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# $^{1}H+^{13}C$ NMR and mass spectrometric elucidation of *Dalbergia sissoo* leaf derived mycoendophyte, *Aspergillus tubingensis*and its antifungal evaluation

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*Aspergillus tubingensis*, a mycoendophyte isolated for the first time from *Dalbergia sissoo*, has been identified and described on the basis of macro and micro-morphological features and ITS sequence based molecular phylogenetic analysis. The fungal endophyte shows antifungal potential against *Rhizoctonia solani*and *Fusarium oxysporum*, investigated though a range of assays, such as dual culturing, food poisoning technique, disc diffusion assay, and broth dilution method. $^{1}H+^{13}C$ NMR and chromatographic profiling (GC-MS/MS and LC-MS) of the crude extract has also been done for their compound assessment. The study highlights the significance of the fungal endophytein encompassingnumerous bioactive metabolites that possess antifungal applications.

**Keywords :** Fungal endophyte,GC-MS/MS,LC-MS,metabolites,NMR, pathogens, phylogeny

## INTRODUCTION

Mycoendophytes are known to be ubiquitous, occurring in almost all plants, grasses,trees,and algae (Hyde and Soytong 2008). Plants harbouring endophytes are considered to be resistant and sturdy as they are supposed to be involved in distinct mechanisms (Busby *et al.* 2016; Rachel *et al.* 2022). Endophytes possess the capability to synthesize various bioactive compounds (Abdul-Razek *et al.* 2020; Falade *et al.* 2021) including secondary metabolites such asalkaloids, phenolics, lignans, quinones, flavonoids, peptides, steroids, and isocoumarins (Khalil *et al.* 2021) that are known to possess antimicrobial, anti-cancerous, and antimalarial activities (Dramae*et al.* 2022), beneficial for the pharmaceutical industry (Tiwari and Bae, 2022). Genus *Aspergillus*, specifically the black aspergilla (*A. tubingensis* and *A. niger*), is well known to adapt in extreme environments (Miada*et al.* 2018), as they are abundantly present, resist varied pH ranges, grow rapidly (Nielsen *et al.* 2009) and are utilized as a preferable option in the fermentation industry for the production of enzymes and secondary metabolites (dos Santos *et al.* 2016).The morphology based identification of the species is usually supported with the molecular data, chemotaxonomy, metabolite profiling, and the chromatographic techniques (Xanthopoulou *et al.* 2019) because of the ambiguous phenotypic traits that may lead tothe uncertainty in their phylogeny (Nielsen *et al.* 2009).

Metabolites extracted from the microbial originare highly beneficial from medicinal and agricultural point of view(Cao *et al.* 2019). Recent report indicated *A. tubingensis*as a good biocontrol agent against pathogens including, *Botrytis cinerea* (Zhao *et al.* 2018), *Alternaria alternata*, *A.brassicae*, *Colletotrichum lagenarium*, *Fusarium oxysporum*, *Gaeumannomy-cesgraminis, Penicillium digitatum*,*Valsamali*(Xu *et al.* 2020), and *Rhizoctoniasolani* (Kriaa*et al.* 2015).Moreover, biopesticides have gained much consideration in the recent decades as an effective alternative in place of conventional synthetic pesticides in order to manage plant diseases (Qin *et al.* 2021).

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Only a few of endophytic fungi isolated from the medicinal plants have been evaluated and even these have not been muchexploredfor their bioactive metabolites (Uzma *et al.*2019).Though *A. tubingensis* has been reported as an endophyte from various plants species (Huang *et al.* 2010; Puri *et al.* 2021; Mohamed*et al.* 2022), yet *Dalbergia* has not been explored in this regard till now. Hence, the current study was conducted with an aim of evaluationof the antifungal potential (against *R. solani* and *F. oxysporum*)of*A. tubingensis*isolated from *D. sissoo* along with the detection of itsbioactive compounds using $^{1}H+^{13}C$ NMR and mass spectrometrictechniques (GC–MS/MS, LC–MS/MS).

## MATERIALS AND METHODS

### *Leaf tissue*

Asymptomatic leaf samples of *Dalbergia sissoo* were gathered from Bir Gurdialpura Wildlife Sanctuary (76°10'E and 76°15'E longitude and 30°00'N latitude) during the summer season i.e. July, 2025. Until its further processing, the leaf samples were shifted to the laboratory packed in a polythene bag, kept under deep freeze condition and analyzed within 24 hour.

### *Isolation of the fungal endophyte and purification*

In order to get rid of the epiphytic and phylloplane microflora, the healthy *D. sissoo* leaves underwent surface sterilization following Larran *et al.*(2007). Leaves were initially immersed in 70% ethanol for 60 seconds, and then soaked in 3% sodium hypochlorite (NaOCl) solution for about 120 seconds, repeatedly washed in 70% ethanol for 120 seconds again and finally washed in sterile water. The air dried tissues were given an incision of 10mm and were transferred to PDA medium. Leaf sample without any kind of disinfectant treatment was marked as a negative control whereas the tissue following chemical treatment without any cut served as a positive control for the study. The agar plates were incubated at 28ºC±2ºC for about 9 days in the dark conditions. Upon the daily observation, the mycelium out of the excised portion of the leaf tissue was cultured onto the PDA/MEA/YEA medium and subsequently sub-cultured for purification.

