2023)
Highbush Cranberry (HC)	*Viburnum trilobum*	Exhibits antioxidant, antibacterial, anti-inflammatory, and cytotoxic effects	(Sharifi-Rad et al., 2021)

1.2 Medicinal plant sterilization

Plant tissues were surface sterilized following a protocol adapted from Arnold et al. (2007) and Hamzah et al. (2018), with modifications based on tissue type. Leaf samples were rinsed under distilled water for 5 min, immersed in 70% ethanol for 1 min, rinsed in sterile distilled water, treated with 1% sodium hypochlorite (bleach) for 1 min, rinsed again, then immersed in 70% ethanol for an additional 1 min, followed by a final rinse of distilled water. Root samples, due to their thicker structure, were treated with longer exposure: 70% ethanol for 3 min, 1% bleach for 2 min, and a second 70% ethanol wash for 3 min, with distilled water rinses between and after each step. All samples were processed in a Class II biosafety cabinet and allowed to air dry on sterile Petri dishes.

Leaf segments were cut into ~0.5–1 cm squares, while root samples were cut into ~1–1.5 cm pieces with a longitudinal incision to expose internal tissue.

1.3 Fungal endophyte isolation

Following surface sterilization, tissue segments were plated onto 90 × 15 mm Petri dishes containing one of four media types: malt extract agar (MEA), potato dextrose agar (PDA), MEA + ampicillin, and PDA + ampicillin, with a final antibiotic concentration of 50 μg/mL. Plates were sealed and incubated at ambient room temperature (22 ± 2 °C) for up to five weeks. Emerging fungal colonies were monitored daily and differentiated based on macroscopic morphological characteristics. Distinct morphotypes were identified according to colony color, texture (e.g., powdery versus filamentous growth), radial growth rate, margin characteristics, and pigmentation observed on both the obverse and reverse sides of the agar. Morphologically distinct fungal colonies were sub-cultured onto fresh agar plates to obtain pure isolates. Any isolates initially grown on ampicillin-supplemented media were subsequently transferred to antibiotic-free media prior to antibacterial screening to ensure that no observed activity resulted from residual ampicillin.

1.4 Fungal endophyte identification

Genomic DNA was extracted from fresh fungal mycelium using a modified cetyltrimethylammonium bromide (CTAB) protocol previously described in Hausner et al. (1992) and optimized for next generation sequencing (Wai and Hausner, 2021). Whole-genome sequencing was performed by MicrobesNG (Birmingham, UK) using Illumina short-read technology.

Illumina reads and contigs were uploaded onto the Galaxy (usegalaxy.eu; Afgan et al. 2018). The reads were initially assessed using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and assemblies were generated with SPAdes vs. 3.14.0 (setting the "--careful" option and assembly graph option, Bankevich et al. 2012). Bandage (Wick et al. 2015) was used to examine the assembly graph files generated from SPAdes to aid in the recovery of potential mitogenome contigs. In addition, GetOrganelle was used to recover mitogenome-derived reads and to generate mitogenome assemblies (Jin et al., 2020) v1.7.5, with the organelle type set to fungus mitogenome (i.e., -F fungus_mt).

The assembled mitogenome contigs were annotated using the MFannot program (Lang et al. 2023; https://megasun.bch.umontreal.ca/cgi-bin/dev_mfa/mfannotInterface.pl; setting "Genetic Code" to 4) and RNAweasel (Prince et al. 2022; https://megasun.bch.umontreal.ca/cgi-bin/RNAweasel/RNAweaselInterface.pl; setting to predict tRNAs, and group I and group II introns.

The isolated mitochondrial sequences were used as BLASTn queries against the NCBI GenBank nucleotide database to identify closely related taxa and the potential hits provided guides for nuclear marker selection.

Taxonomic identity was assessed using three commonly applied fungal molecular markers: the internal transcribed spacer (ITS) region, the β-tubulin gene, and complete mitochondrial genome sequences. Reference FASTA sequences for these markers were obtained from NCBI based on literature and database searches. Marker sequences were imported into the Galaxy Europe platform (usegalaxy.eu) and used as queries to identify contigs with similar sequences. The recovered "hits" were used to search in NCBI to find

similar sequences and for the top hits the sequence identity and query coverage were recorded.

1.5 Screening for antibacterial activity

Antibacterial activity of fungal endophytes was assessed using the agar plug diffusion method, following the protocol of Onn et al. (2016) with modifications. Overnight bacterial cultures (see Table 2) were prepared by inoculating 5 mL of LB broth in sterile test tubes and incubating at 37 °C for 16–18 hours in a New Brunswick Excella E24 incubator shaker, set to 220 rpm. Initial antimicrobial screening using the agar plug diffusion assay was performed against a broader panel of bacterial strains including both Gram-positive and Gram-negative pathogens, to assess general antibacterial activity. Bacterial suspensions were then standardized to a 0.5 McFarland standard using a DensiCHEK densitometer, diluted using 0.85% NaCl solution (physiological saline). A uniform bacterial lawn was established by swabbing the standardized suspension across the surface of LB agar plates. One-week-old fungal endophyte cultures were sampled by cutting 1 cm diameter agar plugs and placed centrally on a freshly streaked LB plate, later incubated at 37 °C for 24 hours. Zones of inhibition (cm) produced by fungal endophytes were measured to assess antibacterial activity. Only fungal isolates that show inhibitory effects toward the bacterial strains in the preliminary screening were selected for further analysis.

Table 2. Bacterial strains used for antimicrobial screening assays.

Strain ID[1]	Bacterial Species	Gram Reaction	Description	References
AB258†/‡	*Acinetobacter baumannii*	Gram-negative	Efflux-sensitive derivative of A. baumannii ATCC 17978 containing deletions in adeIJK, adeFGH, and adeAB	(Kornelsen et al., 2021)
PA075‡	*Pseudomonas aeruginosa*	Gram-negative	Efflux-sensitive strain containing deletions in *mexAB-oprM, mexCD-oprJ, mexEF-oprN, mexJK, mexXY,* and *opmH*	(Kumar et al. 2006)
MRSA†/‡	*Staphylococcus aureus*	Gram-positive	Methicillin-resistant clinical isolate	(Turner et al., 2019)
AB030†	*Acinetobacter baumannii*	Gram-negative	MDR clinical isolate	(Fernando et al., 2013)
PA082†	*Pseudomonas aeruginosa*	Gram-negative	MDR clinical isolate	(Singh et al., 2013)
ATCC 17978†	*Acinetobacter baumannii*	Gram-negative	Wild type strain	(Piechaud and Second 1951)
PA01†	*Pseudomonas aeruginosa*	Gram-negative	Wild type strain	(Holloway 1955)
ATCC 4352‡	*Klebsiella pneumoniae* subsp. *pneumoniae*	Gram-negative	Reference strain widely used for antiseptic and quality-control testing	(Brown & Wilson, 1972)

[1] † Included in primary screening using agar plug diffusion. ‡ Included in downstream antibacterial disc diffusion assays.

MDR = multidrug resistant, ATCC = American type culture collection

2. Crude Extraction and Metabolite Characterization

2.1 Fermentation and extraction of fungal crude

For each strain, 250 mL of sterile liquid medium (either malt extract broth or potato dextrose broth) was prepared in a 1 L Erlenmeyer flask to initiate static fermentation for secondary metabolite production. A single flask was used per fungal isolate during the initial fermentation. Agar plugs from actively growing solid fungal cultures were aseptically transferred into the flasks, that were covered with aluminum caps, and incubated at 22 ± 2 °C under static conditions for approximately 14 days. Fungal growth was monitored visually, and fermentation was considered complete when a dense surface mat of mycelium formed.

Extraction of secondary metabolites from each fungal culture was performed following vacuum filtration of the fermented broth through a ceramic Buchner funnel. The recovered spent media was acidified to pH 2.5–3.0 using HCl. An equal volume of ethyl acetate was added to the acidified filtrate (1:1, v/v). After phase separation, the upper organic layer, containing the extracted metabolites, was collected, while the lower aqueous layer was retained for an additional extraction using fresh ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure using a Büchi Rotavapor R-300 rotary evaporator (110 rpm, 125 mbar) at 40 °C to remove the ethyl acetate solvent. The remaining crude extract was rinsed from the flask with methanol and transferred to a glass vial using a sterile serological pipette. Extracts were allowed to air dry overnight to remove residual solvent, sealed, and stored at −20 °C until further analysis.

2.2 Antibacterial disc diffusion assay

Antibacterial activity of fungal crude extracts was evaluated using a disc diffusion assay following Sharma et al. (2017) with modifications. Sterile 6 mm filter paper discs were placed onto LB agar plates with overnight bacterial lawns of the test organisms (Table 2). For downstream analyses, a subset of representative strains was selected to focus on clinically relevant and antibiotic-resistant pathogens, enabling a more targeted evaluation of therapeutic potential while maintaining coverage of both Gram-positive and Gram-negative pathogens. Each disc was loaded with 7 µL of crude extract at a concentration of 1 g/mL prepared in methanol. Methanol-loaded discs served as controls. Plates were incubated at 35 ± 2 °C for 24 h, after which antibacterial activity was determined by measuring the diameter of the zone of inhibition surrounding each disc.

2.3 Determining MIC

Minimum inhibitory concentrations (MICs) of the purified fungal extract fractions were determined using the broth microdilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2019) and adapted from Gill et al. (2023). Bacterial cultures grew overnight in 5 mL of LB broth and adjusted to a 0.5 McFarland standard (10^8 CFU/mL) in 0.85% NaCl using a DensiCHEK densitometer. The adjusted cultures were diluted in Mueller–Hinton Broth (MHB) to a final bacterial density of approximately 5×10^5 CFU/mL. 50 uL of purified fractions were prepared in two-fold serial dilutions from 512 to 16 µg/mL in a 96-well microtiter plate, and each well was inoculated with the 50 uL of bacterial suspension. Plates were incubated at 37 °C for 16–20 h. Ciprofloxacin and methanol were included as positive and negative controls, respectively. The MIC was recorded as the lowest extract concentration at which no visible bacterial growth was observed.

