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BIOACTIVITY-GUIDED METABOLITE PROFILING OF *NODULISPORIUM VERRUCOSUM*, AN ENDOPHYTIC FUNGI ISOLATED FROM *TAXUS WALLICHIANA*

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ABSTRACT

Endophytic fungi associated with medicinal plants are important sources of biologically active secondary metabolites. *Taxus wallichiana* Zucc., a medicinal plant native to the Himalayan region, harbors diverse endophytic fungi with potential biotechnological and pharmaceutical applications. This study aimed to investigate the bioactivities and metabolite composition of *Nodulisporium verrucosum* (T3I) isolated from *T. wallichiana*. The isolate (T3I) was identified as *N. verrucosum* based on morphological characteristics and internal transcribed spacer (ITS) sequence analysis. Its crude extract was evaluated for antimicrobial and antioxidant activities, total phenolic and flavonoid contents, and metabolite composition using liquid chromatography–mass spectrometry (LC–MS/MS)-based profiling. The extract showed selective antimicrobial activity against *Enterococcus faecalis*, *Staphylococcus* aureus, and *Candida albicans*, with inhibition zones of 12.33 ± 0.57, 8.67 ± 0.57, and 10.67 ± 0.57 mm, respectively. Antioxidant activity was concentration-dependent, with DPPH and ABTS IC_{50} values of 162.5 and 25.37 µg/mL, respectively. Total phenolic and flavonoid contents were 13.37 ± 1.47 mg GAE/g and 1.20 ± 0.04 mg QE/g, respectively. LC–MS profiling revealed diverse putatively annotated metabolites, including phenolic acids, flavonoids, coumarins, alkaloids, and other fungal metabolites. Overall, *N. verrucosum* demonstrated notable antioxidant and selective antimicrobial activities, highlighting its potential as a source of biologically active secondary metabolites.

Keywords: *Taxus wallichiana*, Endophytic fungi, *Nodulisporium verrucosum*, Bioactivity, LC-MS/MS

1 INTRODUCTION

Living quietly inside plant tissues without causing any immediate harm, endophytic fungi are a varied assortment of microorganisms that frequently build mutualistic partnerships to boost the host plant's chemical defense mechanisms [1]. High-value medicinal plants, such as *T. wallichiana*, serve as significant ecological niches for these fungi, which act as "microfactories" for a vast array of novel bioactive secondary metabolites [2]. Among these, the genus *Nodulisporium* the asexual morph of several teleomorphic genera including *Hypoxylon* and *Xylaria* has gained prominence for its exceptional metabolic versatility and therapeutic potential [3].

N. verrucosum is a particularly notable species within this genus, characterized by its ability to adapt to a wide range of hosts and environmental stressors [4]. While it has been isolated from chemically complex plants such as *Vanda tessellata*, *Tithonia diversifolia*, and *Cannabis sativa*, its presence in medicinal species like *T. wallichiana* highlights its role as a prolific producer of host-mimetic bioactive compounds [4–6]. The secondary metabolism of *N. verrucosum* is remarkably diverse, yielding compounds with potent antidiabetic, antibiofilm, and insecticidal properties [7]. The antidiabetic potential of *N. verrucosum* is underscored by its ability to produce high-potency inhibitors of key metabolic enzymes, including -amylase, -glucosidase, and dipeptidyl peptidase-IV (DPP-IV). Key metabolites responsible for these activities include 2-methylcinnamic acid, an insulin-mimetic agent, and tetraneurin-A, a sesquiterpene lactone that regulates carbohydrate digestion [4]. Furthermore, the species produces tributyrin, a short-chain triglyceride that exhibits strong anti-virulence activity against *Streptococcus pyogenes* by inhibiting biofilm formation without driving antimicrobial resistance.

Beyond clinical applications, the broader genus *Nodulisporium* is globally recognized for producing nodulisporic acids (NAs), a class of highly decorated indole diterpenes (IDTs). These compounds, such as nodulisporic acid A (NAA), possess potent insecticidal activity against blood-feeding arthropods by targeting arthropod-specific chloride channels, maintaining an exceptional safety profile in mammals. Additionally, *N. verrucosum* demonstrates industrial utility through the secretion of psychrozymes cold-active amylases and proteases capable of maintaining catalytic efficiency in extreme trans-Himalayan environments [5].

Despite the growing interest in endophytic fungi as sources of biologically active secondary metabolites, the bioactive and metabolomic potential of *N. verrucosum*, particularly as an endophyte of *T. wallichiana*, remains poorly explored. Therefore, the present study aimed to characterize an endophytic *N. verrucosum* isolated from *T. wallichiana* through morphological and molecular approaches and to investigate its bioactive potential. Specifically, the study evaluated the antimicrobial and antioxidant activities, total phenolic and flavonoid contents, and metabolite profile of the fungal crude extract. Metabolite profiling was further performed to tentatively identify bioactive secondary metabolites. This study provides insights into the bioactive potential of *N. verrucosum* and expands the understanding of endophytic fungi associated with *T. wallichiana* as potential sources of biologically relevant metabolites.

