



Therapeutic potential and metabolite profiling of fungal endophytes from *Withania somnifera* (L.) Dunal

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Abstract

Endophytic fungi associated with *Withania somnifera* (L.) Dunal represent a promising source of bioactive compounds, some of which may be identical to or distinct from those produced by the host plant. However, their metabolic diversity and therapeutic potential, influenced by geography and environment, remain underexplored. The crude extracts of the fungal endophytic isolates were tested for their antioxidant activity, antidiabetic potential and anti-inflammatory efficacy by established biochemical assays. When compared with other endophytic isolates, namely W2P, W1R, W2S, W1L and W2LA, W2LB consistently showed the highest activity in phosphomolybdenum and ferric reducing antioxidant power (FRAP) assays, suggesting substantial antioxidant potential. Endophytic isolate W1L, on the other hand, demonstrated the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and high anti-inflammatory activity. Internal transcribed spacer (ITS) rDNA sequencing identified two potent fungal endophytic isolates, W1L and W2LB as *Daldinia korfii* and *Diaporthe perseae*. The extract's liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) profiling revealed a broad range of phenolics, terpenoids, alkaloids, alkanes, fatty acids and other pharmaceutically significant compounds. This study adds to the diversity of fungal endophytes associated with *W. somnifera* by identifying less frequently found species, including *D. perseae*, *D. korfii* and indicates that the endophytes of *W. somnifera* enhance the phytochemical diversity of their host, contributing to anti-inflammatory, antidiabetic and antioxidant activities. Hence, the experiment supports further purification and analysis for drug development.

Keywords: ashwagandha; bioactive compounds; *Daldinia korfii*; *Diaporthe perseae*; endophytic fungi; secondary metabolites; *Withania somnifera*

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1. Introduction

Endophytes inhabit plant tissues asymptotically, forming a diverse group of symbiotic microbes. The presence of endophytes boosts agricultural output (1). Apart from the above attribute, various other important plant physiological functions such as nitrogen fixation, phytoremediation, hormone signalling, secondary metabolite synthesis and metabolic regulation are all actively mediated by endophytic bacteria and fungi (2). The co-evolution of symbiotic microbes with their host plants has enabled these microorganisms to become a significant source of bioactive compounds (3). *Withania somnifera* (L.) Dunal, widely recognised as ashwagandha or Indian ginseng, has been an important component in Ayurvedic medicine for more than 3000 years, valued for its diverse therapeutic benefits (4). It is a prominent herb, which has long been utilised to address a range of health conditions, including inflammatory disorders, rheumatism, bronchopulmonary ailments, cancer and mental ailments. The primary bioactive components which reinforce its therapeutic value are withanolides and tropane alkaloids (5). Endophytes have been shown to synthesise bioactive

compounds traditionally attributed to their host plants. Moreover, they can increase the expression of genes responsible for the production of phytochemicals as evidenced by increased withanolide production in *W. somnifera* upon endophytic association (6, 7). The endophytes isolated from medicinal plants have reported the occurrence of bioactive metabolites, namely flavonoids, phenolics, carbohydrates, saponins, tannins and terpenoids (8). Hence, further investigation in this domain is both necessary and promising. This study aimed to isolate and characterise fungal endophytes from *W. somnifera* with the assistance of internal transcribed spacer (ITS) sequencing, assess their anti-inflammatory, antidiabetic and antioxidant properties and profile their secondary metabolites using liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses. This comprehensive method sheds light on the medicinal potential of endophytic fungi associated with *W. somnifera* and its potential application in sustainable drug discovery.

2. Materials and Methods

Collection of plant tissues

Medicinal shrub, *W. somnifera* was purchased from a nursery near the Lalbagh Botanical Garden, Bengaluru, Karnataka, India, (12.9422 ° N, 77.5894 ° E) during January 2025. The plant tissues (leaf, stem, petiole and root) selected were healthy and free from any infections. Samples were carefully harvested and transferred into pre-labelled zip-lock covers. Immediately after collection, the tissues were transferred to the laboratory.

Isolation of fungal endophytes

To eliminate dust and surface debris, the plant samples were first cleaned under running tap water. Following that, the samples were exposed to a surface sterilisation regimen that included a 1 min immersion in 2 % sodium hypochlorite solution, distilled water wash followed by 70 % ethanol and a final rinse in sterile distilled water. The segments that had been surface-sterilised were aseptically dried on sterile filter paper. Following surface sterilisation, the plant tissues were cut into small parts, using sterile blades and were aseptically placed onto potato dextrose agar (PDA) plates, incorporated with 100 µg/mL streptomycin to inhibit bacterial contamination. For 5–7 days, the plates were maintained at 25 ± 2 °C. Fungal colonies that emerged from the tissue segments' margins were routinely observed. To generate pure cultures, distinct colonies were sub-cultured onto new PDA plates. For further investigations, these pure fungal isolates were maintained on PDA plates at 4 °C (8).

Laboratory-scale fermentation of the endophytic fungi

To obtain adequate mycelial biomass, active-growing cultures of the 6 endophytic fungal isolates were initially grown on fresh PDA plates and cultured for seven days at 28 °C. This was followed by the inoculation of 5 × 5 mm mycelial plugs, from these mature cultures into 250 mL Erlenmeyer flasks, containing 50 mL of potato dextrose broth (PDB), supplemented with 100 µg/mL streptomycin to inhibit bacterial contamination. Six replicate flasks were inoculated with each fungal strain and maintained at 28 °C for two weeks under static conditions (9).

Preparation of crude endophytic fungal extract

After filtering the fungal mycelium out of the fermentation broth, it was dried at 40 °C in a hot-air oven until its weight remained constant. The dried biomass was then powdered and refluxed using methanol as solvent to extract the intracellular metabolites, focussing solely on the compounds within the biomass (10). A rotary evaporator (RE-100 S, D Lab Scientific) was used to concentrate the resultant crude methanolic extract. For further analysis, the dried residue was weighed, reconstituted in methanol and stored at 4 °C.

Estimation of total phenolic content

Folin-Ciocalteu method with minor modification was used to determine the total phenolic content (TPC) (11, 12). A volume (1.5 mL) of Folin-Ciocalteu reagent (1 : 1 dilution) was combined with a 0.5 mL portion of the fungal extract (1 mg/mL) namely, W2P, W1R, W2S, W2LB, W1L and W2LA and incubated for 10 min. After adding 1 mL of 7.5 % sodium carbonate, the mixture was incubated in the dark for 2 hr. Absorbance was measured at 765 nm using a Shimadzu UV-1800 spectrophotometer. The experiments were carried out in triplicate and the results are reported as mg gallic acid equivalents per g of extract (mg GAE/g).

Antioxidant activity

DPPH radical scavenging assay

The extract's free radical scavenging activity was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay with minor modification (12). A 1 mg/mL solution of the methanolic extract was prepared and 1 mL of this solution was mixed with 1 mL of 0.1 mM DPPH reagent. The mixture was incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm using a Shimadzu UV-1800 spectrophotometer. Experiments were performed in triplicate and the percentage of radical scavenging activity was calculated.

Percentage of free radical scavenging activity =

$$\left[\frac{A_0 - A_1}{A_0} \right] \times 100 \quad \text{Eq. (1)}$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

FRAP assay

The reductive potential of the methanolic extract (1 mg/mL) was determined using the ferric reducing antioxidant power (FRAP) analysis with minor modification. After adding 1 mL aliquot with 2.5 mL of 1 % potassium ferricyanide and 2.5 mL of 0.2 M phosphate buffer (pH 6.6), the mixture was incubated at 50 °C for 20 min. Further, 2.5 mL of trichloroacetic acid (10 %) was added and the mixture was centrifuged at 1000 rpm for 10 min using a DLAB D1524R centrifuge. An aliquot of 2.5 mL of the supernatant was mixed with 500 µL of 0.1 % ferric chloride and 2.5 mL of distilled water. Absorbance was measured at 700 nm using a Shimadzu UV-1800 spectrophotometer (13). The antioxidant capacity, expressed as milligrams of ascorbic acid equivalents per gram of extract (mg AAE/g), was determined in triplicate.