2.4 LC–MS/MS Analysis and Fractionation of Fungal Extracts

To characterize the secondary metabolites, present in the fungal extracts, crude extracts were first analyzed using liquid chromatography–mass spectrometry (LC-MS/MS). The resulting mass spectral data were uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform to generate molecular networks and compare detected features against reference spectral libraries for preliminary metabolite annotation. This also served as a dereplication step that enabled the rapid identification of previously reported compounds and prioritized novel metabolites of interest for further purification.

Following LC-MS/MS-based dereplication, crude extracts from candidate isolates were subjected to flash column chromatography to separate individual metabolites. Fractionation was performed using a silica gel stationary phase and an organic solvent gradient consisting of hexane, ethyl acetate, and methanol of increasing polarity. Eluted fractions were collected sequentially and monitored by thin layer chromatography (TLC) using hexane: ethyl acetate (7:3, v/v) as the developing solvent system. Developed plates were visualized under ultraviolet (UV) illumination to assess the presence and separation of distinct metabolites. Fractions displaying distinct TLC profiles were retained for further purification and downstream chemical analysis.

2.5 Statistical analysis

Because the primary objective of this study was exploratory screening for antibacterial activity among fungal endophyte isolates, results were interpreted descriptively. Antibacterial activity was evaluated based on the presence or absence of zones of inhibition and relative differences in inhibition zone size among isolates. Consequently, no formal statistical analyses were performed.

Results:

Fungal Isolation and Diversity

A total of 264 fungal endophytes were isolated from surface-sterilized tissues across the seven medicinal plant species based on colony morphology. Fungal isolation success varied among hosts, ranging from 21 isolates from dandelion and creeping blue bell, to 66 isolates from lily of the valley (Figure 1).

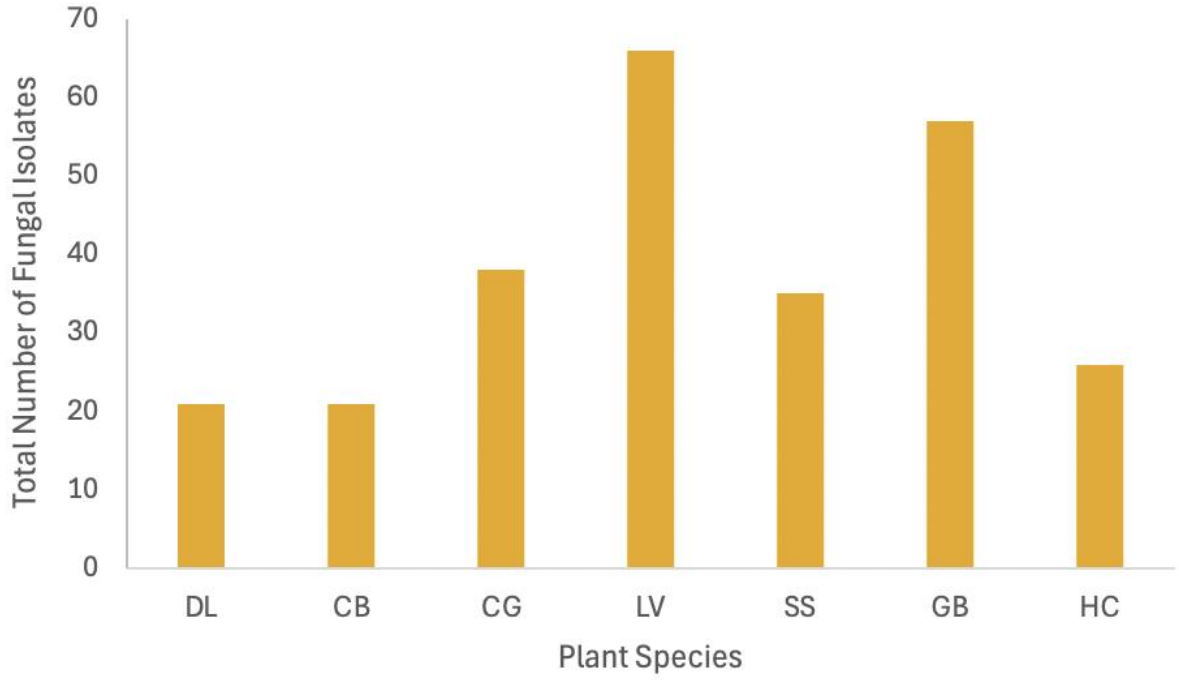

Figure 1. Total number of fungal endophytes isolated from each medicinal plant species. Bars represent the number of morphologically distinct fungal isolates (n = 264) recovered from each host plant. Plant abbreviations: *Taraxacum officinale* (dandelion, DL), *Asarum canadense* (canadian ginger, CG), *Campanula rapunculoides* (creeping bluebells, CB), *Arctium lappa* (greater burdock, GB), *Viburnum trilobum* (highbush cranberry, HC), *Convallaria majalis* (lily of the valley, LV), *Aralia nudicaulis* (sarsaparilla, SS).

Antibacterial Activity of Endophytic Fungal Isolates

Of the 264 isolates, 58 (22%) produced measurable antibacterial activity, as observed by the presence of zone of inhibitions, against the antibiotic-sensitive AB258 strain, which lacks major efflux pumps and is therefore useful for detecting antibacterial

metabolites that might otherwise be exported by resistant strains. The distribution of antibacterial isolates varied substantially among host plants: greater burdock contributed the most active isolates (20), while Canadian ginger yielded only one (Figure 2). Across all host plants, antibacterial activity was tissue biased. From the antibacterial fungal isolates, 74.1% originated from root tissues, whereas only 25.9% were isolated from leaf tissues (Figure 2). This indicates that roots were the predominant source of endophytes exhibiting antibacterial activity in the AB258 screening assay.

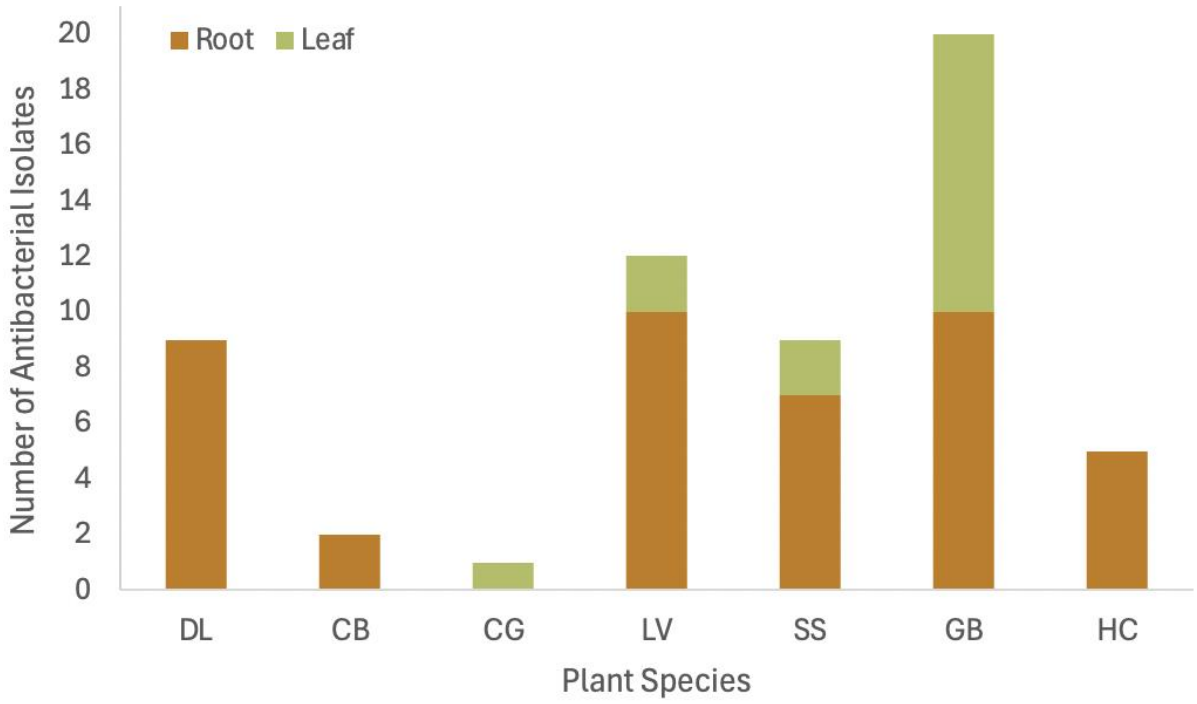

Figure 2. Tissue origin (root vs. leaf) of AB258-active fungal endophytes. Bars show the number of isolates exhibiting antibacterial activity against the sensitive AB258 strain (n = 58), separated by tissue of origin. Root-derived isolates are shown in brown and leaf-derived isolates in green. Plant abbreviations: *Taraxacum officinale* (dandelion, DL), *Asarum canadense* (canadian ginger, CG), *Campanula rapunculoides* (creeping bluebells, CB), *Arctium lappa* (greater burdock, GB), *Viburnum trilobum* (highbush cranberry, HC), *Convallaria majalis* (lily of the valley, LV), *Aralia nudicaulis* (sarsaparilla, SS).

Twenty-four of the 58 isolates (41.4%) inhibited additional bacterial strains (see Table 2), showing that a substantial proportion of the AB258-active fungi produced metabolites with broader-spectrum antibacterial properties. Among the 24 isolates, 15 (62.5%) produced stronger ZOIs of 1.5 cm or greater, while 9 isolates (37.5%) produced smaller zones (<1.5 cm). To streamline downstream work and prioritize isolates with comparatively higher antibacterial activity, only the 15 strongest isolates were selected for crude extract production.

All 15 fungal extracts inhibited at least one bacterial strain, with zones of inhibition (ZOIs) ranging from 1.0–3.6 cm across the panel (Table 3; Figure 3). Several isolates exhibited broad-spectrum activity: DL16 (ZOIs 2.6–3.6 cm), GB29 (2.2–3.4 cm), and GB2 (1.7–2.9 cm) inhibited all four test strains. Moderate multi-strain inhibition was observed for DL18, LV6, and GB41. The efflux-sensitive *Pseudomonas aeruginosa* strain PA0750 was the most resistant, inhibited strongly by only DL16 and GB29 with ZOIs of 3.6 cm and 3.4 cm, respectively. In contrast, *Acinetobacter baumannii* AB258 and MRSA were inhibited by 13 of 15 extracts, with ZOIs ranging from 1.0–3.3 cm and 1.0–3.2 cm, respectively. *Klebsiella pneumoniae* was inhibited by 12 of 15 extracts, with ZOIs ranging from 1.0–2.6 cm (Table 3). A complete set of crude extract inhibition plates is provided in the Appendix (Figure A1).