2 MATERIALS AND METHODS

2.1 Isolation and identification

The isolation of fungus (T3I) was done from bark of *T. wallichiana* collected from Taplejung, Nepal [26]. The collected sample was sterilized and the bark segments were inoculated onto Modified Melin-Norkrans medium (MMN) [8,9] following incubation at 27 ± 3°C for approximately 8 days.

Morphological identification of the fungi was done using the pure isolate culture plates and microscopy was performed using Lacto Phenol cotton blue (LPCB)dye at10x, 40x and 100x magnification [10,11]. Molecular identification was performed universal internal transcribed spacer (ITS) primers (ITS1 & ITS4). Genomic DNA (gDNA) was extracted using Fungal/ Bacterial Mini-Prep Kit Quick DNA™ obtained from Zymo Research Company, with minor modifications then amplified using PCR following [12]. The obtained product was sequenced using Sanger sequencing by Macrogen, Korea. The sequence was compared against the GenBank database using nucleotide BLAST. The phylogeny tree is constructed comparing our sequences with similar sequence from NCBI GenBank.

2.1.1 Fungal crude extraction

The extracellular extraction was carried out for extract of *N. verrucosum*. The mycelial plugs of 6mm each were plugged out from the pure culture and inoculated in an Erlenmeyer flask(250ml) containing120ml of Potato Dextrose Broth (PDB) and kept at 150 rpm in a shaking incubator for about 14 days. After 2 weeks the liquid filtrate was separated from mycelia by a cotton cheese cloth and then equal amount of ethyl acetate was added to filtrate, shaken well and separated using separating funnel. The process is repeated for two times. The filtrate was filtered using Whatman's filter no.1, after that solvent was evaporated using rotary evaporator. The obtained extract is store in $4^{o}C$ until further use [12-14].

2.2 Antimicrobial activity of Fungal Crude Extract

Antimicrobial activity of fungal extract was performed using agar well diffusion method against five ATCC(American Type Culture Collection) bacterial strains : two Gram-negative bacteria; *Klebsiella pneumoniae* (ATCC 13883) and *Escherichia coli* (ATCC 25922), two Gram-positive bacteria; *Staphylococcus aureus* (ATCC 12600) and *Enterococcus faecalis* (ATCC 19433), with one fungus *Candida albicans* (ATCC 10231).The bacterial strains were initially cultured and grown overnight 37°C in Mueller-Hinton broth(MHB) whereas, *C. albicans* was grown in PDB at 27°C for 48 hours [15]. All test strains were maintained to a McFarland turbidity standard of 0.5 McFarland standard (1×10^8 CFU/ml). Pathogens were spread on Mueller-Hinton (MH) agar allowed to dry for a few minutes. Once the plates dried, wells were created using a sterile borer with an inner diameter of 6mm. In one well, 20 µL of DMSO was added as a negative control. In the next well, 20 µL of fungal extract prepared in DMSO (25 mg/mL) was added. 10 µg Gentamicin (Ezy MIC strip discs, HIMEDIA) was used as a positive control for the bacterial plates, while for *C. albicans*, 24 µg of Fluconazole (Ezy MIC strip discs, HIMEDIA) was used. The plates were allowed for proper diffusion of extract and then incubated at 37°C for 24 hours, whereas plates with *C. albicans* were incubated for 48 hours at 27°C. The assay for each test pathogen was carried out in triplicate. The zones of inhibition (ZOI) diameter were measured in mm and compared with the standards Gentamicin and Fluconazole for the respective plates.

2.2.1 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the fungal extract of *N. verrucosum* was determined against the microorganisms used in the agar well diffusion assay [12]. Test strains were cultured in MHB and adjusted to a turbidity equivalent to 0.5 McFarland. MIC determinations were performed in sterile 96-well microplates, and all assays were conducted in triplicate. Each well was filled with 100 µL of MHB. The fungal extract (1000 µg/mL) was added to the first row (100 µL per well) and mixed by pipetting 5–6 times. Two-fold serial dilutions were performed across the plate to obtain concentrations ranging from 1000 µg/mL to 7.81 µg/mL, with 100 µL transferred for each dilution. Bacterial suspensions were diluted 1:20 in MHB, and 10 µL of the diluted inoculum was added to each well. Plates were sealed and incubated at 37°C overnight. After incubation, 20 µL of 0.125% 2,3,5-triphenyltetrazolium chloride (TTC) prepared in sterile distilled water was added to each well, and plates were incubated for an additional 2–4 hours. Wells exhibiting viable bacterial growth reduced TTC and developed a red coloration, whereas wells without growth remained colorless. The MIC was defined as the lowest concentration of extract at which no color change was observed. Wells containing MHB and inoculum but no extract were included as growth controls [16-17].

2.2.2 DPPH(2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

The antioxidant activity of the fungal extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. Test concentrations for extract ranged from 12.5 to 500 µg/mL and L-ascorbic acid at the same concentrations was used as a positive control [18]. A DPPH stock solution (0.04 mg/mL) was prepared in methanol. For each assay well, 50 µL of sample or control solution was combined with 150 µL of the DPPH reagent (sample: DPPH = 1:3) in 96-well plates. Blanks were prepared by mixing methanol and DPPH reagent at the same 1:3 ratio. All measurements were performed in triplicate. The reaction mixtures were incubated in the dark at room temperature for 30 minutes, after which absorbance was measured at 517 nm using a BioTek Epoch microplate reader [19].