Phosphomolybdenum assay

The total antioxidant capacity of the extracts was assessed via the phosphomolybdenum assay. A volume of 200 µL of methanolic extract (5 mg/mL) was added to 2 mL of a reagent solution containing 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulfuric acid. Incubation was carried out for 90 min at 95 °C in a temperature-controlled water bath. After incubation, samples were cooled to room

temperature and absorbance was measured at 765 nm using a Shimadzu UV-1800 spectrophotometer (14). The calibration curve was established using ascorbic acid as the reference standard. Each analysis was carried out in triplicate and the antioxidant activity was expressed as mg AAE/g.

Antidiabetic activity

α -Amylase inhibition assay

The α -amylase inhibitory potential of the extract was determined using the 3,5-dinitrosalicylic acid (DNS) colorimetric assay adapted for spectrophotometric analysis with minor modification (13). A volume of 200 μ L of α -amylase solution (0.4 mg/mL) in Tris-buffered saline (TBS, pH 6) was combined with 500 μ g of the sample and the mixture was incubated at 30 °C for 10 min to facilitate enzyme-extract interaction. Following incubation, 200 μ L of 1 % soluble starch (substrate) was added and the reaction mixture was maintained at room temperature for 3 min. The enzymatic reaction was terminated by adding 200 μ L of DNS reagent and the mixture was subsequently heated at 85–90 °C for 10 min to allow colour development. Upon cooling to room conditions, the mixture was diluted with 5 mL of distilled water. The absorbance of the resulting chromogenic complex was recorded at 540 nm using a Genesis 10S ultraviolet-visible (UV-Vis) spectrophotometer. Experiments were conducted in triplicate to ensure reproducibility. Acarbose (500 μ g) served as the positive control. The percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \left[\frac{A_0 - A_1}{A_0} \right] \times 100 \quad \text{Eq. (2)}$$

where, A_0 and A_1 represent the absorbance values of the control and test samples respectively.

Anti-inflammatory assay

Bovine serum albumin denaturation assay

The anti-inflammatory potential of the extract was assessed via the protein denaturation inhibition method with minor modification (13, 15). The reaction mixture consisted of 1 mL of 0.1 % (w/v) bovine serum albumin (BSA), 1 mL of TBS (pH 6.5) and 500 μ g of the test sample. The mixture was initially maintained at 37 °C for 15 min to simulate physiological conditions, followed by thermal induction of protein denaturation at 60 °C for 10 min in a controlled water bath (Model EPS/WB-S2/SS, EPS Biosolutions). Once the temperature was lowered to room temperature, absorbance was measured at 660 nm using a Genesis 10S UV-Vis spectrophotometer. Diclofenac sodium (500 μ g) was utilised as the positive control. The percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \left[\frac{A_0 - A_1}{A_0} \right] \times 100 \quad \text{Eq. (3)}$$

where, A_0 and A_1 correspond to the absorbance values of the control and test samples respectively. All

experiments were conducted in triplicate to ensure reproducibility.

Morphological and molecular characterisation

Fungal isolates exhibiting notable bioactivity were subjected to both classical and molecular identification methods. Macroscopic features including colony morphology, pigmentation, margin type, elevation and surface texture were recorded for phenotypic classification. For microscopic examination, fungal structures were stained using lactophenol cotton blue and observed under a compound microscope. Hyphal architecture was evaluated to support identification of the endophytes (16).

For molecular characterisation, isolates were cultured on PDA for 6 days, following which genomic DNA was extracted. The ITS region of ribosomal DNA was amplified by polymerase chain reaction (PCR) using the universal fungal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Bidirectional sequencing was carried out using the BigDye Terminator (BDT) v3.1 cycle sequencing kit on ABI 3730xl genetic analyser. The resulting forward and reverse sequence data were assembled into a consensus sequence using Aligner software.

The consensus ITS sequence was compared with sequences in the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database using basic local alignment search tool (BLASTn) to determine sequence similarity. The top ten matches exhibiting the highest identity scores were selected for alignment using ClustalW. A phylogenetic tree and genetic distance matrix were subsequently generated using molecular evolutionary genetic analysis (MEGA) version 11 to infer evolutionary relationships (17).

Analysis of bioactive compounds from fungal isolates using liquid chromatography-mass spectrometry

Methanolic fungal extracts exhibiting significant bioactivity were subjected to LC-MS analysis. The analysis was performed using an Agilent Q-ToF G6540B coupled to an Agilent 1260 Infinity II high-performance liquid chromatography (HPLC) in positive electrospray ionisation mode (+ESI). Chromatographic separation was achieved on an Agilent Eclipse XDB-C18 column (3 $\times$ 150 mm, 3.5 μ m) maintained at 40 °C. A 15 μ L aliquot of the methanolic extract was injected. Separation of the compounds was achieved using a mobile phase consisting of solvent A (water with 0.1 % formic acid) and solvent B (acetonitrile with 0.1 % formic acid) supplied at a flow rate of 0.3 mL/min. Chromatographic separation was achieved using the following gradient elution program: 0 min, 95 % A; 2 min, 95 % A; 25 min, 5 % A; 28 min, 5 % A; 28.1 min 95 % A, 30 min 95 % A.

The mass spectrometer was operated under the following conditions: A capillary voltage of 3500 V, drying gas flow rate of 8 L/min, nebuliser pressure set at 35 psi and gas temperature maintained at 300 °C. Mass

spectra were acquired in full scan mode over an m/z range of 100–1700 for a total analysis time of 30 min. Accurate mass and isotope patterns were compared to the METLIN personal compound database and library (PCDL B.08.00) in order to identify the compounds (18). Tentative identification was assigned when a peak matched the library spectrum with high similarity score.

Analysis of bioactive compounds from fungal isolates using gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry analysis was performed using a Shimadzu GC-2010 gas chromatograph interfaced with a GCMS-QP2010 SE mass spectrometer and equipped with an AOC-20i auto-injector. An Rxi-5Sil MS column (30 m $\times$ 0.25 mm diameter $\times$ 0.25 μ m film thickness) was used. A sample volume of 1 μ L was introduced using a split injection mode (split ratio 4.0) with the injection port temperature maintained at 280 °C. The auto-injector protocol included 3 pre-solvent, 3 post-solvent and 3 sample rinses to prevent cross-contamination. The carrier gas flow was controlled under constant linear velocity at 36.1 cm/sec, yielding a column flow rate of 0.99 mL/min and a purge flow of 3.0 mL/min. The oven temperature program was as follows: initial temperature 50 °C held for 1 min, increased at 10 °C/min to 250 °C and held for 10 min, followed by a further increase at 2 °C/min to 280 °C and held for 20 min. The mass spectrometer was operated in electron ionisation mode (EI) in full scan mode, acquiring data over a mass-to-charge (m/z) range of 40–550 with an event time of 0.30 sec and a scan speed of 2000. The ion source and interface temperatures were maintained at 230 °C and 290 °C respectively, with a solvent cut time of 3.50 min to protect the detector. Mass spectral patterns were compared with the National Institute of Standards and Technology (NIST) 2017 database to identify the compounds. Tentative identification was assigned when a peak matched the library spectrum with a high similarity score.

Statistical analysis

Data analysis was performed using International Business Machines Statistical Package for the Social Sciences (IBM SPSS) Statistics, version 24.0. Results are presented as mean $\pm$ standard error (SE) from three independent replicates. Significant differences were assessed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), with significance set at $p < 0.05$.

3. Results and Discussion

Total phenolic content of the endophytic fungal extracts

Results were quantified based on a standard calibration curve of gallic acid within the range of 20–100 μ g/mL. The TPC of the endophytic fungal extracts varied across isolates, ranging from 8.38–23.41 mg GAE/g. The extract

of isolate W2P exhibited the highest phenolic content, followed by W1L. The lowest TPC was observed in W2S, highlighting the substantial differences in the phenolic content among isolates. The TPC values of all fungal isolates are presented in Table 1.