Table 3. Zone of inhibition (ZOI) values for fifteen crude fungal extracts tested against the four-strain bacterial panel defined in Table 2. Extracts were prepared at 1 g/mL in methanol, with methanol serving as the solvent control. Values represent ZOI diameters (cm) measured using disc diffusion assays. Plant abbreviations: *Taraxacum officinale* (dandelion, DL), *Asarum canadense* (canadian ginger, CG), *Campanula rapunculoides* (creeping bluebells, CB), *Arctium lappa* (greater burdock, GB), *Viburnum trilobum* (highbush cranberry, HC), *Convallaria majalis* (lily of the valley, LV), *Aralia nudicaulis* (sarsaparilla, SS).

Fungal Strain ID	Zone of Inhibition (cm)			
	Bacterial Species			
	Efflux-sensitive *Pseudomonas aeruginosa* (PA075)	Efflux-sensitive *Acinetobacter baumannii* (AB258)	Methicillin-resistant *Staphylococcus aureus* (MRSA)	*Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC 4352)
DL 2	0.0	2.5	1.8	0.0
DL 16	3.6	3.3	2.6	2.6
DL 18	1.8	2.6	3.2	1.6
LV 6	1.8	2.8	1.8	2.0
LV 12	1.1	2.5	1.6	1.4
HC 12	0.0	1.0	1.1	0.0
GB 2	1.8	2.6	2.9	1.7
GB 3	1.3	2.0	2.2	1.2
GB 10	1.4	2.2	1.8	1.7
GB 21	1.3	1.5	1.0	1.0
GB 26	1.5	2.2	1.6	1.4
GB 29	3.4	3.0	2.2	2.2
GB 39	1.9	2.2	1.3	1.0
GB 41	1.5	2.6	2.4	1.8
GB 52	1.3	1.8	1.6	1.2

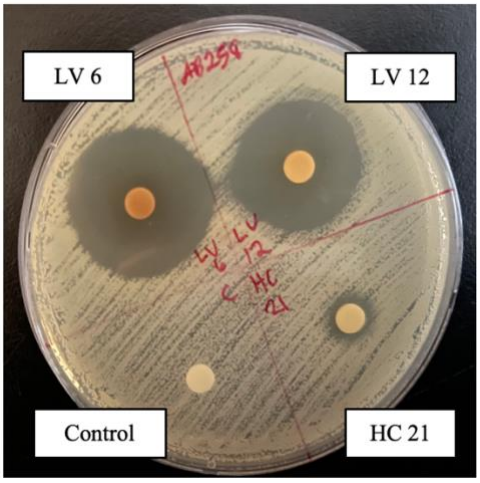

Figure 3. Representative disc diffusion assay showing antibacterial activity of a crude fungal extract against the sensitive AB258 strain. The figure shows an example plate from crude extracts derived from *Viburnum trilobum* (highbush cranberry, HC), and *Convallaria majalis* (lily of the valley, LV), illustrating how inhibition zones (ZOIs) were measured to quantify antibacterial activity. Each crude extract was prepared at a concentration of 1 g/mL in methanol, and methanol alone served as the solvent control. Clear zones surrounding the extract-loaded discs indicate inhibition of bacterial growth.

Based on the panel screening results, three isolates, DL16, LV6, and GB2, were selected for liquid chromatography–mass spectrometry (LC–MS) characterization. These isolates were prioritized because they exhibited strong and consistent multi-strain antibacterial activity, produced relatively large zones of inhibition, and originated from different host plants and tissues, thereby maximizing the chemical diversity captured for downstream analysis.

Although GB29 showed one of the strongest inhibition zones, it displayed morphological features like DL16. Both GB29 and DL16 demonstrated rapid growth on PDA, covering most of the plate surface within 14 days. On the obverse surface, colonies appeared grey-green and distinctly powdery in texture, consistent with heavy sporulation, while the reverse side exhibited pronounced yellow–orange pigmentation visible through the agar (Figure 4). The two isolates

displayed highly comparable colony morphology, including similar sporulating surfaces and reverse pigmentation patterns. This shared phenotype suggests that GB29 and DL16 may be taxonomically related and/or possess overlapping biosynthetic capacities. Due to time constraints, only one representative was advanced; therefore, DL16 was selected over GB29 because it produced slightly larger ZOIs and represented the stronger candidate of the two.

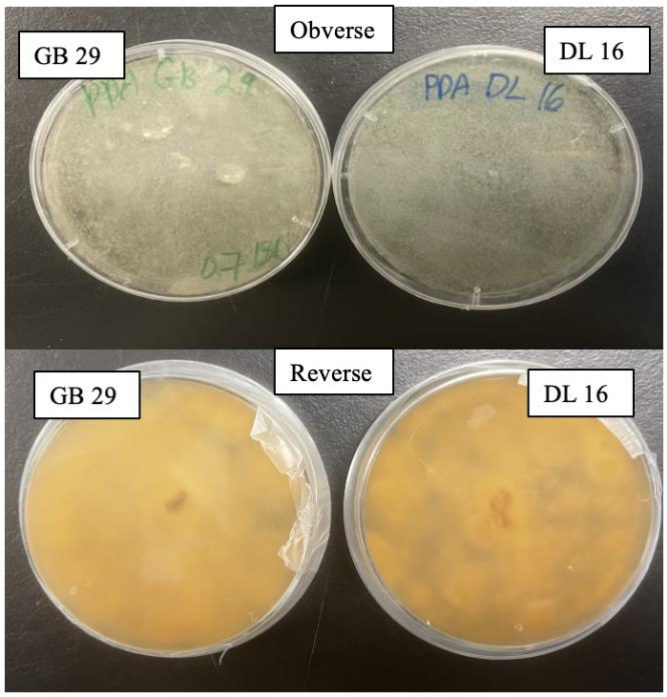

Figure 4. Colony morphology of fungal endophyte isolates GB29 and DL16 grown on potato dextrose agar (PDA). Obverse (top) and reverse (bottom) views of isolates after incubation at 25°C for 14 days. On the obverse surface, both GB29 and DL16 formed dense, powdery grey-green colonies indicative of abundant sporulation, with diffuse colony margins and near-complete plate coverage. On the reverse side, both GB29 and DL16 display strong yellow–orange pigmentation visible through the agar.

Molecular Identification of Candidate Endophytic Fungi

For each of the three candidate isolates (DL16, GB2, and LV6) whole-genome assemblies were analyzed using three molecular markers: the complete mitochondrial

genome, the internal transcribed spacer (ITS) region, and the β-tubulin gene. Taxonomic identity was assessed based on percent identity and query coverage obtained from BLASTn comparisons against the NCBI GenBank database.

For isolate DL16, all three markers showed complete concordance. The mitochondrial genome sequence exhibited 100% identity and 100% query coverage to *Penicillium digitatum* (Table 4). Similarly, both ITS and β-tubulin sequences showed 100% identity and 100% query coverage to *P. digitatum*. The consistent agreement across mitochondrial and nuclear markers supports unambiguous identification of DL16 as *Penicillium digitatum*.

Table 4. BLASTn results for multilocus sequence analysis of isolate DL16 using mitochondrial, internal transcribed spacer (ITS), and β-tubulin markers. The table shows the top database match, maximum score, total score, query coverage (%), E-value, percent identity (%), accession length, and accession number for each marker.

Marker	Scientific Name	Max Score	Total Score	Query Coverage (%)	E value	Percent Identity (%)	Accession Length	Accession number
Mitocondrion	*Penicillium digitatum*	24083	24083	100	0	100	28978	NC_015080.1
ITS	*Penicillium digitatum*	989	989	100	0	100	547	OR468972.1
β-tubulin	*Penicillium digitatum*	1336	1336	100	0	100	723	KC175444.1

Isolate GB2 also demonstrated full agreement among markers with regards to species designation (Table 5). The mitochondrial genome showed 100% identity and 100% query coverage to the mtDNA of *Trichoderma reesei*. The ITS and β-tubulin loci likewise returned 100% identity and 100% query coverage to *T. reesei*. These results consistently support the assignment of GB2 to *Trichoderma reesei*.

Table 5. BLASTn results for multilocus sequence analysis of isolate GB2 using mitochondrial, internal transcribed spacer (ITS), and β-tubulin markers. The table shows the top database match, maximum score, total score, query coverage (%), E-value, percent identity (%), accession length, and accession number for each marker.

Marker	Scientific Name	Max Score	Total Score	Query Coverage (%)	E value	Percent Identity (%)	Accession Length	Accession number
Mitocondrion	*Trichoderma reesei*	11119	11119	100	0	100	42130	NC_003388.1
ITS	*Trichoderma reesei*	1197	1197	100	0	100	648	MW514156.1
β-tubulin	*Trichoderma reesei*	342	342	100	10^{-89}	100	231	OM669565.1

For isolate LV6, the recovered mitochondrial sequence showed 100% identity and 100% query coverage to reference genomes annotated as *Alternaria alternata* complex in GenBank (Table 6). To further assess taxonomic placement, ITS and β-tubulin loci were extracted from the nuclear genome assembly and subjected to BLASTn analysis. Both markers returned 100% identity and 100% query coverage to entries annotated as *Alternaria alternata*. However, the ITS sequence also produced equivalent 100% identity matches to several closely related members of the *Alternaria alternata* species complex. Similar results were observed for β-tubulin, which matched multiple *Alternaria* species with identical identity and coverage values. Due to this lack of resolution among closely related taxa, species-level assignment based solely on ITS and β-tubulin was not possible, whereas the complete mitochondrial genome provided a highly specific match to *Alternaria alternata* reference sequences.

Table 6. BLASTn results for multilocus sequence analysis of isolate LV6 using mitochondrial, internal transcribed spacer (ITS), and β-tubulin markers. The table shows the top database match, maximum score, total score, query coverage (%), E-value, percent identity (%), accession length, and accession number for each marker.