The Percentage Inhibition (I%) of L-Ascorbic acid(standard) and the fungal extract, was calculated using the formula:

$$Abs_{Control} - Abs_{sample}\,)/Abs_{control} * 100$$

Where Abs_{sample} is the absorbance of the test samples, and $Abs_{control}$ is the absorbance of the control.

A calibration curve was created between the percentage inhibition (I%) and the sample concentrations,then inhibition concentration (IC_{50}) was subsequently determined [20].

2.2.3 ABTS (2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)) assay

The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation scavenging assay was performed following [21] with minor modifications. ABTS radical cations were generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate in deionized water and allowing the mixture to stand in the dark at room temperature for 12–16 h [22]. The resulting solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm, measured using a UV–Vis spectrophotometer. Fungal extract and L-ascorbic acid (positive control) were prepared at concentrations of 500–12.5µg/mL. For each assay, sample or control (volume) was mixed with ABTS reagent in a 1:3 ratio in 96-well plates, and all measurements were performed in triplicate. After incubation in the dark for 45 minutes, absorbance was recorded at 734 nm using a BioTek Epoch microplate reader. Percent inhibition was calculated and plotted to generate the standard curve. The scavenging activity percentage (%) of the standard as well as the fungal extracts were calculated using the given formula:

ABTS scavenging (%) = $(Abs_{Control} - Abs_{sample})/Abs_{control} * 100$

Where Abs_{sample} is the absorbance of the test samples and $Abs_{control}$ is the absorbance of the Control.

The IC_{50} value (50% inhibition of ABTS radicals) was determined from the calibration curves generated using different concentrations of the test samples.

2.2.4 Total Phenolic Content (TFC)

Total phenolic content (TPC) was determined by the Folin–Ciocalteu colorimetric assay in 96-well plates [23] Gallic acid (160–5 µg/mL) was used for the calibration curve. Fungal extract was prepared at 1 mg/mL in methanol. In each well, 20 µL of standard or sample was mixed with 100 µL Folin–Ciocalteu reagent (1:10 v/v in distilled water), incubated in the dark for 10 min, then 80 µL Na2CO3 (75 g/L) was added and incubated in the dark for 1 h. Measurements were performed in triplicate; absorbance was read at 750 nm on a BioTek Epoch microplate reader using methanol as blank.

The phenolic content was calculated from the gallic acid calibration curve and expressed as µg gallic acid equivalents (GAE) per mg of dry extract using the equation:

$$Total\ Phenolic\ Content\ (TPC) = \frac{C \times V}{m}$$

where C is the concentration read from the standard curve (mg/mL), V is the volume of extract used (mL), and m is the mass of dry extract dissolved per mL (g).

2.2.5 Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric assay with sodium acetate modified from [24]. Quercetin (5–160 µg/mL) served as the calibration standard. A 1 mg/mL extract stock was prepared in methanol; 100 µL was mixed with 400 µL methanol, then 100 µL 10% AlCl3 and 100 µL 1 M sodium acetate were added. After 45 min incubation in the dark, 200 µL of the reaction mixture was transferred to 96-well plates and absorbance measured at 510 nm (BioTek Epoch). All measurements were performed in triplicate; TFC was calculated from the quercetin standard curve and expressed as mg quercetin equivalents (QE) per g dry extract using the equation:

$$Total\ Flavonoid\ Content\ (TFC) = \frac{C \times V}{m}$$

where C is concentration from the quercetin curve (mg/mL), V is volume of extract used (mL), and m is mass of dry extract per mL (g); results expressed as mg QE/g dry extract.

2.2.6 HR-LCMS

High-resolution LC–MS/MS analysis was performed on a QTOF mass spectrometer (Agilent G6550A, Agilent Technologies) with an electrospray ionization (ESI) source in positive and negative modes [25]. Chromatographic separation was achieved on a Hypersil GOLD C18 column (100 × 2.1 mm, 3 µm) at 40 °C. Mobile phases were 0.1% formic acid in water (A) and acetonitrile (B) with the gradient: 5% B (0–1 min), 5→100% B (1–25 min), 100% B (25–30 min), re-equilibration to 5% B (30–35 min). Injection volume was 5 µL and flow rate 0.3 mL min^{-1}. MS data were acquired in AutoMS/MS (data-dependent) mode over m/z 120–1200 with a scan rate of 1 Hz. Instrument settings included VCap 3.5 kV, fragmentor 175 V, nebulizer 35 psig, drying gas 13 L min^{-1} at 250 °C, and sheath gas 11 L min^{-1} at 300 °C. Metabolite identification used accurate mass (≤5 ppm) and MS/MS spectral matching against public databases (KEGG, HMDB, METLIN, LMP, CAS). Data acquisition and processing were performed with Agilent MassHunter software.