The phenolic content present in the above isolates is significant, as these compounds are well known for their antioxidant capabilities. Along with flavonoids, phenolic compounds contribute to a broad spectrum of biological activities, including antimicrobial effects, metal chelation and antioxidant properties (19). Natural phenolic compounds include flavonoids, phenolic acids, chalcones, tannins, lignins, lignans, stilbenes, among other related compounds (20). The highest TPC (23.41 mg GAE/g) is comparable with the phenolic content of 35 mg GAE/g, exhibited by the methanolic extract of *Muyocopron sahnii*, an endophyte of *Gynura procumbens* (Lour.) Merr. (21). The fungal endophyte *Daldinia eschscholtzii* isolated from *Psidium guajava* L., exhibited a phenolic content of 43.85 mg GAE/g (19). Isolate W1L, identified as *Daldinia korfii* exhibited the second highest TPC of 18.91 mg GAE/g. The strain W2LB, identified as *Diaporthe perseae*, exhibited a TPC of 17.88 mg GAE/g. In comparison, the ethyl acetate extracts of *Diaporthe neotheicola* KaS-6 and *Diaporthe macadamiae* KaS-4, both endophytes of *Kirkia acuminata* Oliv. showed TPC values of 26.56 and 12.71 mg GAE/g respectively (22). Overall, these results highlight the diversity and potential of the isolates as sources of phenolic compounds.

Table 1. Total phenolic content of fungal endophytes

Fungal endophytic code	Total phenolic content (mg GAE/ g crude extract $\pm$ SE)
W2P	23.41 $\pm$ 0.49 ^a
W1R	12.68 $\pm$ 1.21 ^c
W2S	8.38 $\pm$ 0.82 ^c
W2LB	17.88 $\pm$ 3.68 ^b
W1L	18.91 $\pm$ 0.90 ^{ab}
W2LA	8.61 $\pm$ 0.27 ^c

Results are presented as mean $\pm$ SE ($n = 3$). Different letters indicate statistically significant differences at $p < 0.05$. The analysis showed 5 degrees of freedom, between groups and 12 degrees of freedom within groups.

Antioxidant activity

DPPH radical scavenging activity of the endophytic fungal extracts

The DPPH free radical scavenging activity varied between 32.44–88.17 % as illustrated in (Fig. 1A). The highest inhibition was recorded for *D. korfii* (W1L), followed by *D. perseae* (W2LB) while the lowest activity was observed in the endophyte, W2P.

A common method for assessing antioxidant activity is the DPPH assay, which relies on the reduction of DPPH (14). The reducing agents in the extracts reduce DPPH, resulting in a colour change from violet to yellow (23). Similar observation have been reported for

the fungal endophyte of *W. somnifera*, WSF-6, which exhibited the highest antioxidant activity of 81.25 % (24). Likewise, *D. eschscholtzii*, a fungal endophyte isolated from *P. guajava*, exhibited 80.30 % inhibition of free radicals (19). Azaphilones isolated from the endophytic fungus *D. perseae*, obtained from the Chinese mangrove *Pongamia pinnata* (L.) Pierre, demonstrated significant radical scavenging activity, with IC₅₀ values between 7.3–16.08 µg/mL, emphasising the fungus's robust capacity to neutralise free radicals (25).

Ferric reducing antioxidant activity of the endophytic fungal extracts

The FRAP values were calculated using a standard calibration curve of ascorbic acid (20–100 µg/mL). As illustrated in Fig. 1B, the endophytic fungi's FRAP values were found to be between 18.36–55.48 mg AAE/g. The isolate *D. perseae* (W2LB) displayed the highest activity, followed by *D. korffii* (W1L). The extract of W2S showed the lowest FRAP value.

The conversion of Fe³⁺ (ferricyanide) to Fe²⁺ (ferrous) ions is measured using the FRAP assay, which is driven by electron or hydrogen donating antioxidants (14, 23). Consistent with the DPPH assay, the FRAP results reinforce the antioxidant capabilities of *D. perseae* (W2LB) and *D. korffii* (W1L). The elevated FRAP values correlate with the diverse chemical profile of phenolic compounds, as well as alkaloids and terpenoids, identified through LC-MS analyses. Cultivation of the endophytic fungus *Diaporthe fraxini* ED2 in two distinct media resulted in marked differences in FRAP values, recorded as 53.88 and 188.41 µg AAE/mg extract respectively (26). In the present study, as all the isolates were cultured under identical conditions, it is evident that metabolomic

diversity and strain-specific bioactivity contribute to variations in the FRAP values expressed by the different fungal endophytic isolates.

Phosphomolybdenum antioxidant activity of the endophytic fungal extracts

Quantification of total antioxidant activity (TAA) was performed using a standard calibration curve of ascorbic acid over the concentration range of 20–100 µg/mL. The TAA of the endophytic fungal extracts ranged from 32.19–58.11 mg AAE/g (Fig. 1B). The highest activity was exhibited by the isolate, *D. perseae* (W2LB), followed by W1R, whereas the lowest TAA was observed in the extract of W2S.

The assay is based on the reduction of Mo(VI) to Mo(V), by antioxidants in the extract, forming the green phosphate Mo(V) complex (14). *Daldinia eschscholtzii* exhibited a higher TAA of 309.23 mg AAE/g than that observed in the present study (19). A significant correlation was observed between phenolic content and antioxidant activity in W2LB and W1L, except for W1R, indicating a possible role of the non-phenolic compounds in scavenging the free radicals (27).

Antidiabetic activity

α-Amylase inhibition activity of the fungal endophytes

The α-amylase inhibitory activity of the extracts at 500 µg ranged from 22.11–45.16 % (Fig. 2). By measuring the inhibition of α-amylase, the antidiabetic efficacy was evaluated. Among the extracts, W2P exhibited the highest inhibitory activity, whereas the W2S extract showed the lowest inhibition. The ethyl acetate extract of the endophyte *Diaporthe* sp. isolated from *Ocimum sanctum* Linn. exhibited notable α-amylase inhibitory activity, with an IC₅₀ value of 40.73 µg/mL (28).

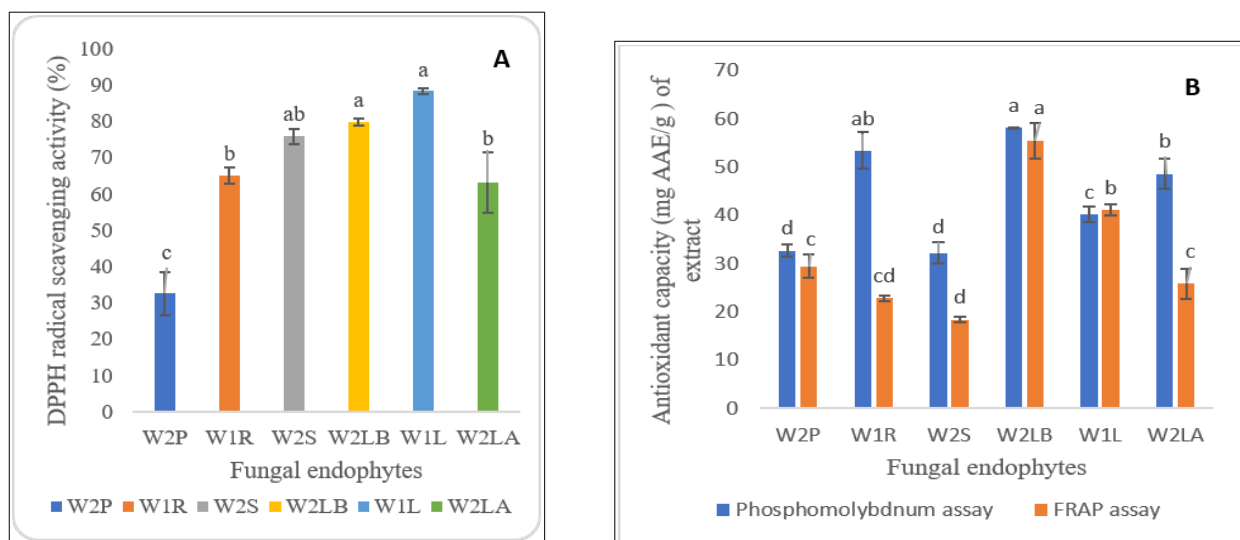


Fig. 1. Antioxidant assays of fungal endophytic extracts. (A) DPPH free radical scavenging activity expressed as percentage inhibition; (B) total antioxidant activity and ferric reducing antioxidant power of the extracts expressed, as mg AAE/g of the extract. Results are presented as mean ± SE (n = 3). Different letters indicate statistically significant differences at $p < 0.05$. The analysis showed 5 degrees of freedom, between groups and 12 degrees of freedom within groups.

DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing antioxidant power.

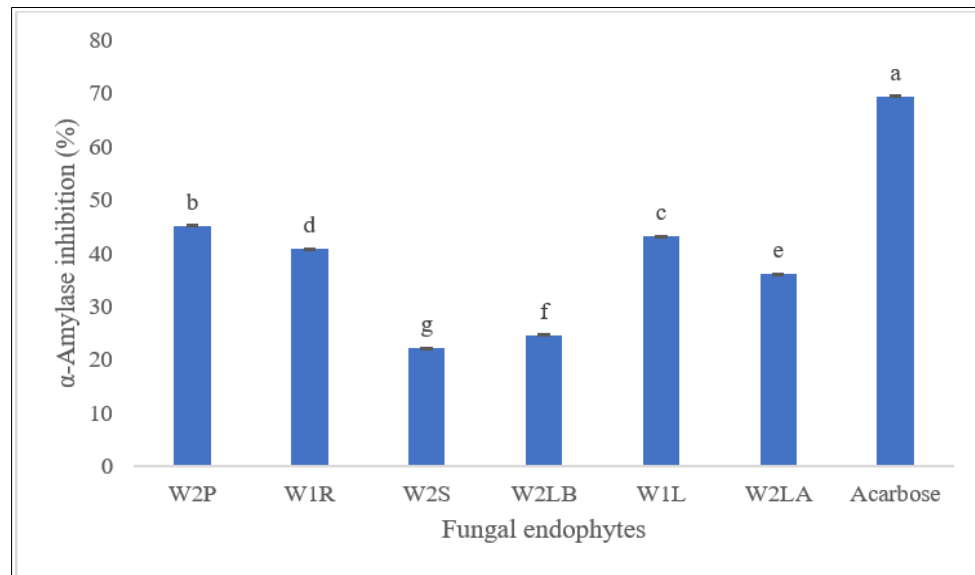


Fig. 2. α-Amylase inhibition assay.

Results are presented as mean $\pm$ SE ($n = 3$). Statistically significant differences ($p < 0.05$) are indicated by distinct letters. Degrees of freedom, between groups = 6 and within groups = 14.

Similarly, the endophytic isolate *Aspergillus luchuensis* Inui of *Acacia nilotica* (L.) Willd. ex Delile demonstrated 81 % inhibitory efficacy against α-amylase (29). At a concentration of 200 µg/mL, endophytes isolated from *Syzygium cumini* (L.) Skeels exhibited α-amylase inhibition ranging from 29.62–62 % (30). In the current study, the observed inhibition of 45.16 % indicates considerable α-amylase inhibitory activity, although it is lower than that of the standard drug acarbose (69.41 %). This emphasises that the bioactivity may be influenced by specific fungal isolates or variations in extraction conditions.

Anti-inflammatory assay

Bovine serum albumin denaturation activity of the fungal endophytes

The anti-inflammatory activity of the extracts was evaluated using the BSA denaturation method. Percentage of BSA denaturation inhibition ranged from 19.95–66.43 % in 500 µg of the crude extract as illustrated in Fig. 3. Among the isolates, W2LA exhibited the highest inhibition, whereas W2LB showed the least activity. *Aspergillus fumigatus* AUMC 8872 exhibited an activity of 62.7 %, whereas *Emericella nidulans* AUMC 8854 showed significantly stronger inhibition of BSA denaturation, reaching 95 % (15). According to a previous study, endophytic *Bacillus pacifica* NCLALC2 from *Curcuma longa* L. exhibited higher inhibition than diclofenac sodium, suggesting strong protein-stabilising potential, while *Fusarium fujikuroi* PCLCUT2 showed 71.96 % inhibition, highlighting strain-specific variations in anti-inflammatory efficacy (31). The standard control, diclofenac sodium exhibited a higher mean inhibition of BSA denaturation of 67.82 %. Among the fungal isolates, W1R, W1L and W2LA exhibited inhibition

comparable to that of the standard control, suggesting strong protein-stabilising potential in these extracts and highlighting their promise for further anti-inflammatory investigation. The low anti-inflammatory activity of W2LB could be attributed to the presence of co-extracted components. Further fractionation of the biologically active molecules from the crude extract may further improve the potency (32).

Identification of the fungal endophytes

Endophytic isolates W1L and W2LB, which showed strong antioxidant activities, were selected for morphological and molecular characterisation. Endophytic isolate W1L also displayed notable anti-inflammatory and antidiabetic activities. Both the endophytes were filamentous and filiform, with a cottony texture. Endophytic isolate W1L was white in colour, whereas W2LB had a white and greyish colouration, as summarised in Table 2 and illustrated in Fig. 4. Microscopic examination revealed that both fungi had septate hyphae.

Amplification of the ITS region, using ITS1 and ITS4 primers generated PCR amplicons of approximately 600 bp. The fungal endophyte W1L showed 100 % similarity to sequences in the National Center for Biotechnology Information (NCBI) GenBank database and was identified as *D. korffii*, while isolate W2LB showed 99.37 % sequence similarity and was identified as *D. perseae* (Table 3; Fig. 5A, B). The consensus sequence of W1L is presented in Fig. 6 and the evolutionary relationship of the isolates was assessed by phylogenetic analysis as given in Table 4 and Fig. 7. The consensus sequence of W2LB is illustrated in Fig. 8. Phylogenetic analysis of *D. perseae* is presented in Table 5 and Fig. 9.

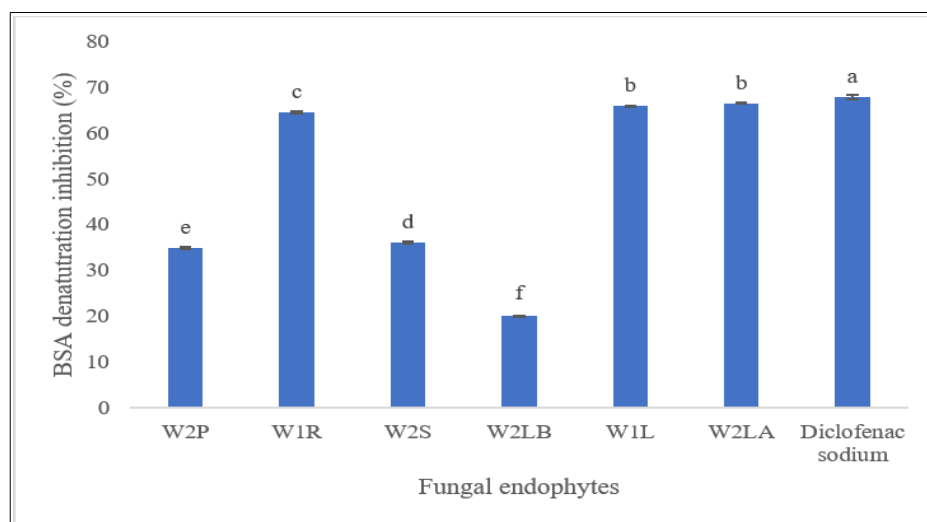


Fig. 3. Bovine serum albumin (BSA) denaturation assay.

Results are presented as mean $\pm$ SE ($n = 3$). Statistically significant differences ($p < 0.05$) are indicated by distinct letters. Degrees of freedom, between groups = 6 and within groups = 14.