Marker	Scientific Name	Max Score	Total Score	Query Coverage (%)	E value	Percent Identity (%)	Accession Length	Accession number
Mitocondrion	*Alternaria alternata*	36967	36967	100	0	100	57449	PV822414.1
ITS	*Alternaria alternata complex*	857	857	100	0	100	533	KT375656.1
β-tubulin	*Alternaria alternata complex*	1225	1225	100	0	100	809	MG558003.1

LC–MS/MS Analysis and Chromatographic Fractionation of Bioactive Extracts

LC–MS/MS analysis followed by molecular networking on the GNPS platform revealed a diverse range of putative secondary metabolites across the three candidate fungal isolates. A total of 29 putative metabolites were detected in both the GB2 and LV6 extracts, while 30 putative metabolites were detected in the DL16 extract. Of these, 24 metabolites in GB2 and LV6, and 26 metabolites in DL16, were annotated through comparison with GNPS spectral libraries, while the remaining features could not be confidently assigned and were classified as uncharacterized compounds. Detailed lists of both annotated and uncharacterized metabolites for each extract are provided in Appendix Tables A1–A6. The annotated metabolites represent a range of compound classes previously reported to exhibit diverse biological activities, including antibacterial, antioxidant, and anticancer properties.

Crude extracts from the three candidate isolates (GB2, LV6, and DL16) were prepared for downstream chemical analysis. However, due to insufficient

crude extract yield, isolate LV6 could not be subjected to further chromatographic purification. The remaining extracts were therefore evaluated to determine their suitability for additional fractionation.

Thin-layer chromatography (TLC) was subsequently performed to assess compound separation within the fractions obtained from these crude extracts. The TLC profile of the GB2 fraction revealed multiple distinct bands under UV illumination, with at least three clearly separated features observed on the chromatographic plate (Figure 5), suggesting the presence of several chemically distinct compounds. In contrast, fractions derived from the DL16 extract showed minimal band separation, with only a limited number of detectable bands observed on the TLC plate.

Figure 5. Thin-layer chromatography (TLC) analysis of a fraction derived from the GB2 crude extract developed using a hexane:ethyl acetate (7:3, v/v) solvent system. Differences in band number and migration distance reflect variation in compound polarity among the detected metabolites.

Based on these observations, the GB2 extract was prioritized for further purification. Additional flash column chromatography was therefore performed to further resolve the chemical constituents present in the GB2 extract. This secondary fractionation yielded three pure fractions, which were collected for future chemical characterization.

Minimum inhibitory concentrations (MICs) were determined for three purified fractions (GB2 B, GB2 C, and GB2 D) against methicillin-resistant *S. aureus* (MRSA) strain, and two Gram-negative *A. baumannii* strains, AB258 and ATCC 17978 (Table 7). All three fractions exhibited similar inhibitory activity across the strains examined. For the MRSA strain, each fraction produced an MIC of 256 μg mL^{-1}. In contrast, lower MIC values were observed for the AB258 and ATCC 17978 strains, with all three fractions showing MICs of 128 μg mL^{-1} (Table 7). No differences in inhibitory activity were observed among the three fractions across the bacterial strains tested.

Table 7. Minimum inhibitory concentrations (MICs) of purified fractions GB2 B, GB2 C, and GB2 D against methicillin-resistant *Staphylococcus aureus* (MRSA) and two *Acinetobacter baumannii* strains (AB258 and ATCC 17978). MIC values are expressed in μg mL^{-1} and indicate the lowest concentration required to inhibit visible bacterial growth.

Purified Fractions	Minimum Inhibitory Concentrations (μg/mL)		
	Bacterial Species		
	Wild-type *Acinetobacter baumannii* (ATCC 17978)	Efflux-sensitive *Acinetobacter baumannii* (AB258)	Methicillin-resistant *Staphylococcus aureus* (MRSA)
GB2 B	128	128	256
GB2 C	128	128	256
GB2 D	128	128	256

Discussion:

Fungal Diversity and Host Influence

Fungal endophytes are taxonomically diverse and ecologically widespread, colonizing nearly all plant lineages across a broad range of environments. Their diversity and distribution are shaped by multiple ecological factors, including host identity, soil chemistry, moisture availability, geographic location, and climatic conditions (Ayala et al., 2023; Huusko et al., 2017; Oita et al., 2021). Given that nearly 300,000 terrestrial plant species may each harbor multiple endophytic fungi, the global diversity of fungal endophytes is estimated to include several million species (Mishra et al., 2019).

In this study, 264 morphologically distinct fungal endophytes were recovered from seven medicinal plant species, indicating that all host plants examined support diverse and culturable endophytic communities. It should be noted, however, that culture-based isolation approaches capture only the culturable fraction of endophytic communities, and

a proportion of endophytes may remain undetected due to their inability to grow in pure culture outside host tissues (Akram et al., 2023). Additionally, morphotype classification was based solely on macroscopic colony characteristics. As morphological traits can vary with growth conditions and may overlap among taxa, this approach may have resulted in underestimation or overestimation of true isolate diversity. Molecular identification would be required to confirm taxonomic distinctiveness among the 264 morphologically defined isolates.

The number of isolates recovered varied substantially among hosts, ranging from 21 isolates in dandelion and creeping bluebell to 66 in lily of the valley. The variation in endophyte isolation success among hosts observed in this study is consistent with previous multi-host surveys demonstrating that plant species differ widely in the abundance and diversity of their endophytic fungi. Studies sampling multiple hosts have similarly found that some plants yield only a small number of endophytes, whereas others harbor highly diverse communities of fungal endophytes (U'Ren et al., 2012; Wang et al. 2019). These host-dependent differences are commonly attributed to species-specific traits, including tissue structure and phytochemical composition, that influence fungal colonization success, in addition to local environmental conditions (AlSharari et al., 2022; Caruso et al., 2020).

Consistent with these ecological patterns, greater burdock yielded the highest number of antibacterial isolates (20) among the plants screened in this study. One possible explanation for the disproportionately high number of antibiotic-producing endophytes isolated from greater burdock may relate to differences in environmental context between sampling locations. Greater burdock was sampled from a relatively natural and

undisturbed habitat at Buns Creek, whereas the other candidate plants were obtained from managed garden environments. Plants growing in relatively natural or undisturbed environments are often exposed to a broader diversity of environmental microorganisms compared with plants cultivated in managed systems (Harrison & Griffin, 2020). Because many fungal endophytes are horizontally transmitted from surrounding microbial communities, environmental context can strongly influence the diversity and composition of plant endophyte assemblages (Christian et al., 2016; Pirttila et al., 2015). Studies comparing plants grown under different management regimes have demonstrated that endophytic fungal abundance and diversity are often significantly higher in less intensively managed environments, such as organic or natural ecosystems, than in conventional or highly managed systems (Xia et al., 2019). These findings suggest that plants growing in microbially rich habitats may host more diverse endophyte communities, increasing the likelihood of isolating strains capable of producing bioactive secondary metabolites.

Investigating the distribution of endophytic fungi across plant tissues is essential for identifying reservoirs of bioactive diversity. The phyllosphere (above-ground tissues such as leaves and stems) and the rhizosphere (root-associated tissues) differ markedly in their microbial communities. Previous studies show that fungal biodiversity is typically higher in the rhizosphere than in the phyllosphere because root tissues provide a more stable, long-lived, and nutrient-rich environment for colonization (Jin et al., 2015; Hartmann et al., 2008). The rhizosphere is nutrient-rich, with up to 40% of photosynthetic products released from plant roots entering the surrounding soil, supporting diverse microbial populations (Contreras-Cornejo et al., 2009).

The results of this study closely reflect these ecological patterns. Of the 58 isolates that inhibited *A. baumannii* AB258, 74.1% originated from roots, while only 25.9% were recovered from leaves, indicating that most bioactive isolates were associated with below-ground tissues. This strong root bias is consistent with reports that rhizosphere-associated fungi exhibit both higher diversity and greater metabolic potential than phyllosphere endophytes (Jin et al., 2015; Qian et al., 2019; Su et al., 2010). Several ecological mechanisms may be creating this root-associated bias. In contrast to below-ground tissues, leaf-associated endophyte communities experience persistent environmental stressors, including exposure to UV radiation, limited nutrient availability, and temperature fluctuations over daily cycles. These conditions can restrict fungal establishment and persistence in leaf tissues, contributing to the generally lower diversity of leaf-associated endophytic fungi relative to those inhabiting root-associated environments (Qian et al., 2019; Remus-Emsermann & Schlechter, 2018). Additionally, the greater abundance of endophytic fungi in roots may be influenced by differences in tissue longevity. Typically, roots persist for substantially longer periods than leaves and stems, increasing the opportunity for endophytes to be acquired from the surrounding environment. This pattern aligns with a predominantly horizontal mode of endophyte transmission, in which fungi are recruited from the environment over time, leading to higher endophyte abundance in older, longer-lived root tissues (Su et al., 2010).

Consistent with this expectation, several of the most potent antibacterial isolates identified in this study, including DL16, GB29, and GB 2, were recovered exclusively from root tissues. The alignment between our results and prior literature suggests that root tissues offer greater bioprospecting potential, as their higher endophyte richness yields a

larger and more chemically diverse set of fungi capable of producing antibacterial metabolites relevant to drug discovery.

Clinical Relevance of the Antibacterial Screening Panel

To evaluate the antibacterial potential of isolated endophytic fungi against clinically relevant and antimicrobial-resistant pathogens, the screening panel was designed to include both Gram-positive and Gram-negative species associated with high morbidity, mortality, and treatment failure.

Methicillin-resistant *S. aureus* (MRSA) was included as a representative antibiotic-resistant Gram-positive pathogen of major clinical importance. MRSA is both a common commensal organism and a leading cause of serious infections, including bacteremia, endocarditis, skin and soft tissue infections, and hospital-acquired infections. Despite declining incidence in some regions, MRSA remains associated with substantial morbidity and mortality worldwide (Turner et al., 2019).

P. aeruginosa strains (PA01 and PA082) were selected as opportunistic Gram-negative pathogens frequently implicated in healthcare-associated infections, particularly among immuno-compromised patients and individuals with chronic conditions such as cystic fibrosis, chronic obstructive pulmonary disease, and cancer. *P. aeruginosa* is well known for its high resistance to many antibiotics. A major factor is the bacterium's outer membrane, which has low permeability compared with many other Gram-negative bacteria, limiting the passive entry of antibiotics into the cell, even in strains lacking major efflux pumps (Krūmiņa et al., 2026).