3 RESULTS

3.1 Identification

The fungus T3I first identified by morphologically and microscopy as shown in Figure 1. The sequence showed high percentage of identity (100%) with *N. verrucosum* (OR826642.1) from GeneBank [26]. The identified fungus has been successfully deposited to GenBank with the accession number PV648446. The phylogeny tree (Figure 2) was constructed on Phylogeny v2.0 [27]. Sequence alignment was computed by Mafft v7.526 [28] and alignment trimming was done using ClipKit v2.11.4 [29]. Phylogenetic tree inference was performed with IQ-Tree v2.4.0 [30].

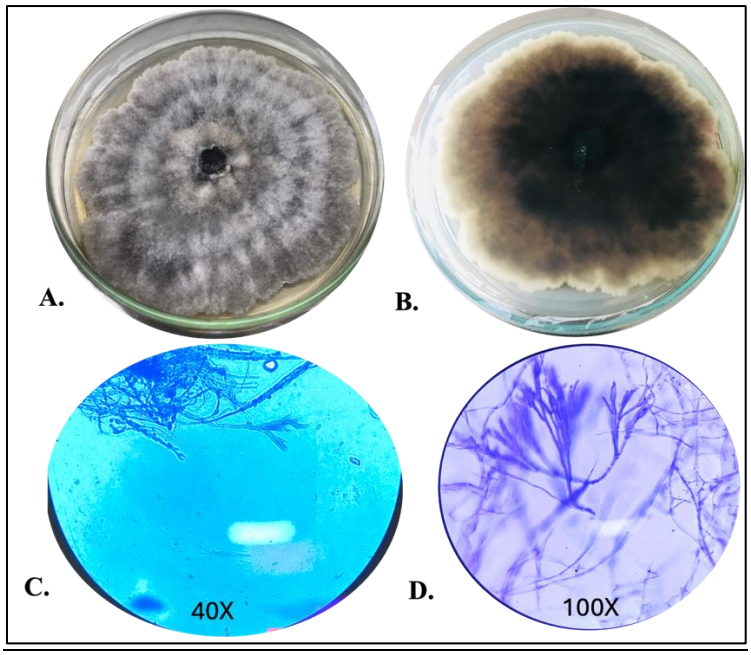


Figure 1: Macroscopic and microscopic characteristics of the fungal isolate. (A) Colony morphology on the upper surface of the culture medium showing radial zonation and concentric growth patterns; Floccose to cottony, with a dense aerial mycelium. (B) reverse colony morphology showing dark brown to black pigmentation; (C-D) microscopic morphology showing septate, branched hyphae and branched conidiophore structures under light microscopy.

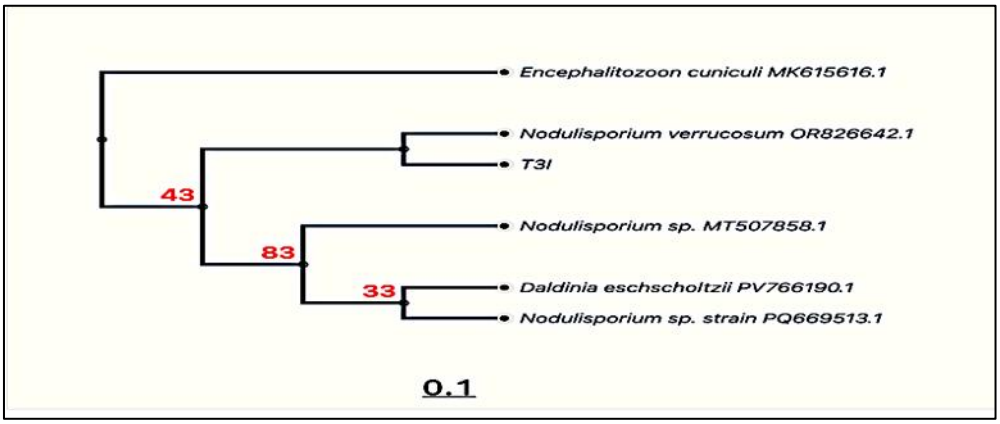


Figure 2: Phylogenetic tree showing relationship of our strain *N. verrucosum* (T3I), with similar sequences taken from GenBank by BLASTn search. The tree was constructed using online platform Phylogeny (v2.0) and *Encephalitozoon cuniculi* (MK615616.1) was taken as outgroup. Bootstrap values are based on 1000 replicates.

3.2 Antimicrobial assay

The fungal extract demonstrated selective antimicrobial activity (Figure 3), showing varying degrees of inhibition predominantly against Gram-positive bacteria and the fungal pathogen. Among the bacterial strains tested, the highest susceptibility was observed in *E. faecalis* with a zone of inhibition of 12.33 ± 0.57 mm, followed by *Staphylococcus* sp. (8.67 ± 0.57 mm). Both susceptible Gram-positive strains exhibited a MIC of 1000 µg/mL. Conversely, the extract showed no inhibitory activity (NA) against the tested Gram-negative pathogens, *K. pneumoniae* and *E. coli*, via the disc diffusion method, though *K. pneumoniae* displayed an MIC threshold at 1000 µg/mL. In terms of antifungal potential, the extract exhibited noteworthy activity against *C. albicans*, generating a zone of inhibition of 10.67 ± 0.57 mm and a corresponding MIC value of 1000 µg/mL. Although the standard commercial reference drugs; gentamicin for bacteria and fluconazole for fungi produced expectedly larger zones of inhibition, the broad-spectrum baseline activity of the crude extract underscores its therapeutic potential as a natural antimicrobial agent.