Table 2. Morphological characteristics of the selected fungal endophytes

Fungal isolate	Colour	Form	Margin	Elevation	Texture
W1L	White	Filamentous	Filiform	Flat	Cottony
W2L B	White and grey	Filamentous	Filiform	Flat	Cottony

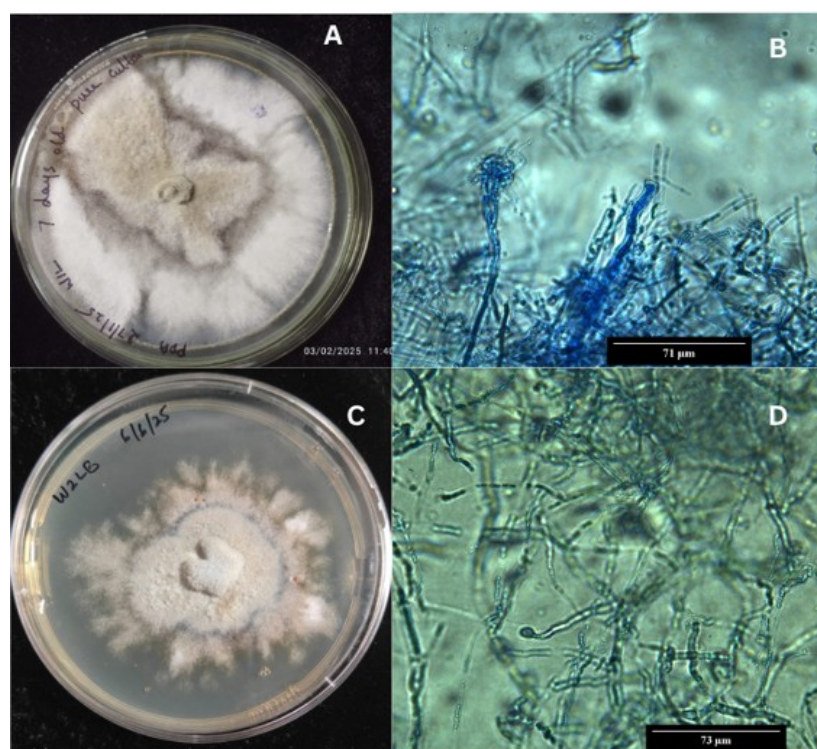
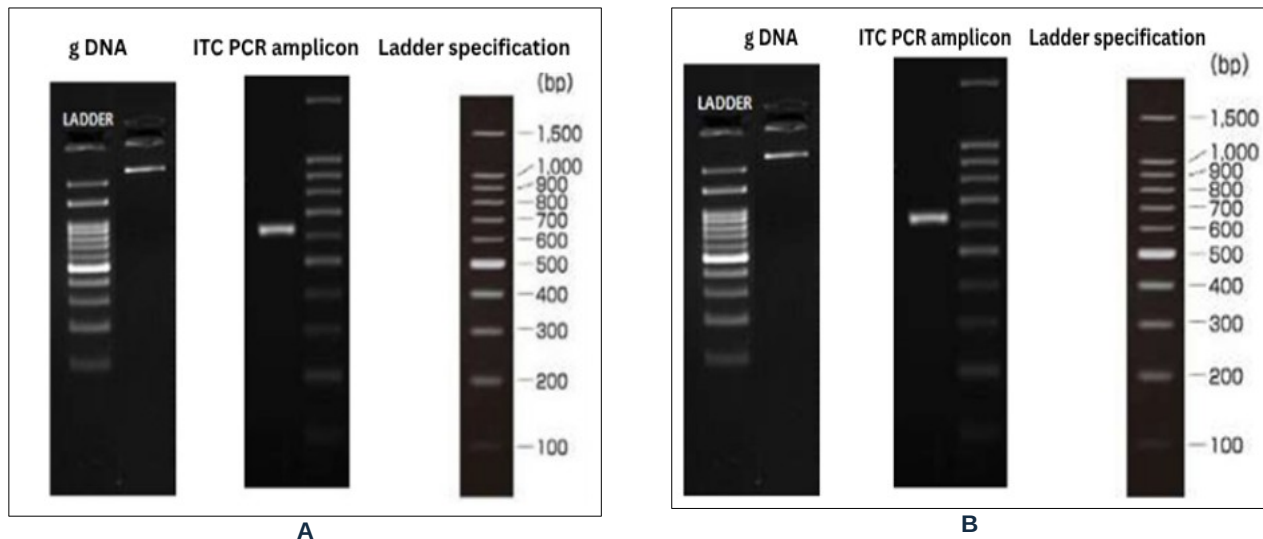


Fig. 4. Morphological characterisation of endophytic fungal isolates W1L and W2LB. (A) colony morphology of isolate W1L on potato dextrose agar; (B) microscopic appearance of isolate W1L stained with lactophenol cotton blue; (C) colony morphology of W2LB on potato dextrose agar; (D) microscopic appearance of isolate W2LB stained with lactophenol cotton blue.

Table 3. Basic local alignment search tool analysis of internal transcribed spacer rDNA sequences of the selected fungal endophytes

Sl. No.	Endophytic fungal code	Closest species	Sequence identity (%)	Accession number
1	W1L	<i>Daldinia korfii</i>	100.00	PV855807
2	W2LB	<i>Diaporthe perseae</i>	99.37	PV855818

**Fig. 5.** Electropherogram of the polymerase chain reaction amplified internal transcribed spacer rDNA gene region of the fungal isolates. (A) W1L; (B) W2LB.**>D. korfii**

TCCGTAGGTGAACCTGCGGAGGGATCATTACTGAGTTATCTAACTCCAACCCTATGTGAACTTACCGCCGTTGCCTCGGCGGG
 CCGCGTTTCGCCCTGTAGTTTACTACCTGGCGGGCGCGCTACAGGCCCGCCGGTGGACTGCTAACTCTGTTATATATACGTATCT
 CTGAATGCTTCAACTTAATAAGTTAAAACCTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT
 AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCATTAGTATTCTAGTGGGCATGCCTGT
 TCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGTCTCCAGGGCCCTAGTTCCTCCAAAGTCATCGG
 CGGAGTCGGAGCGTACTCTCAGCGTAGTAATACCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAACCCCTATATC
 TTTAGTGGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACCTTAAGCATATCAATAAGCGGAGGA

Fig. 6. Consensus sequence of the W1L fungal isolate.**Table 4.** Sequences producing significant alignments with W1L

Description	Max score	Total score	Query cover (%)	E-value	Percentage identity (%)	Accession number
<i>Daldinia korfii</i> isolate 7_SD310_3_572bp	1057	1057	100	0.00	100	MK209004.1
<i>Daldinia</i> sp. isolate hlb31	1057	1057	100	0.00	100	MK311339.1
Fungal isolate designated as MEFX01	1057	1057	100	0.00	100	OP508398.1
<i>Daldinia korfii</i> isolate LYN-UPM S10	1051	1051	100	0.00	99.83	MW157269.1
<i>Daldinia eschscholtzii</i> strain 3P0	1051	1051	100	0.00	99.83	OP237139.1
<i>Daldinia eschscholtzii</i> strain C202N	1051	1051	100	0.00	99.83	OP237400.1
<i>Daldinia eschscholtzii</i> isolate PPCF1	1051	1051	100	0.00	99.83	MW898677.1
<i>Nodulisporium verrucosum</i> isolate DHR AC2R2C	1051	1051	100	0.00	99.83	OR826642.1
<i>Daldinia eschscholtzii</i> isolate CFL7	1051	1051	100	0.00	99.83	MN180849.1
<i>Daldinia</i> sp. isolate hlb17	1051	1051	100	0.00	99.83	MK311338.1