Clinical isolates of *A. baumannii* (AB258, AB030, and ATCC17978) were included due to their role as opportunistic pathogens responsible for severe hospital-

acquired infections and their well-documented multidrug resistance. Clinical isolates commonly overexpress resistance-nodulation-division (RND) efflux pumps, which actively expel antimicrobial agents and contribute to treatment failure (Fernando et al., 2013). *A. baumannii* AB258 is the antibiotic sensitive strain used for the preliminary screening.

K. pneumoniae was included to highlight clinically significant pathogens associated with antimicrobial resistance (AMR) and hospital-acquired infections. Globally, it is a major contributor to AMR-related mortality, one of only six pathogens linked to over 100,000 AMR-related deaths in 2021 (Robles Aguilar et al., 2024). *K. pneumoniae* has also emerged as a frequent cause of secondary infections in critically ill COVID-19 patients, with co-infection rates up to 43% in ICU admissions, most commonly due to *K. pneumoniae*, and associated with high mortality (Koupaei et al., 2024).

Taxonomic Identity and Bioactive Potential of Candidate Endophytes

Fungal species identification has traditionally relied on a combination of spore morphology, chemotype, and molecular markers (Adeyemo & Schmidt-Heydt, 2024). However, morphological characteristics and secondary metabolite profiles can vary widely depending on environmental conditions and may not accurately reflect phylogenetic relationships, therefore molecular markers have become the most reliable approach for fungal taxonomy.

The internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster is considered the universal DNA barcode for fungi because of its high copy number, ease of amplification, and sufficient variation for species-level identification in many fungal

groups (Schoch et al., 2012). Additional loci, such as the β-tubulin gene, are often used to provide improved phylogenetic resolution in certain genera where ITS alone may not adequately distinguish closely related species (Glass & Donaldson, 1995). Mitochondrial genomes and mitochondrial markers can also contribute to fungal taxonomy and phylogenetics because they are typically conserved in structure yet accumulate mutations at rates that can help resolve evolutionary relationships among closely related taxa (Aguileta et al., 2014).

For isolate LV6, although mitochondrial sequences showed complete identity to *Alternaria alternata*, nuclear markers (ITS and β-tubulin) failed to resolve the isolate to a single species. Both loci produced identical top matches to multiple *Alternaria* taxa. This likely reflects the well-documented taxonomic complexity of the *Alternaria alternata* species complex, in which closely related species share identical ITS and β-tubulin sequences.

Common nuclear markers, including ITS, β-tubulin, TEF1, and ATPase, often fail to distinguish closely related Alternaria species (Zhang et al., 2020). Even when multiple loci are analyzed together, isolates assigned to different species can be indistinguishable, highlighting the limited resolution of traditional barcodes within the *A. alternata* species complex. Broader studies also show that single-gene markers frequently lack sufficient discriminatory power, and accurate identification often requires multilocus approaches (Adeyemo & Schmidt-Heydt, 2024

In contrast, mitochondrial genomes provide well-supported phylogenetic relationships among Alternaria species and contain structural features such as gene rearrangements, introns, and repetitive elements, that offer additional phylogenetic signal

beyond single nuclear loci (Liang et al., 2026). Because mitochondrial identity agrees with nuclear marker placement, the isolate is conservatively assigned to the *Alternaria alternata* complex, a group of closely related, morphologically similar species often indistinguishable using standard barcodes. Future multilocus or genome-scale analyses would likely improve taxonomic resolution.

A. alternata is widely reported as a plant-associated fungus that can occur as both a pathogen and an asymptomatic endophyte within the *Ascomycota* phylum (DeMers, 2022). This species has a broad ecological distribution and is frequently isolated from plant tissues without visible disease symptoms, indicating its ability to colonize hosts in a non-pathogenic lifestyle. Endophytic isolates of *A. alternata* have been reported from numerous plant species, including crop plants such as strawberry, apple, rapeseed, soybean, rice, and citrus (DeMers, 2022).

Endophytic *A. alternata* is also recognized as a source of diverse bioactive secondary metabolites with potential pharmaceutical and agricultural applications. Several studies have reported antibacterial compounds produced by endophytic *A. alternata*, including polyketides active against phytopathogenic bacteria such as *Xanthomonas oryzae* and *Ralstonia solanacearum* (Zhao et al., 2021). Extracts from endophytic isolates have also demonstrated antibacterial activity against clinical bacterial strains and cytotoxic activity against a human lung carcinoma cell line (A549), indicating potential anticancer properties (Ashoka & Shivanna, 2023). Additionally, *A. alternata* extracts have shown measurable antibacterial activity against bacteria including *Bacillus subtilis*, *S. aureus*, *Escherichia coli*, *K. pneumonia*, and *P. aeruginosa*, along with significant antioxidant activity (Kusmiati et al., 2024; Sabreen et al., 2015).

Multilocus sequence analysis consistently identified isolate GB2 as *Trichoderma reesei*, with 100% identity and query coverage across mitochondrial and nuclear markers. *Trichoderma reesei* is recognized as a filamentous fungus that can occur in multiple ecological niches, including soil, saprophytic environments, and as an endophyte within plant tissues (Onofre et al., 2014). Endophytic isolates of *T. reesei* have been recovered from plants such as *Solanum surattense*, where the fungus was shown to promote wheat growth and improve plant tolerance under salt stress conditions (Ikram et al., 2019). Similarly, endophytic *T. reesei* isolates obtained from banana tissues have demonstrated antagonistic activity against *Fusarium oxysporum* f. sp. *cubense*, causal agent of Panama wilt (Savani et al., 2021).

T. *reesei* is also known to produce a wide range of secondary metabolites with diverse biological activities. Studies have demonstrated that extracts of *T. reesei* exhibit antibacterial activity against several bacterial pathogens, including *S. aureus*, *E. coli*, and *P. aeruginosa* (Wigati et al., 2024). Endophytic isolates of *T. reesei* have also shown broad-spectrum antibacterial activity against phytopathogenic bacteria such as *Xanthomonas oryzae*, *Pseudomonas syringae*, *Agrobacterium tumefaciens*, and *Ralstonia solanacearum*, with bioactive extracts containing compounds such as phenolics, flavonoids, and organic acids that may contribute to both antibacterial and antioxidant activities (Ikram et al., 2019).

Based on multilocus sequence analysis, isolate DL16 was identified as *Penicillium digitatum*, with 100% identity and query coverage across mitochondrial and nuclear markers. The genus *Penicillium*, comprising more than 200 species, represents one of the largest and most extensively studied groups of fungi and is well recognized as an

important source of antibiotics and other bioactive secondary metabolites. Many *Penicillium* species occur as endophytes, colonizing plant tissues asymptomatically and producing metabolites that may protect their host plants against environmental stresses or microbial pathogens, with potential applications in agriculture, biotechnology, and pharmaceutical development (Toghueo & Boyom, 2020). However, *P. digitatum* is not typically described as an endophyte; rather, it is best known as the causal agent of green mold disease and a major post-harvest pathogen of citrus fruits (Abd Ali et al., 2023).

P. digitatum is also capable of producing bioactive secondary metabolites. For example, the metabolite bis-(2-ethylhexyl) phthalate isolated from *P. digitatum* has demonstrated antibacterial activity against both Gram-positive and Gram-negative bacterial pathogens (Abd Ali et al., 2023). In addition, this species produces several indole alkaloid secondary metabolites, including tryptoquialanines, tryptoquivalines, and fumiquinazolines, which have been detected during infection of citrus hosts (Costa et al., 2019). Some of these compounds, such as tryptoquialanine A, have been shown to possess biological activity, including insecticidal effects, suggesting that *P. digitatum* may represent a source of diverse bioactive natural products (Costa et al., 2019).

Chemical Characterization of Antibacterial Metabolites

LC–MS/MS analysis coupled with GNPS molecular networking revealed numerous putative secondary metabolites across all three fungal extracts. While the majority of detected features were annotated through spectral library matching, several metabolites remained uncharacterized. Specifically, five uncharacterized features were detected in both the GB2 and LV6 extracts, while four uncharacterized metabolites were identified in the DL16 extract. In natural product discovery workflows, such unmatched

spectral features are of particular interest, as they may represent previously undescribed secondary metabolites not currently represented in spectral databases. Fungal endophytes are well recognized sources of structurally diverse natural products with a wide range of biological activities, including antibacterial, antioxidant, and anticancer compounds (Newman and Cragg, 2020; Keller, 2019). Molecular networking platforms such as GNPS facilitate the rapid prioritization of potentially novel compounds by highlighting metabolites that lack reference spectra in existing libraries (Wang et al., 2016; Nothias et al., 2020). Therefore, the uncharacterized metabolites detected in these extracts represent promising targets for future structural elucidation and bioactivity screening to determine whether they possess previously unreported chemical scaffolds or biological activities.

The relatively high MIC values observed for fractions GB2 B, GB2 C, and GB2 D indicate that these purified compounds exhibit limited antibacterial potency when tested individually. However, the earlier disc diffusion assays demonstrated clear zones of inhibition, suggesting that the antibacterial phenotype may not be attributable to a single compound alone.

One possible explanation is that the bioactivity observed in crude extracts results from synergistic interactions among multiple metabolites, which may be reduced or lost following chromatographic separation (Liu et al., 2025). Synergistic antimicrobial interactions are well documented, where the combined activity of two or more compounds produces greater inhibitory effects than individual agents alone. Such interactions can enhance pathogen killing, disrupt multiple cellular targets, or reduce the concentration required for antimicrobial activity (Baym et al., 2016).

Compounds that exhibit limited antimicrobial activity individually may still play an important role in combination therapies, where interactions among metabolites can produce synergistic effects that enhance overall antibacterial efficacy (Woods, & Read, 2023). Additionally, these metabolites may function as antibiotic adjuvants, compounds that enhance the efficacy of existing antimicrobials rather than acting as strong antibiotics independently (Dhanda et al., 2023). Identifying metabolites with adjuvant properties represents an increasingly important strategy for addressing antimicrobial resistance, as such compounds can restore or enhance the effectiveness of existing antibiotic therapies. Future work should therefore evaluate both synergistic interactions among these fractions and their capacity to potentiate conventional antibiotics, which may reveal combinatorial effects that reduce MIC values and enhance antibacterial activity.