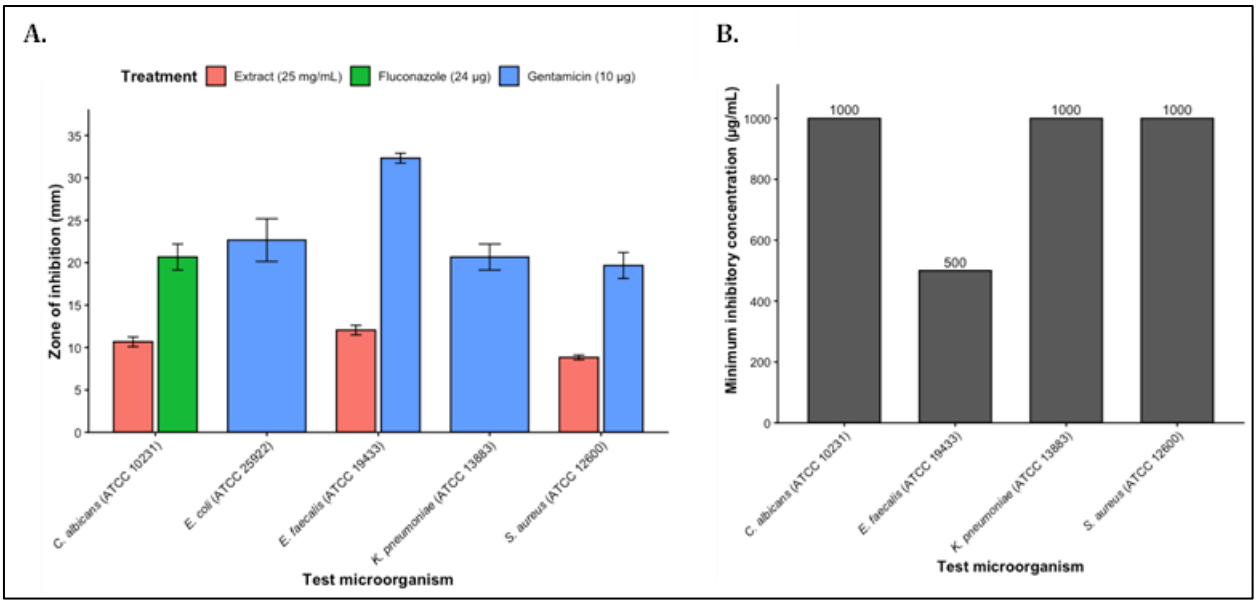


Figure 3: Antimicrobial activity of the *N. verrucosum* extract against selected bacterial and fungal pathogens. A. Bars represent the zone of inhibition (mm), expressed as mean ± standard deviation (SD), for the fungal extract (25 mg/mL) and reference B. Figure Minimum inhibitory concentration (MIC) of the *N. verrucosum* extract against selected bacterial and fungal pathogens. MIC values are expressed in µg/mL. NA indicates no detectable antimicrobial activity or MIC not determined.

3.3 In Vitro Antioxidant Evaluation

The antioxidant potential of the *N. verrucosum* crude extract was systematically evaluated using two distinct radical scavenging mechanisms: the DPPH and ABTS assays, with ascorbic acid serving as the positive control. Both assays revealed a distinct, concentration-dependent increase in radical scavenging activity across the tested range of 0 to 500 μg/mL. In the DPPH assay, *N. verrucosum* extract demonstrated a progressive radical-scavenging curve, yielding an IC_{50} value of 162.5 μg/mL and reaching approximately 70% inhibition at the highest tested concentration (Figure 4A). While the crude extract required a higher concentration to achieve equivalent radical inhibition compared to the pure standard, these results confirm that *N. verrucosum* possesses notable in vitro antioxidant potential, likely driven by its bioactive secondary metabolites. Interestingly, the extract showed significantly higher efficacy in the ABTS assay, closely approaching the potency of the standard reference (Figure. 4B). The *N. verrucosum* extract followed a highly competitive curve, yielding a remarkably low IC_{50} value of 25.37 μg/mL and stabilizing at over 85% inhibition at higher concentrations.