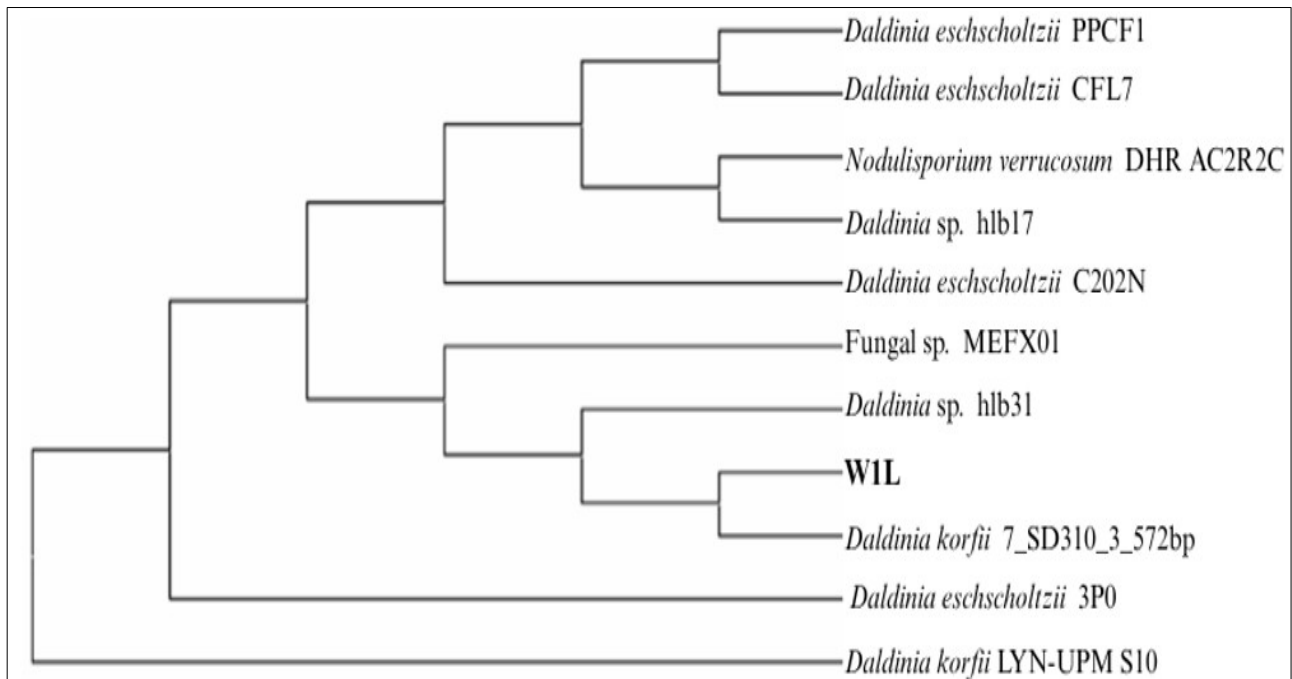


Fig. 7. The phylogenetic tree represents the evolutionary relationship of fungal isolate W1L with its closest related species.

> *D. perseae*

CTGGAGTTTACTACCTGGCGTGTGCGCTACAGGCCCGCCGGTGGACTGCTAAACTCTGTTATATATACGTATCTCTGAATGCTT
CAACTTAATAAGTTAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCGTGTCGAGCGTCA
TTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGTCTCCAGGGCCTAGTTCGCCAAAGTCATCGGCGGAGTCGGA
GCGTACTCTCAGCGTAGTAATACCATTCTCGCTTTTGCAGTAGCCCCGCGGCTTGCCGTAAAACCCCTATATCTTTAGTGGTTG
ACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA TAAGCGGAGGAA

Fig. 8. Consensus sequence of the W2LB fungal isolate.

Table 5. Sequences producing significant alignments with W2LB

Description	Max score	Total score	Query cover (%)	E-value	Percentage identity (%)	Accession number
<i>Diaporthe perseae</i> isolate HNNUZCJ-084	867	867	100	0.00	99.37	OM281084.1
<i>Daldinia eschscholtzii</i> isolate Manjari Medika (MM4)	867	867	100	0.00	99.37	OM265282.1
<i>Daldinia eschscholtzii</i> isolate 1-1-1-1	867	867	100	0.00	99.37	MF579574.1
<i>Daldinia eschscholtzii</i> voucher MEL:2382728	867	867	100	0.00	99.37	KP012909.1
<i>Daldinia eschscholtzii</i> strain C14	867	867	100	0.00	99.37	JX178946.1
<i>Daldinia</i> sp. strain HD-3	867	867	100	0.00	99.37	PQ249056.1
<i>Daldinia eschscholtzii</i> isolate HNNUZCJ-083	867	867	100	0.00	99.37	OM281083.1
<i>Daldinia eschscholtzii</i> isolate TGM23	867	867	100	0.00	99.37	OQ683794.1
<i>Sporothrix</i> sp. strain G18	867	867	100	0.00	99.37	MT729849.1
<i>Daldinia eschscholtzii</i> isolate DS43	867	867	100	0.00	99.37	KY432343.1

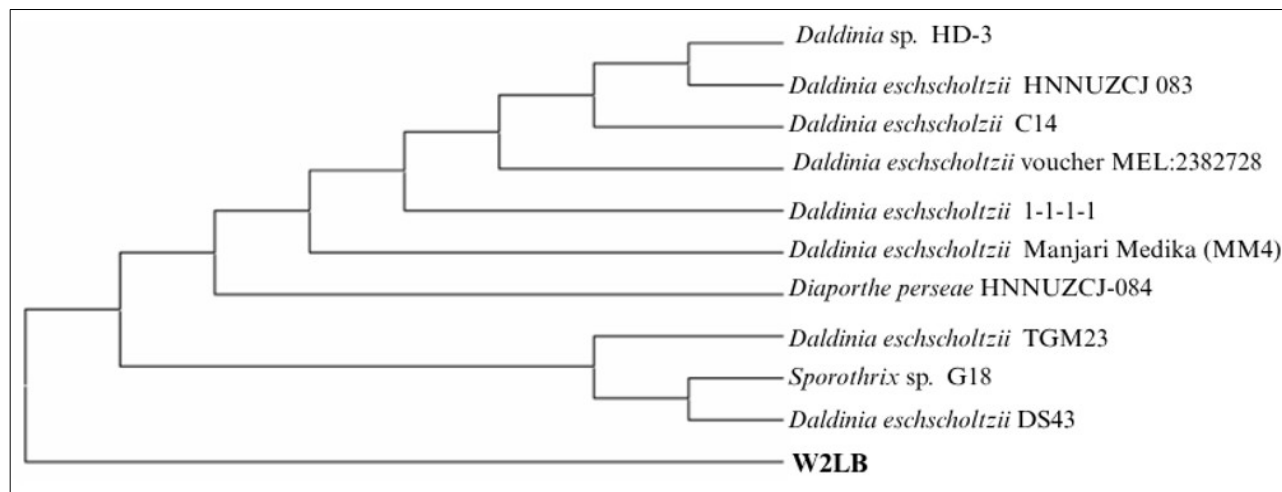


Fig. 9. The phylogenetic tree represents the evolutionary relationship of fungal isolate W2LB with its closest related species.

Identification of bioactive compounds in fungal isolates by liquid chromatography-mass spectrometry analysis

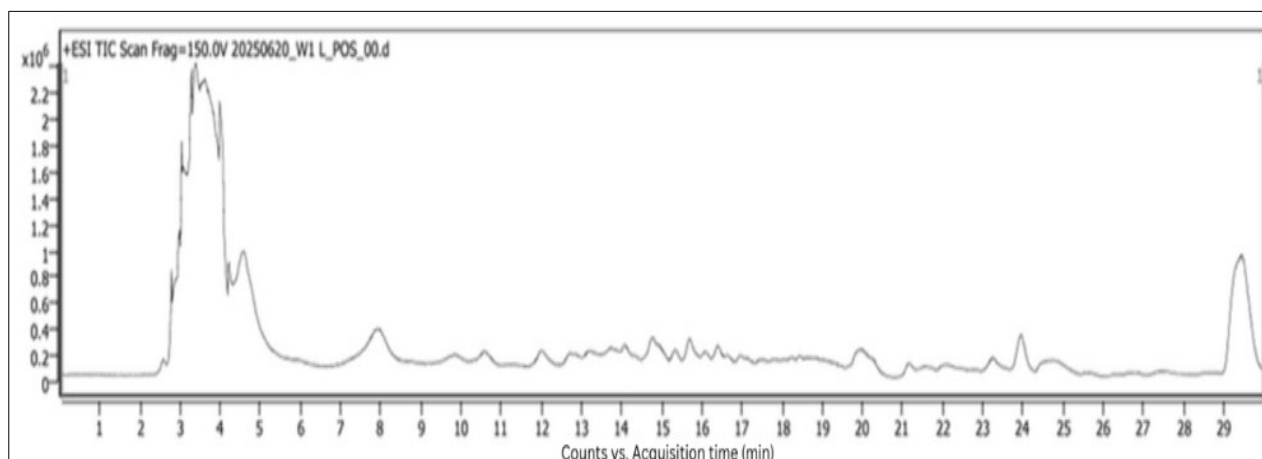
Liquid chromatography-mass spectrometry analysis of W1L and W2LB led to the tentative identification of multiple compounds, as illustrated by the chromatograms A and B (Fig. 10). The analysis revealed an extensive range of chemical categories, encompassing phenolic compounds, alkaloids, terpenoids, aromatic derivatives, organic acids, amino acid derivatives, lipid derivatives and esters.