Although the discovery of entirely novel fungal genera is often prioritized due to their potential to harbor unique and unexplored chemistry, well-characterized genera such as *Alternaria*, *Penicillium*, and *Trichoderma* continue to yield previously uncharacterized compounds, underscoring their ongoing relevance for antimicrobial discovery (Bao et al., 2025; Pathak et al., 2020).

Filamentous fungi have historically yielded clinically important antibiotics, yet genomic and metabolomic evidence indicates that their biosynthetic capacity is far from exhausted (Correia et al., 2023; Pathak et al., 2020). Recent estimates suggest that fungi may produce over 1.4 million secondary metabolites, the vast majority of which remain uncharacterized (Riedling & Rokas, 2025). In this context, the isolates examined here represent promising systems for further investigation, not only as sources of individual antibacterial compounds but also for their potential to exhibit synergistic interactions and

antibiotic adjuvant activity. Exploring both the chemical diversity and combinatorial effects of these metabolites may reveal novel strategies to enhance antibacterial efficacy and address antimicrobial resistance.

Conclusion:

Overall, this study demonstrates that medicinal plants harbor diverse communities of fungal endophytes capable of producing antibacterial metabolites. These findings contribute to the growing body of evidence that fungal endophytes represent an underexplored reservoir of natural products with potential applications in antimicrobial drug discovery.

Future work should focus on structural characterization of the bioactive metabolites present in GB2 fractions using analytical techniques such as LC–MS and NMR spectroscopy, which is essential for resolving molecular structures and confirming compound identities. Optimization of culture conditions may also improve metabolite yields from promising isolates, including LV6 and DL16, enabling further chemical investigation. In addition, molecular identification and phylogenetic analysis will be important to more precisely resolve the taxonomic placement of the LV6 isolate within the *Alternaria* complex, which contains many closely related and difficult-to-distinguish species. Future studies should also evaluate the potential for synergistic interactions among purified fractions and their capacity to act as antibiotic adjuvants, including testing combinations of metabolites and co-administration with existing antibiotics to assess whether such interactions enhance antibacterial efficacy. Together, these approaches will help clarify both the chemical diversity and taxonomic identity of these endophytes and further evaluate their potential as sources of novel antimicrobial compounds.

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Appendix:

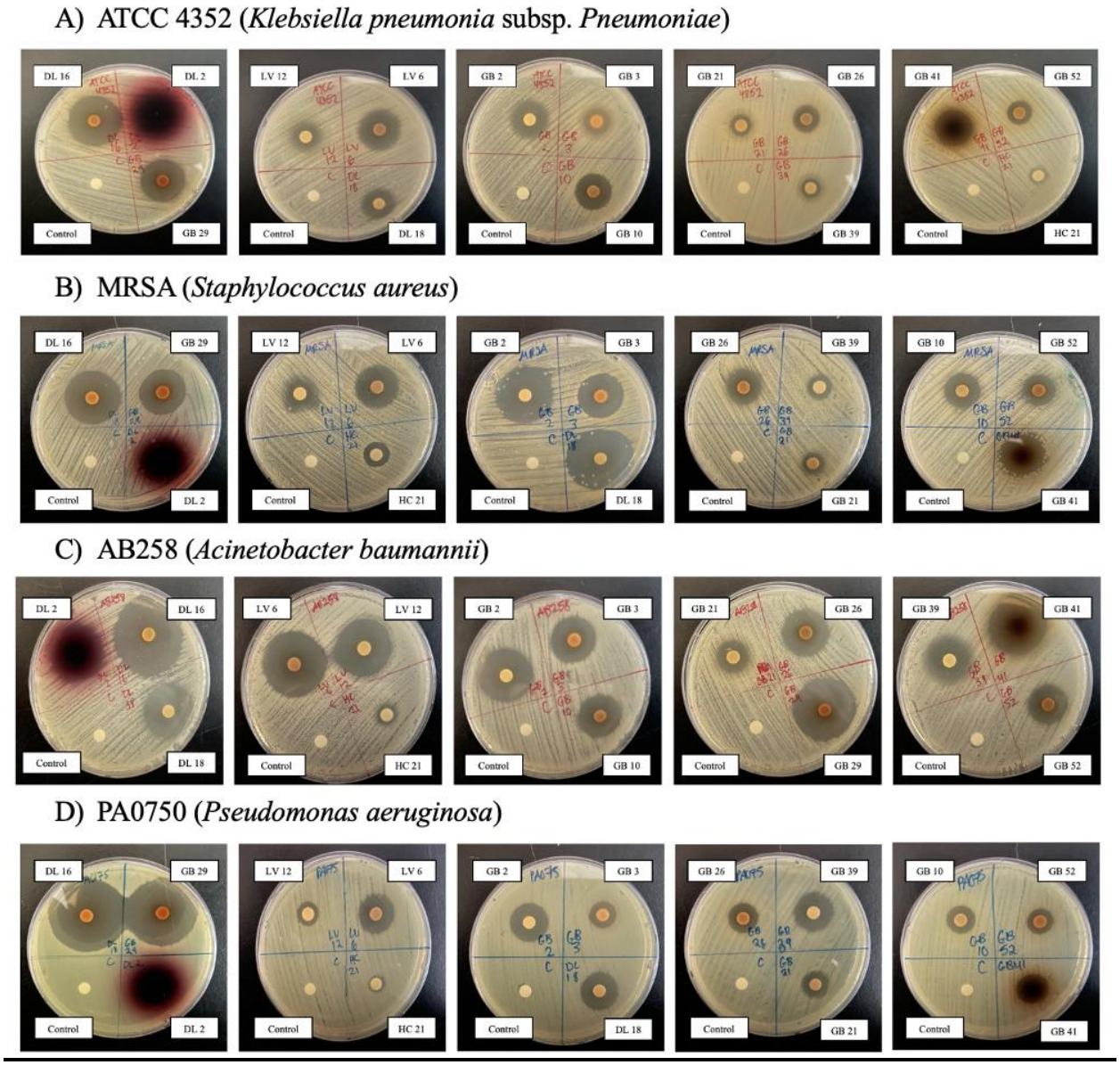

Figure A1. Complete collection of crude extract inhibition plates for all 15 antibacterial fungal isolates. Disc diffusion assays were performed against the four bacterial strains outlined in Table 3, and the resulting zones of inhibition were quantified to generate the values reported in Table 4. *K. pneumoniae* was added to refocus the screening panel toward clinically relevant pathogens associated with antimicrobial resistance and hospital-acquired infections. Recent global estimates show that *K. pneumoniae* is one of only six bacterial pathogens responsible for over 100,000 AMR-related deaths in 2021 (Robles Aguilar et al, 2024).

Table A1. Putatively characterized metabolites detected in GB2 fungal crude extracts through LC–MS/MS analysis and molecular networking using the GNPS platform. Metabolite annotations were assigned through spectral library matching, and associated molecular formulas and reported biological activities were compiled from the literature

Feature ID	Annotation	Molecular Formula	Description	Reference
1	(2E)-3-(2,4-dichlorophenyl)-1-naphthylprop-2-en-1-one	$C_{19}H_{13}ClO$	Halogenated chalcone derivative with reported antibacterial activity, mediated by interactions with bacterial DNA gyrase.	(Dammene Debbih et al., 2024)
3	3-Acetylaconitine	$C_{36}H_{49}NO_{12}$	Aconitine-type diterpenoid alkaloid with reported cytotoxicity against P388 leukemia cells, and anti-inflammatory effects reported in osteoarthritis models.	(He et al., 2011; Xiao et al., 2024)
6	Cinchonine	$C_{19}H_{22}N_2O$	Quinoline alkaloid closely related to quinine, with known antimalarial, antioxidant, and anti-inflammatory activity, and forms a stable complex with human transferrin, suggesting potential neuroprotective effects.	(Shamsi et al., 2025)
7	Citric acid	$C_6H_8O_7$	Tricarboxylic acid with reported antioxidant and anti-inflammatory activity, shown to reduce lipid peroxidation, inflammation, and liver damage. Also reported to exhibit anti-biofilm and anti-capsular activity against multidrug-resistant *Acinetobacter baumannii.*	(Abdel-Salam et al., 2014; El-Soudany et al., 2024)

8	Daidzein	$C_{15}H_{10}O_4$	Isoflavone with reported antioxidant, anti-inflammatory, and estrogenic activity. Shown to modulate estrogen receptors and inflammatory pathways, with evidence for anticancer, cardiovascular, and neuroprotective effects	(Praisthy et al., 2025)
9	Evista (Raloxifene)	$C_{28}H_{27}NO_4S$	Selective estrogen receptor modulator with clinical use in osteoporosis prevention. Reported antioxidant and neuroprotective effects in animal models, and anticancer activity in cell systems.	(Hao et al., 2025; Konyaliogl u et al., 2007; Morishima et al., 2008)
10	Gitoxigenin	$C_{23}H_{34}O_5$	Cardiac steroid (cardenolide) with cardiotonic activity via inhibition of Na^+/K^+-ATPase, with additional evidence for cytotoxic and anticancer effects.	(Bedir et al., 2021; Hashimoto et al., 1986)
12	Hydroquinidine	$C_{20}H_{26}N_2O_2$	Quinidine derivative with antiarrhythmic activity via sodium channel blockade.	(Isbister et al., 2025; Mazzanti et al., 2017)
13	Isoscopoletin	$C_{10}H_8O_4$	Coumarin derivative reported to exhibit anti-tumor activity, with additional antioxidant and anti-inflammatory activity effects.	(Ruan et al., 2024)
14	Lecanoric acid	$C_{16}H_{14}O_7$	Depside compound reported to exhibit diverse bioactivities, including cytotoxic (anticancer), antibacterial, antimycobacterial,	(Vo et al., 2024)

			antiviral, and antioxidant effects.	
15	Quinclorac	$C_{10}H_5Cl_2NO_2$	Quinoline-based herbicide that interferes with plant growth hormone (auxin) signaling.	(Deng et al., 2021)
16	13S-HOTrE	$C_{18}H_{30}O_3$	Lipoxygenase-derived oxylipin from α-linolenic acid with reported anti-inflammatory activity via NLRP3 inflammasome inhibition.	(Kumar et al., 2016)
17	17-HDoHE	$C_{22}H_{32}O_3$	Oxidized DHA-derived specialized pro-resolving mediator and biosynthetic precursor to 17-oxo-DHA, which exhibits anti-inflammatory activity, including inhibition of the NLRP3 inflammasome.	(Cipollina et al., 2016; Egawa et al., 2016)
19	1-(2,4,5-Trimethoxyphenyl)propane-1,2-diol	$C_{12}H_{18}O_5$	A natural phenylpropanoid reported from *Piper clusii*, which has been synthesized from β-asarone.	(Sinha et al., 2001)
24	Neochlorogenic acid	$C_{16}H_{18}O_9$	Phenolic compound which possesses multiple pharmacological activities containing antioxidant and anti-inflammatory effects through mediating the AMPK/Nrf2 signaling pathway in A549 cells.	(Gao et al., 2020)
25	Pinolidoxin	$C_{18}H_{26}O_6$	Tetrasubstituted nonenolide fungal metabolite that acts as a phytotoxin.	(De Napoli et al., 2000)