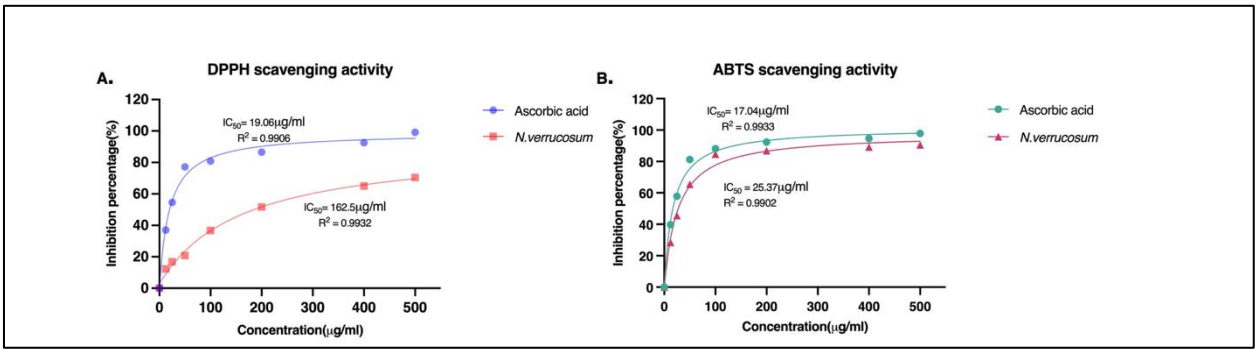


Figure 4: Antioxidant activity (A) DPPH radical scavenging activity of *N. verrucosum* extract and ascorbic acid (positive control). (B) ABTS radical scavenging activity of *N. verrucosum* extract and ascorbic acid. IC_{50} values and coefficients of determination (R^2) were obtained from nonlinear regression analysis.

3.4 Total Phenolic Content and Total Flavonoid Content

The Total Phenolic Content (TPC) of the fungal extract was quantified spectrophotometrically using a gallic acid standard calibration curve. Utilizing this calibration model, the TPC of the fungal extract was determined to be 13.37 $\pm$ 1.47 mg GAE/g. The Total Flavonoid Content (TFC) of the fungal extract was determined spectrophotometrically using

a quercetin standard calibration curve. The TFC of the fungal extract was calculated to be 1.2 ± 0.04 mg of quercetin equivalents per gram of dry extract (mg QE/g).

3.5 LCMS/MS (Liquid Chromatography Mass Spectroscopy)

High-resolution LC-MS/MS analysis identified a chemically diverse set of metabolites. In negative-ion mode 71 unique compounds were annotated and in positive-ion mode 65 unique compounds, with putative identifications accepted when the library match score was ≥70 based on retention time, m/z, and MS/MS fragmentation pattern (Figure 5). The annotated metabolites spanned multiple structural classes. Major classes included phenolic acids (gallic acid, vanillic acid, isoferulic acid), flavonoid glycosides (cynaroside A), polyphenolic and lignan-related metabolites (urolithin B, enterodiol), nitrogen-containing secondary metabolites (strictosamide; pyrido[3,4-c]psoralen; 1,4-dimethylpyrrolo[1,2-a]pyrazine; fusarochromanone), coumarin derivatives (fraxidin), iridoid glycosides (genipin 1-β-gentiobioside), fatty acid derivatives (corchorifatty acid A), glycosylated lactones (pantoyllactone glucoside), and phosphorylated sugar-derived metabolites (fructoselysine 6-phosphate) (Table: 1). Several of these compounds have previously been associated with bioactivities and may therefore contribute to the bioactivity observed for the fungal extract.