From the extract of W1L, 14 compounds were identified, as shown in Table 6. Notable compounds include 6-deoxyfagomine which has reported to alleviate endogenous metabolic disorders (33, 34).

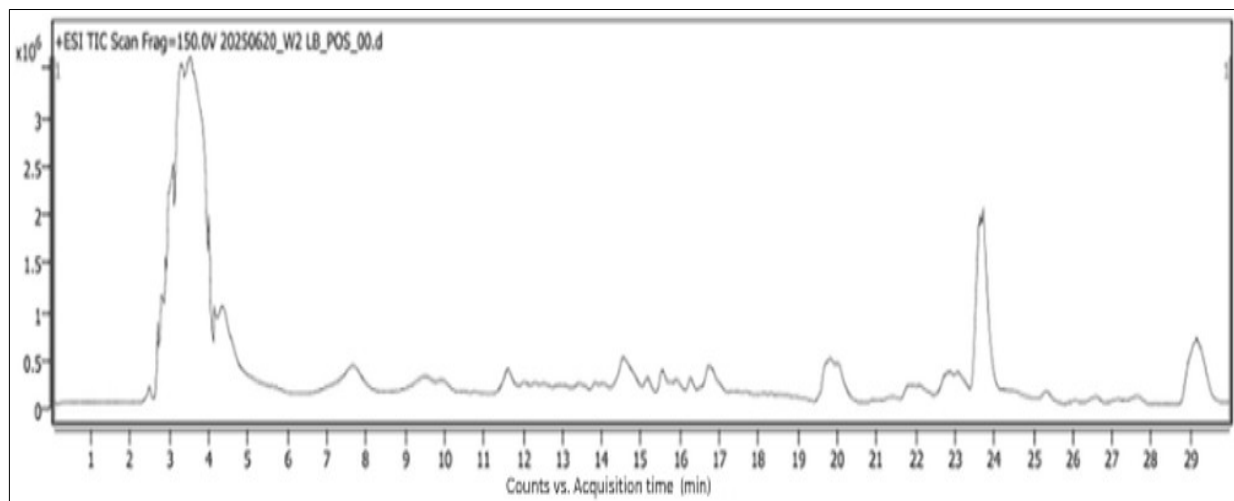
Norvaline, one of the compounds identified, has the capacity to suppress arginase activity. Being a neuroprotective compound, norvaline could be potentially used as a therapeutic intervention for neurodegenerative diseases, such as Alzheimer's (35). Norvaline has also been reported to lower blood pressure in experimental models (36). Similarly,

lentiginosine, an alkaloid identified in this extract, has been previously reported as an amyloglucosidase inhibitor, while 4-hydroxyacetophenone, paeonol and kynurenic acid have been associated with anti-inflammatory potential (37–40). Notably, norvaline and kynurenic acid have previously been reported from fungal endophytes lending support to their fungal origin in the present extract (41, 42).

Analysis of the W2LB extract revealed 18 compounds (Table 7). Among the metabolites identified in our endophytic extracts, hydroxyisocaproic acid and 3-hydroxydodecanedioic acid belong to chemical classes with reported biological activities. A fungal endophyte-derived metabolite, 2-hydroxyisocaproic acid has been associated with anti-inflammatory and 3-hydroxydodecanedioic acid has been reported to induce an antitumour immune response (43–45). Lachnophyllum ester, common to both W1L and W2LB, has been reported to possess antioxidant activity in addition to antifungal effects and was previously isolated from the medicinal plant, *Baccharis trinervis* (Lam.) Pers. (46).



A



B

Fig. 10. Liquid chromatography-mass spectrometry chromatograms of the fungal isolates. (A) W1L; (B) W2LB.

Table 6. Compounds identified in liquid chromatography-mass spectrometry analysis of W1L

Sl. No.	Compound	Chemical class	Retention time (min)	Molecular formula	Molecular weight (g/mol)
1	Norvaline	α -Amino acid	3.106	C ₅ H ₁₁ NO ₂	117.15
2	1,4-Dideoxy-1,4-imino-D-ribitol	Pyrrolidine alkaloid	3.426	C ₅ H ₁₁ NO ₃	133.15
3	Nicotinic acid	Pyridine monocarboxylic acid	4.388	C ₆ H ₅ NO ₂	123.11
4	6-Deoxyfagomine	Piperidine alkaloid	4.693	C ₆ H ₁₃ NO ₂	131.17
5	4-Hydroxyacetophenone	Phenolic compound	4.784	C ₈ H ₈ O ₂	136.15
6	Lentiginosine	Indolizidine alkaloid	5.075	C ₈ H ₁₅ NO ₂	157.21
7	Paeonol	Phenolic compound	7.891	C ₉ H ₁₀ O ₃	166.17
8	Tiglic acid	Monocarboxylic acid	9.829	C ₅ H ₈ O ₂	100.12
9	Kynurenic acid	Quinoline carboxylic acid	12.679	C ₁₀ H ₇ NO ₃	189.17
10	Huperzine B	Alkaloid	13.656	C ₁₆ H ₂₀ N ₂ O	256.34
11	Lachnophyllum ester	Fatty acid ester	14.815	C ₁₁ H ₁₂ O ₂	176.21
12	Lauric acid	Fatty acid	15.357	C ₁₂ H ₂₄ O ₂	200.32
13	3-Hydroxy- β -ionone	Norisoprenoid	24.827	C ₁₃ H ₂₀ O ₂	208.30
14	Myrtenal	Monoterpenoid	29.417	C ₁₀ H ₁₄ O	150.22

Table 7. Compounds identified in liquid chromatography-mass spectrometry analysis of W2LB

Sl. No.	Compound	Chemical class	Retention time (min)	Molecular formula	Molecular weight (g/mol)
1	Norvaline	α -Amino acid	3.509	C ₅ H ₁₁ NO ₂	117.15
2	Hydroxyisocaproic acid	Hydroxy carboxylic acid	4.468	C ₆ H ₁₂ O ₃	132.16
3	Cerulenin	Epoxydodecadienamide	4.539	C ₁₂ H ₁₇ NO ₃	223.27
4	4-Hydroxyacetophenone	Phenolic compound	4.583	C ₈ H ₈ O ₂	136.15
5	Anthranilic acid	Aminobenzoic acid	5.768	C ₇ H ₇ NO ₂	137.14
6	3-hydroxydodecanedioic acid	Hydroxy carboxylic acid	12.049	C ₁₂ H ₂₂ O ₅	246.30
7	Tiglic acid	Monocarboxylic acid	12.784	C ₅ H ₈ O ₂	100.12
8	Pilosine	Alkaloid	13.250	C ₁₆ H ₁₈ N ₂ O ₃	286.33
9	3-butyldiene-7-hydroxyphthalide	Phthalide	14.242	C ₁₂ H ₁₂ O ₃	204.22
10	Huperzine B	Alkaloid	14.330	C ₁₆ H ₂₀ N ₂ O	256.34
11	Lachnophyllum ester	Fatty acid ester	14.533	C ₁₁ H ₁₂ O ₂	176.21
12	Lauric acid	Fatty acid	15.212	C ₁₂ H ₂₄ O ₂	200.32
13	Citronellyl β -sophoroside	Terpene glycoside	15.549	C ₂₂ H ₄₀ O ₁₁	480.5
14	(S)-isosclerone	Naphthalenone polyketide	15.854	C ₁₀ H ₁₀ O ₃	178.18
15	6-methoxymellein	Dihydroisocoumarin	16.742	C ₁₁ H ₁₂ O ₄	208.21
16	LysoPE(0:0/18:1(11Z))	Glycerophospholipid	20.090	C ₂₃ H ₄₆ NO ₇ P	479.6
17	Militarinone A	Pyridone alkaloid	23.013	C ₂₆ H ₃₇ NO ₆	459.6
18	Carvone	Monoterpene ketone	29.152	C ₁₀ H ₁₄ O	150.22

Identification of bioactive compounds in fungal isolates by gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry analysis was performed for the tentative identification of the volatile compounds present in the hexane extracts of W1L and W2LB, as illustrated by the chromatograms shown in Fig. 11A and B. Gas chromatography-mass spectrometry analysis of the hexane extract of the endophytic fungus revealed a predominance of long-chain alkanes. The compounds identified in W1L and W2LB are presented in Table 8 and 9 respectively. Notably, octadecane, heneicosane and eicosane, identified in the present study, have also been reported as constituents of hexane extracts from endophytic

fungus, *Aspergillus flavus* YRB2 (47). Eicosane has previously exhibited modest free radical scavenging activity, robust anti-inflammatory, analgesic and antipyretic activities (48, 49). In addition, tetracosane, one of the compounds identified has been reported to induce apoptosis in AGS gastric cancer cells (50). The W2LB additionally showed greater diversity in compounds, with 7,9-di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione, previously reported among bioactive metabolites of *Cannabis sativa* L. derived endophytic fungi with antidiabetic activity and hexadecane, 2,6,10,14-tetramethyl-, a natural diterpene previously reported from the leaf essential oil of *Terminalia catappa* L. (51, 52). The presence of these therapeutically significant compounds may account for the extract's biological activities.