26	Pyrenophorol	$C_{16}H_{24}O_6$	Sixteen-membered diolide fungal metabolite, phytotoxic, with anthelmintic activity and antifungal effects against *Microbotryum violaceum*.	(Ashok et al., 2018)
27	Decylbenzenesulfonic acid	$C_{16}H_{26}O_3S$	Alkylbenzenesulfonate surfactant commonly used in detergent and cleaning formulations, where it functions as a hydrotrope to modify solubility and viscosity.	(Burns, 1999)
28	Leu-Pro	$C_{11}H_{20}N_2O_3$	A dipeptide composed of leucine and proline.	(Pubchem, 2026)
29	Testosterone	$C_{19}H_{28}O_2$	Steroid androgen that acts as an androgen receptor agonist to promote male sex organ development and secondary sexual characteristics.	(Azhagiya Singam et al., 2019)

Table A2. Putatively characterized metabolites detected in LV6 fungal crude extracts through LC–MS/MS analysis and molecular networking using the GNPS platform. Metabolite annotations were assigned through spectral library matching, and associated molecular formulas and reported biological activities were compiled from the literature.

Feature ID	Annotation	Molecular Formula	Description	Reference
1	Soyasaponin I	$C_{48}H_{78}O_{18}$	Pentacyclic triterpenoid saponin and trisaccharide derivative that has been been shown to alleviate hypertensive intracerebral hemorrhage in experimental models by inhibiting the renin–angiotensin–aldosterone system, suggesting	(Li et al., 2023)

			potential therapeutic applications.	
2	Aphyllic acid	$C_{15}H_{26}N_2O_2$	Pentacyclic triterpenoid reported to possess bronchospasmolytic activity, inhibit vagus nerve–mediated cardiac impulse transmission, and attenuate the toxic effects of anticholinesterase agents.	(Otargaliev et al., 1977)
3	Citric acid	$C_6H_8O_7$	Tricarboxylic acid with reported antioxidant and anti-inflammatory activity, shown to reduce lipid peroxidation, inflammation, and liver damage. Also reported to exhibit anti-biofilm and anti-capsular activity against multidrug-resistant *Acinetobacter baumannii.*	(Abdel-Salam et al., 2014; El-Soudany et al., 2024)
5	13S-HOTrE	$C_{18}H_{30}O_3$	Lipoxygenase-derived oxylipin from α-linolenic acid with reported anti-inflammatory activity via NLRP3 inflammasome inhibition.	(Kumar et al., 2016)
6	Decylbenzenesulfonic acid	$C_{16}H_{26}O_3S$	Alkylbenzenesulfonate surfactant commonly used in detergent and cleaning formulations, where it functions as a hydrotrope to modify solubility and viscosity.	(Burns, 1999)
7	3-Acetylaconitine	$C_{36}H_{49}NO_{12}$	Aconitine-type diterpenoid alkaloid with reported cytotoxicity against P388 leukemia cells,	(He et al., 2011; Xiao et al., 2024)

			and anti-inflammatory effects reported in osteoarthritis models.	
8	Docosanol	$C_{22}H_{46}O$	Long-chain saturated fatty alcohol used clinically as a topical antiviral agent that inhibits herpes simplex virus entry by blocking membrane fusion.	(Shankar et al., 2022)
11	Altenusin	$C_{15}H_{14}O_6$	Nonsteroidal fungal metabolite from *Alternaria* spp. that exhibits antifungal activity and attenuates nonalcoholic fatty liver disease via activation of the farnesoid X receptor.	(Zheng et al., 2017)
12	Isoarthonin	$C_{25}H_{32}N_2O_4$	Exhibit significant anti-inflammatory activity, potentially through COX-2 inhibition, with reported effects comparable to diclofenac and ibuprofen.	(Chibuye et al., 2024)
13	5,6,2'-Trimethoxyflavone	$C_{18}H_{16}O_5$	Polyphenolic compound found in various plant species that modulates cell signaling and enzyme activity, including inhibition of cytochrome P450, and exhibits anti-inflammatory and antioxidant effects.	(Wollenweber et al., 1990)
14	N-Acetyl-L-phenylalanine	$C_{11}H_{13}NO_3$	Acetylated derivative of L-phenylalanine that occurs as a metabolic intermediate and also functions as a carboxylate ligand in coordination chemistry, forming metal complexes such as	(Wang et al., 2025)

			Mn(II) coordination polymers.	
15	Altersetin	$C_{24}H_{33}NO_4$	*Alternaria*-derived antibacterial metabolite related to equisetin with potent activity against Gram-positive bacteria.	(Hellwig et al., 2003)
16	Epicatechin	$C_{15}H_{14}O_6$	Dietary flavanol linked to blood pressure reduction, cardiovascular and neuroprotective effects, and prevention of cancer, diabetes, and age-related diseases.	(Bernatova, 2018; Takagaki & Nanjo, 2015)
17	Cinchonine	$C_{19}H_{22}N_2O$	Quinoline alkaloid closely related to quinine, with known antimalarial, antioxidant, and anti-inflammatory activity, and forms a stable complex with human transferrin, suggesting potential neuroprotective effects.	(Shamsi et al., 2025)
19	7,8-Dihydroxy-4-methylcoumarin	$C_{10}H_8O_4$	Coumarin derivative with strong antioxidant and radical scavenging activity. It serves as a precursor for bioactive 4-methylcoumarin derivatives and has demonstrated neuroprotective effects, including upregulation of hippocalcin expression.	(Jin et al., 2015)
20	Canrenone	$C_{22}H_{28}O_3$	Steroidal aldosterone antagonist shown to reduce mortality and improve cardiovascular outcomes in patients with chronic heart failure.	(Derosa et al., 2019)

22	Monocrotaline N-Oxide	$C_{16}H_{23}NO_7$	Metabolite of the pyrrolizidine alkaloid monocrotaline that can reconvert to its toxic parent compound and contribute to organ toxicity.	(Lin et al., 2021)
23	Altertoxin I	$C_{20}H_{16}O_6$	*Alternaria*-derived perylenequinone mycotoxin with reported toxic, genotoxic, and carcinogenic effects, and has been linked to esophageal cancer risk.	(Gutsche et al., 2024)
24	12,13-DiHOME	$C_{18}H_{34}O_4$	Oxylipin identified as a thermogenic metabolite that activates brown adipose tissue. Elevated levels are associated with improved metabolic health and potential benefits in obesity and metabolic disease.	(Macêdo et al., 2022)
25	Higenamine	$C_{16}H_{17}NO_3$	Plant-derived alkaloid first isolated from *Aconitum* species and identified as its active cardiotonic component. It has been evaluated as a pharmacologic stress agent for the detection of coronary artery disease.	(Zhang et al., 2017)
25	N-Aceyl-L-tyrosine	$C_{11}H_{13}NO_4$	Soluble acetylated derivative of L-tyrosine used as a tyrosine precursor in parenteral nutrition. It functions as a human metabolite and biomarker and has reported enzyme inhibitory activity.	(Matsumura et al., 2020)

26	Caudatin	$C_{28}H_{42}O_7$	C21 steroidal aglycone isolated from *Cynanchum* species. C21 steroidal glycosides exhibit anticancer activity, and caudatin has been shown to attenuate inflammation by suppressing JNK/AP-1/NF-κB/caspase-1 signaling pathways in activated HMC-1 cells.	(Bailly, 2021; Kim et al., 2023)
27	Cannabidiolic acid	$C_{22}H_{30}O_4$	Non-psychoactive cannabinoid and the acidic precursor of cannabidiol (CBD) found in *Cannabis sativa*. It exhibits anti-inflammatory, anti-emetic, anti-convulsant, and potential anti-carcinogenic effects.	(Formato et al., 2020)
28	Daidzein	$C_{15}H_{10}O_4$	Isoflavone with reported antioxidant, anti-inflammatory, and estrogenic activity. Shown to modulate estrogen receptors and inflammatory pathways, with evidence for anticancer, cardiovascular, and neuroprotective effects	(Praisthy et al., 2025)
29	Dihydroorotate	$C_5H_5N_2O_4^-$	Monocarboxylate anion formed by deprotonation of dihydroorotic acid and serves as an intermediate in de novo pyrimidine biosynthesis.	(Reis et al., 2017)

Table A3. Putatively characterized metabolites detected in DL16 fungal crude extracts through LC–MS/MS analysis and molecular networking using the GNPS platform. Metabolite annotations were assigned through spectral library matching, and associated molecular formulas and reported biological activities were compiled from the literature.