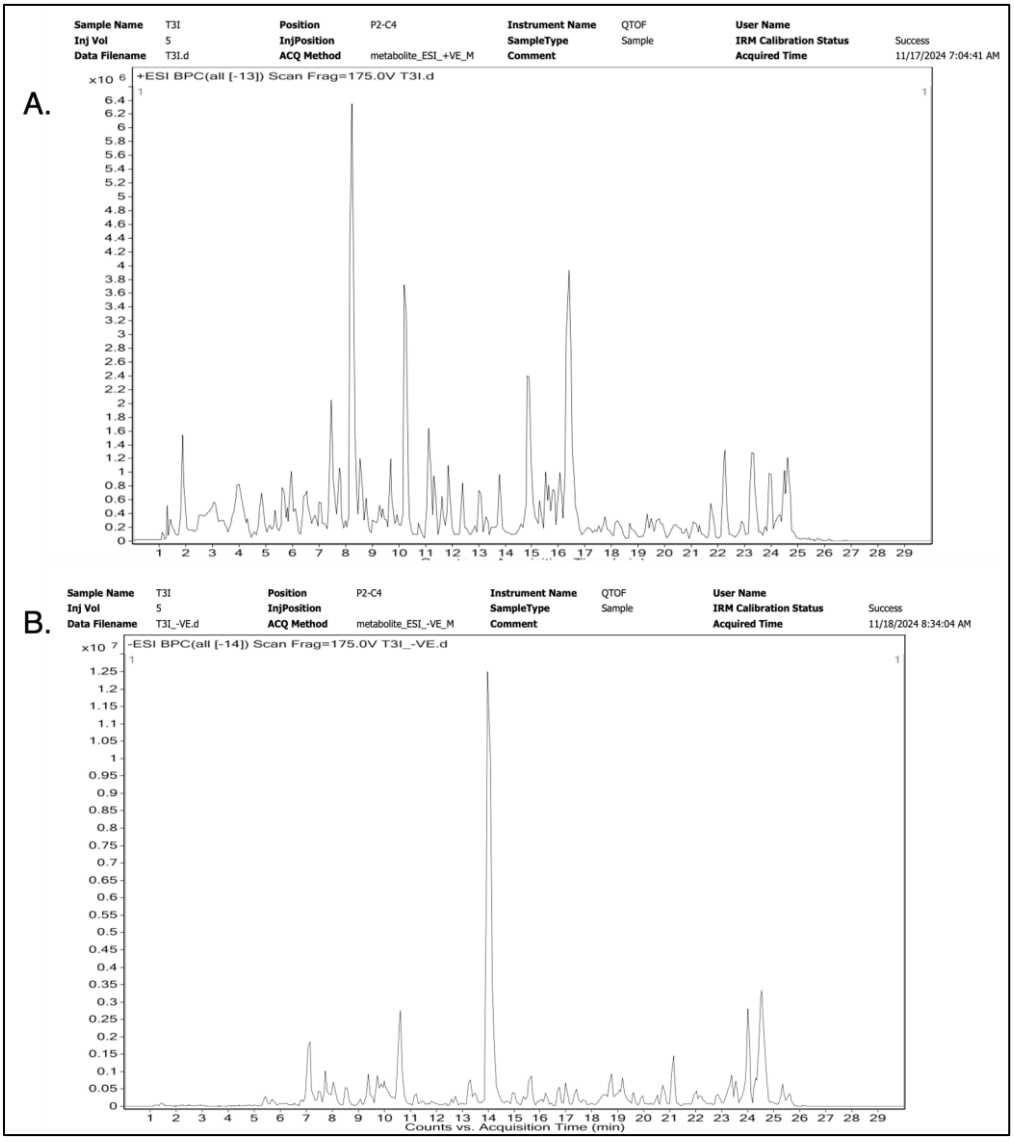


Figure 5: Representative total ion chromatograms (TICs) of the ethyl acetate extract of the endophytic fungus *N. verrucosum*, acquired in (A) positive and (B) negative electrospray ionization modes, illustrating the detected metabolite profiles at different retention times.

Table 1: LC–MS/MS-based identification and characterization of putative metabolites, showing compound names, molecular formulae, match scores, mass-to-charge ratios (m/z), retention times (RT), mass differences (ppm), and ionization polarity.

Compound	Formula	Match score	m/z	RT	Diff (DB, ppm)	Polarity
Gallic acid	C7 H6 O5	83.06	169.0141	23.359	0.25	Negative
Vanillic acid	C8 H8 O4	83.18	167.0351	3.574	-0.23	Negative
Urolithin B	C13 H8 O3	75.64	211.0418	11.282	-7.48	Negative
Strictosamide	C26 H30 N2 O8	72.48	543.1944	21.068	_	Negative
Cynaroside A	C21 H32 O10	95.87	503.2142	19.447	-0.57	Negative
Fraxidin	C11 H10 O5	72.52	221.0475	7.785	-8.5	Negative
Genipin 1-beta-gentiobioside	C23 H34 O15	86.84	609.2063	25.471	-4.62	Negative
1,4-Dimethylpyrrolo[1,2-a]pyrazine	C9 H10 N2	85.61	169.073	6.847	4.01	Positive
Fusarochromanone	C15 H20 N2 O4	70.04	315.1282	7.592	_	Positive
Isoferulic acid	C10 H10 O4	74.64	217.0454	7.839	_	Positive
Enterodiol	C18 H22 O4	71.82	325.1378	16.134	_	Positive
Corchorifatty acid A	C18 H28 O4	73.07	331.1848	22.212	_	Positive

4 DISCUSSION

The identification of isolate T3I as *N. verrucosum* was established through a polyphasic approach, integrating morphological characterization with molecular phylogenetic analysis. The 100% sequence identity with the *N. verrucosum* reference (OR826642.1) provides a robust genetic basis for this classification. While this study provides strong evidence for species placement, contemporary taxonomic standards suggest that reliance on a single genetic marker may be insufficient for distinguishing closely related species or identifying cryptic lineages [31,32]. Future studies should incorporate Multi-Locus Sequence Analysis (MLSA), targeting protein-coding genes such as *rpb2* or *tef1-α* in addition to the ribosomal markers to achieve higher taxonomic resolution [32].

The crude extract of our fungal extract demonstrated antimicrobial activity against *E. faecalis* (12.33 ± 0.57 mm), *Staphylococcus* sp. (8.67 ± 0.57 mm), and *C. albicans* (10.67 ± 0.57 mm), whereas no inhibition was observed against *E. coli* and *K. pneumoniae*. The extract exhibited an MIC of 1000 µg/mL against *E. faecalis*, *Staphylococcus* sp., *K. pneumoniae*, and *C. albicans*, while no MIC could be determined for *E. coli* due to the absence of growth inhibition. This pattern of preferential inhibition of Gram-positive human pathogens is highly consistent with previous research on *Nodulisporium* species isolated from *Nothapodytes foetida* exhibited appreciable antibacterial activity, particularly against Gram-positive pathogens, whereas Gram-negative bacteria were comparatively less susceptible [33,34]. While the extract showed no inhibition against *K. pneumoniae* and *E. coli* in disc diffusion assays, the MIC threshold of 1000 µg/mL for *K. pneumoniae* suggests that the extract may contain bioactive compounds that possess latent activity, which might be restricted by diffusion limits in the agar matrix rather than a total lack of susceptibility [35].