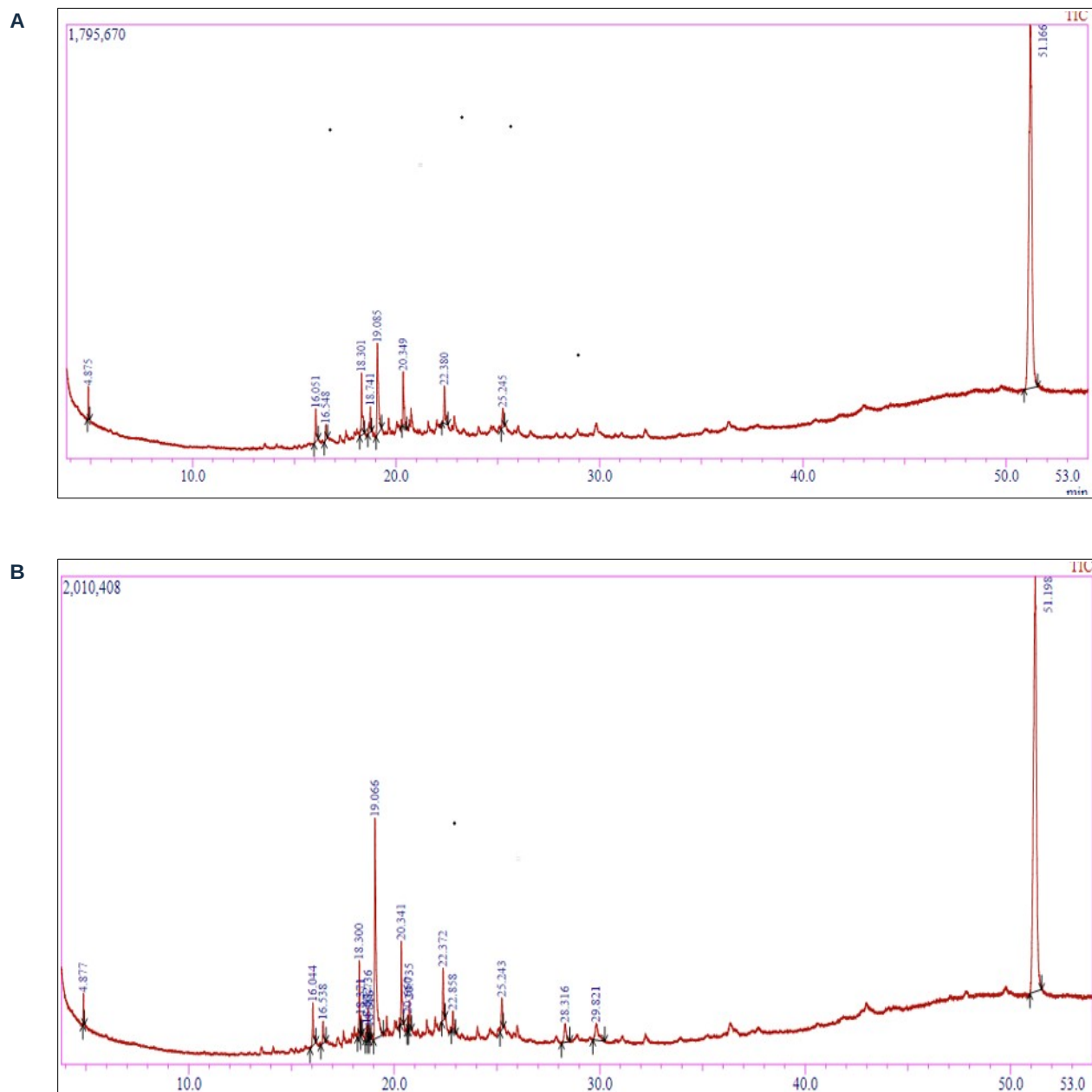


Fig. 11. Gas chromatography-mass spectrometry chromatograms of the fungal isolates. (A) W1L; (B) W2LB.

Table 8. Compounds identified in gas chromatography-mass spectrometry analysis of W1L

Sl. No.	Compound	Retention time (min)	Molecular formula	Molecular weight (g/mol)
1	Hexadecane	16.051	C ₁₆ H ₃₄	226.4
2	Hexadecane,2-methyl-	16.548	C ₁₇ H ₃₆	240.5
3	Octadecane	18.301	C ₁₈ H ₃₈	254.5
4	Nonadecane	18.741	C ₁₉ H ₄₀	268.5
5	Eicosane	20.349	C ₂₀ H ₄₂	282.5
6	Heneicosane	22.380	C ₂₁ H ₄₄	296.6
7	Tetracosane	25.245	C ₂₄ H ₅₀	338.7

Table 9. Compounds identified in gas chromatography-mass spectrometry analysis of W2LB

Sl. No.	Compound	Retention time (min)	Molecular formula	Molecular weight (g/mol)
1	Hexadecane	16.044	C ₁₆ H ₃₄	226.4
2	Heptadecane	16.538	C ₁₇ H ₃₆	240.5
3	Octadecane	18.300	C ₁₈ H ₃₈	254.5
4	Octadecane,2-methyl-	18.371	C ₁₉ H ₄₀	268.5
5	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	18.672	C ₁₇ H ₂₄ O ₃	276.4
6	Eicosane	20.341	C ₂₀ H ₄₂	282.5
7	Hexadecane, 2,6,10,14-tetramethyl-	20.735	C ₂₀ H ₄₂	282.5
8	Heneicosane	22.372	C ₂₁ H ₄₄	296.6
9	Tetracosane	22.858	C ₂₄ H ₅₀	338.7
10	Pentacosane	25.243	C ₂₅ H ₅₂	352.7
11	Nonacosane	29.821	C ₂₉ H ₆₀	408.8

4. Conclusion

This investigation highlights the therapeutic potential of endophytes of *Withania somnifera*. Two of the fungal isolates, *Daldinia korfii* and *Diaporthe perseae* displayed notable antioxidant activity, with the former also exhibiting promising antidiabetic and anti-inflammatory activities. Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) analyses revealed numerous tentatively identified bioactive metabolites belonging to diverse chemical classes. Several of the tentatively identified compounds have been previously reported to exhibit biological activity, underscoring the need for further exploration. Future research should aim to optimise fermentation parameters and employ elicitor-based strategies, to enhance the metabolite production. Subsequent bioassay-guided fractionation and isolation of bioactive compounds will facilitate the identification of the key therapeutic compounds. In addition, exploring synergistic interactions between host-derived and endophyte-derived metabolites may provide further insights into their combined therapeutic potential. Although this study identifies promising medicinal compounds from crude extracts of the endophytes, *D. korfii* and *D. perseae*, further purification, dose-response studies and *in vivo* validation are necessary before any pharmacological or translational claims can be made.

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Authors' contributions

Conceptualisation was carried out by JSA and PN. Methodology, as well as formal analysis and investigation, were performed by JSA. The original draft of the manuscript was prepared by JSA, while review and editing were conducted by PN. Supervision was provided by PN. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest

Authors do not have any conflict of interests to declare.

Ethical issues

None

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