Feature ID	Annotation	Molecular Formula	Description	Reference
1	Homogentisic Acid	$C_8H_8O_4$	Aromatic intermediate in tyrosine degradation reported in *Penicillium commune* and other organisms, which has demonstrated antioxidant and anti-inflammatory activities.	(Wang et al., 2016)
2	Citrate	$C_6H_5O_7^{-3}$	Salt or ester of citric acid, reported antioxidant that functions in primary metabolism and metal chelation.	(Zhang et al., 2025)
3	13S-HOTrE	$C_{18}H_{30}O_3$	Lipoxygenase-derived oxylipin from α-linolenic acid with reported anti-inflammatory activity via NLRP3 inflammasome inhibition.	(Kumar et al., 2016)
4	Decylbenzenesulfonic acid	$C_{16}H_{26}O_3S$	Alkylbenzenesulfonate surfactant commonly used in detergent and cleaning formulations, where it functions as a hydrotrope to modify solubility and viscosity.	(Burns, 1999)
5	3-Acetylaconitine	$C_{36}H_{49}NO_{12}$	Aconitine-type diterpenoid alkaloid with reported cytotoxicity against P388 leukemia cells, and anti-inflammatory effects reported in osteoarthritis models.	(He et al., 2011; Xiao et al., 2024)

6	Planaic acid	$C_{27}H_{36}O_7$	Orcinol depside lichen secondary metabolite identified in *Lethariella* and *Juniperus* rock-inhabiting species with ecological roles including UV protection.	(Huneck et al., 1989)
7	Docosanol	$C_{22}H_{46}O$	Long-chain saturated fatty alcohol used clinically as a topical antiviral agent that inhibits herpes simplex virus entry by blocking membrane fusion.	(Shankar et al., 2022)
8	Quinoxaline-2-carboxylic acid	$C_9H_6N_2O_2$	Nitrogen-containing heterocyclic aromatic compound and core scaffold of quinoxaline-2-carboxylic acid 1,4-dioxide derivatives reported to exhibit antimycobacterial activity against *Mycobacterium* spp.	(Frolova et al., 2023)
9	Agistatin E	$C_{11}H_{16}O_5$	Pyranacetal secondary metabolite originally isolated from a *Fusarium* sp., reported to inhibit cholesterol biosynthesis.	(Steiner et al., 2020)
10	5,6,2'-Trimethoxyflavone	$C_{18}H_{16}O_5$	Polyphenolic compound found in various plant species that modulates cell signaling and enzyme activity, including inhibition of cytochrome P450, and exhibits anti-inflammatory and antioxidant effects.	(Wollenweber et al., 1990)

11	Andrastin A	$C_{28}H_{38}O_7$	Meroterpenoid secondary metabolites produced by several *Penicillium* species that exhibit inhibitory activity against Ras farnesyltransferase, suggesting potential as antitumor lead compounds.	(Matsuda et al., 2013)
12	2-(methyl(2H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)ethanol	$C_8H_{11}N_5O$	Nitrogen-rich pyrazolopyrimidine derivative belonging to a heterocyclic scaffold widely investigated for anticancer activity, including VEGFR-2 inhibition.	(Ruzi et al., 2022)
13	3-Hydroxyphysodic acid	$C_6H_{10}O_5$	Lichen-derived depsidone reported from *Hypogymnia physodes*, demonstrated potent natural antioxidant activity and associated antimicrobial effects.	(Stojanovic et al., 2017; Yilmaz et al., 2005)
14	Cryptostictic acid	$C_{19}H_{16}O_9$	Lichen-derived depsidone isolated from *Hypogymnia physodes*, reported to exhibit antioxidant activity and contribute to antimicrobial effects in methanolic extracts.	(Goga et al., 2021)
16	9-Nitro-20(S)-camptothecin	$C_{20}H_{15}N_3O_6$	Nitro-substituted camptothecin derivative and potent topoisomerase I inhibitor derived from *Camptotheca acuminata*, investigated as an oral anticancer agent for pancreatic cancer and other solid tumors.	(Clark, 2006)

17	Ethofumesate-2-keto	$C_{11}H_{12}O_5S$	Methanesulfonate ester and γ-lactone identified as a metabolite of the herbicide ethofumesate, classified as a marine xenobiotic metabolite and member of the benzofuran family.	(McCulloug h et al., 2016; Saito-Shida et al., 2020)
18	Helenine	$C_{15}H_{20}O_2$	Sesquiterpene lactone, reported to inhibit NLRP3 inflammasome activation by disrupting the NEK7–NLRP3 interaction, demonstrating anti-inflammatory activity.	(Fang et al., 2024)
19	Citrinin hydrate	$C_{13}H_{16}O_6$	hydration product of citrinin isolated from a marine-derived *Penicillium* sp. It was identified in a target-directed microbial screen as an inhibitor of human rhinovirus 3C protease.	(Poddig et al., 1994)
21	Oxymatrine	$C_{15}H_{24}N_2O_2$	Quinolizidine alkaloid isolated from the root of Sophora flavescens. It exhibits antioxidant, anti-inflammatory, antifibrotic, and anticancer activities, and has shown protective effects in cardiovascular diseases.	(Zhang et al., 2017)
23	18Alpha-Glycyrrhetinic Acid	$C_{30}H_{46}O_4$	Triterpenoid with anti-inflammatory and anti-aging activities; shown to reduce pulmonary inflammation and fibrosis in mice.	(Cao et al., 2023)
24	Isoscopoletin	$C_{10}H_8O_4$	Coumarin derivative reported to exhibit anti-tumor activity, with additional antioxidant	(Ruan et al., 2024)

			and anti-inflammatory activity effects.	
25	Fuegin	$C_{15}H_{22}O_4$	Sesquiterpene benzofuran isolated from Polygonum hydropiper. It belongs to the benzofuran class of natural products, a structural group commonly associated with diverse biological activities, although specific bioactivity data for Fuegin itself remains limited.	(Khanam & Shamsuzzaman, 2015; Nakano et al., 1999)
26	Cinchonine	$C_{19}H_{22}N_2O$	Quinoline alkaloid closely related to quinine, with known antimalarial, antioxidant, and anti-inflammatory activity, and forms a stable complex with human transferrin, suggesting potential neuroprotective effects.	(Shamsi et al., 2025)
27	Canrenone	$C_{22}H_{28}O_3$	Steroidal aldosterone antagonist shown to reduce mortality and improve cardiovascular outcomes in patients with chronic heart failure.	(Derosa et al., 2019)
28	Leu-Leu	$C_{12}H_{24}N_2O_3$	Dipeptide of two L-leucine residues; human and *Mycoplasma genitalium* metabolite	(Pubchem, 2026)

29	Caudatin	$C_{28}H_{42}O_7$	C21 steroidal aglycone isolated from *Cynanchum* species. C21 steroidal glycosides exhibit anticancer activity, and caudatin has been shown to attenuate inflammation by suppressing JNK/AP-1/NF-κB/caspase-1 signaling pathways in activated HMC-1 cells.	(Bailly, 2021; Kim et al., 2023)

Table A4. Uncharacterized metabolite features detected in GB2 fungal crude extracts by LC–MS/MS and GNPS molecular networking. These features did not produce confident spectral library matches and therefore remain putatively unidentified. Such metabolites may represent previously undescribed compounds requiring further structural elucidation.

Feature ID	Annotation	Molecular Formula
18	(2S)-2-[[(2S)-2-[[(2S)-2-amino-4-methylpentanoyl]amino]-4-methylpentanoyl]amino]-3-(4-hydroxyphenyl)propanoic acid	$C_{22}H_{32}O_3$
20	(3S,6R,6aR,7S,12aS)-6,6a,7,8-tetrahydroxy-3-methyl-3,4,5,6,7,12a-hexahydro-2H-benzo[a]anthracene-1,12-dione	$C_{19}H_{20}O_6$
21	6-[(8a-carboxy-4,4,6a,6b,11,11,14b-heptamethyl-1,2,3,4a,5,6,7,8,9,10,12,12a,14,14a-tetradecahydropicen-3-yl)oxy]-5-[3,5-dihydroxy-6-(hydroxymethyl)-4-(3,4,5-trihydroxy-6-methyloxan-2-yl)oxyoxan-2-yl]oxy-3,4-dihydroxyoxane-2-carboxylic acid	$C_{48}H_{76}O_{18}$
22	6H-Benzofuro[3,2-c][1]benzopyran-4,9-diol, 6a,11a-dihydro-3-methoxy-, (6aR,11aR)-	$C_{16}H_{14}O_5$

23	17-[2,3-dihydroxy-6-methyl-6-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyheptan-2-yl]-2,3,14-trihydroxy-10,13-dimethyl-2,3,4,5,9,11,12,15,16,17-decahydro-1H-cyclopenta[a]phenanthren-6-one	$C_{33}H_{54}O_{12}$

Table A5. Uncharacterized metabolite features detected in LV6 fungal crude extracts by LC–MS/MS and GNPS molecular networking. These features did not produce confident spectral library matches and therefore remain putatively unidentified. Such metabolites may represent previously undescribed compounds requiring further structural elucidation.

Feature ID	Annotation	Molecular Formula
4	8,8-dimethyl-2,10-dioxo-9H-pyrano[2,3-f]chromen-9-yl) (Z)-2-methylbut-2-enoate	$C_{19}H_{18}O_6$
9	FA 18:1+3O	$C_{18}H_{34}O_5$
10	(2S,3S)-8-[(2R,3S)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-2,3-dihydrochromen-3-yl]-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one	$C_{36}H_{30}O_{16}$
18	(Z)-9-benzylidene-3-methyl-9H-indeno[2,1-c]pyridin-2-ium chloride	$C_{20}H_{15}N{\cdot}HCl$
21	2-(2-furylcarbonylamino)-3-indol-3-ylpropanoic acid	$C_{16}H_{14}N_2O_4$

Table A6. Uncharacterized metabolite features detected in DL16 fungal crude extracts by LC–MS/MS and GNPS molecular networking. These features did not produce confident spectral library matches and therefore remain putatively unidentified. Such metabolites may represent previously undescribed compounds requiring further structural elucidation.

Feature ID	Annotation	Molecular Formula
15	methyl (4R)-4-((3R,5S,7R,9S,10S,13R,17R)-7-acetoxy-3-hydroxy-10,13-dimethyl-12-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoate	$C_{27}H_{42}O_6$
20	2,6-anhydro-, 5-[4-(beta-D-glucopyranosyloxy)-2-methylenebutanoate] 1-(4-hydroxy-2-methylenebutanoate)	$C_{22}H_{34}O_{14}$
22	5-(4-methyl-5-oxo-2H-furan-3-yl)pentanoic acid	$C_{10}H_{14}O_4$
30	Phe-C16:1	$C_{25}H_{39}NO_3$