The antifungal activity against *C. albicans* (MIC 1000 µg/mL) highlights the potential of *N. verrucosum* as a source of novel antifungal agents capable of interfering with fungal cell growth. This is comparable to studies on other endophytic *Nodulisporium* strains, where crude extracts have demonstrated significant inhibitory effects against pathogenic yeasts, likely due to the production of specialized metabolites like polyketides and alkaloids that interfere with fungal ergosterol biosynthesis or cell wall stability [36]. Although the crude extract showed lower potency than standard commercial agents like gentamicin and fluconazole, this difference is expected because the fungal extract represents an unpurified crude metabolite mixture, whereas the positive controls contain purified antimicrobial agents at defined concentrations. Purification and characterization of the active metabolites are needed to significantly enhanced antimicrobial activity [36].

The *N. verrocosum* endophyte exhibited concentration-dependent radical-scavenging activity in both DPPH and ABTS assays, with IC_{50} values of 162.5 and 25.37 µg/mL, respectively. In the context of *Taxus*-associated endophytes, our extract displays stronger DPPH activity than a *Fusarium tricinctum* from *Taxus baccata* (DPPH $IC_{50} \approx 482$ µg/mL) [37] but weaker activity than the most potent *T. wallichiana* isolates (*Alternaria alternata*, *Cladosporium cladosporioides*, *A.*

brassicae), which exhibited DPPH IC_{50} values of ~22–23 µg/mL [38]. The pronounced efficiency of the extract in neutralizing the ABTS radical cation compared to the DPPH radical suggests that the bioactive secondary metabolites present in *N. verrucosum* may preferentially operate via single-electron transfer mechanisms or possess better solubility dynamics in the ABTS reaction medium. This remarkably low IC_{50} value in the ABTS system highlights the presence of highly effective electron- or hydrogen-donating primary antioxidants within the crude extract. Consistent with its moderate antioxidant activity, the *N. verrocosum* extract showed moderate total phenolic but relatively low flavonoid content (13.37 ± 1.47 mg GAE/g and 1.2 ± 0.04 mg QE/g, respectively). In comparison, *T. wallichiana* endophytes (*A. alternata*, *C. cladosporioides*, *A. brassicae*) displayed markedly higher TPC (153–312 mg GAE/g) and TFC (96–644 µg RE/mg) alongside much stronger DPPH activity [38], underscoring the phenolic-rich, high-antioxidant potential of some *Taxus*-associated isolates relative to the *N. verrocosum* endophyte. This prominent phenolic presence strongly correlates with the robust antioxidant and antimicrobial properties observed in the extract. The existing reports on this genus have focused instead on antifungal VOCs, anti-phytopathogenic metabolites such as sporothriolide, and general antimicrobial activities [34]. Thus, the present findings provide among the quantitative evidence that *N. verrocosum* is a moderate phenolic producer with notable ABTS radical-scavenging capacity, expanding the known bioactive potential of *Nodulisporium* endophytes beyond their previously characterized antimicrobial and specialized metabolite profiles and highlighting the diversity of antioxidant mechanisms among fungal endophytes from different host lineages.

The antioxidant and antimicrobial activities of *N. verrucosum* may be associated with its diverse putatively annotated metabolites, including phenolic acids, flavonoids, coumarins, alkaloids, and oxylipins. Phenolic compounds such as gallic acid, vanillic acid, isoferulic acid, and cynaroside have documented antioxidant properties and may contribute to the observed DPPH and ABTS activities [39, 40]. Similarly, strictosamide and related alkaloids have been reported to possess diverse pharmacological activities, including antimicrobial effects [41]. Collectively, these compounds may act additively or synergistically to produce the observed biological activities. However, as the metabolites were tentatively annotated by LC–MS database matching, isolation and structural confirmation are required to establish their direct contribution to the observed activities.

Overall, these results suggest that the biological activities of the crude extract are likely due to the synergistic or additive effects of multiple metabolites rather than a single compound. However, as the compounds were tentatively annotated based on metabolomic database matching, further isolation and structural confirmation using authentic standards and compound-specific bioactivity assays are required to establish their direct contribution to the observed antioxidant and antimicrobial activities.

5 CONCLUSION

The present study demonstrates the bioactive potential of the endophytic fungus *N. verrucosum* isolated from *T. wallichiana*. These findings expand the limited knowledge of the bioactive potential of *N. verrucosum* and highlight *Taxus*-associated endophytic fungi as promising sources of biologically active metabolites. Further multilocus taxonomic confirmation, isolation and structural characterization of individual compounds, and targeted bioactivity studies are required to establish the metabolites responsible for the observed biological effects.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The author declared that no conflict of interest.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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