### *Macro and microscopic observations*

Evaluation pertaining to the colony shape, colour, margins, growth patterns, and the other morphological traits were noted. The colour code was matched with the references given by Korenup and Wanscher (1978). The microscopic details were captured through the Olympus compound microscope, Leica light microscope with equipped cameras for photomicrographs, and SEM for detailed and high resolution images. The final description of the endophyte was compared with the literature (Subramanian,1962; Ellis 1976) and the mycobank for identification of the species. The isolate was deposited at MTCC-IMTECH, Chandigarh with an accession, MTCC 13765.

### *Identification of the genotype*
### *DNA extraction*

CTAB method was employed for the isolation of genomic DNA of the endophytic isolate (Doyle and Doyle, 1990). Approximately, 0.1 g of the pure colony culture was put in a mortar and crushed into powder with 1 ml of the extraction buffer after which the homogenate was placed to 1 mlmicrofuge tube. An eqimolar of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was poured to the microfuge tubes and homogenized by stirring the tubes. Centrifugation at 15,000 rpm for about 15 min was done. The supernatant was gathered in a separate tube and an equi-volume of Chloroform: Isoamyl alcohol (24:1) was poured to the mixture. After that, the DNA was coagulated from the mixture by supplementation of 0.1 µl of 3M sodium acetate pH 7.0 and 0.7 µl of Isopropanol. Again the tubes were centrifuged at 4ºCfollowingthe wash in 90% ethanol prior to air drying. The DNA was immersed and hence dissolved into TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). At last, 5 µl of DNAse free RNAse A (10 mg/ml) was supplemented with the tube in order to remove the RNA.

### *Amplification of the genomic DNA*

1% agrose gel electrophoresis was run to check the quality of the extracted DNA. The desired gene

was amplified by using the purified DNA as a template for PCR reaction.ITS1 (TCCGTAG-GTGAACCTGCGC);ITS4 (TCCTCCGCTT-ATTGATATGC (White *et al.* 1990) primers were used for amplifying the desired genomic DNA. PCR including 1ìl of template the DNA was amplified in 20μl reaction mixinvolving 10X buffer, each deoxynucleotide triphosphate at 0.2 mM, primer at 0.5 μM, and 2 U of the Taq polymerase (R001C TaKaRa Taq™) per ml. PCR cycles included denaturation for 4 mins at 95°C initially, 37 cycles (denaturation, 30 s at 95°C; annealing, 30 s at 50°C; extension, 1 min at 72°C) and last one final extension cycle at 75°C for 12 min.Twenty microliters of the reaction mixtures was analysed onto the 1.0% agarose/Tris-Cl-sodium acetate-EDTA in the presence of 0.5 ìg of EtBr per ml and photography was done under UV illumination. The positive amplicons were then purified by utilizing FavorPrep™ GEL/PCR Purification Kit (Cat No. FAGCK 001).

***Sequencing and molecular phylogenetics***

Sanger's dideoxy method was employed for sequencing the amplified product. The sequences were finally submitted to the NCBI GenBank with the accession no. PX930888, designated as TJED16 and subsequently the BLAST analysis was done. The phylogenetic tree was generated through maximum likelihood analysis using MegaXI software supported by the bootstrap values for enhanced analysis and reliability.

***Crude extract preparation***

5 mm diameter mycelial plugs cultured on PDA were suspended into 1 litre Erlenmeyer flask containing the potato dextrose broth medium, incubated at 28±2ºC for 10 days at 160 rpm in shaking incubator. Liquid broth was filtered through the Whatman filter paper 1, obtaining the mycelial cultures. Consequently, the resulting culture filtrate was extracted twice with 100 mL of methanolic solvent. The obtained extracts were made to concentrate under the vacuum pressure at 45ºC through rota-evaporator. Finally 10mg/mL of the fungal culture extract was prepared for further experimentations (Yehia *et al.* 2020).

***In vitro antifungal assay***

Two fungal phytopathogens, *Rhizoctoniasolani* (MTCC no. 4633) and *Fusarium oxysporum* (MTCC no. 284) were procured from CSIR IMTECH, Chandigarh, India, against which the antifungal activity of the isolated endophyte was assessed.

***Dual culture***

The mycoendophyte and the pathogen were allowed to grow in the same Petri plate, placed 6cm apart upon the PDA at 28±2ºC for atleast 7 days (Luo *et al.* 2015, Liu *et al.* 2025). Width of their respection inhibition zones were measured in mm. The results were evaluated using the formula:

Inhibition rate (%) = 100 - (a/b × 100)

where, a - Colony growth of pathogen with the mycoendophyte, and b - Colony growth of pathogen without the mycoendophyte.

***Food poisoning technique***

According to the protocol outlined by Balouiri*et al.*(2016) and Luo *et al.*(2015), the food poisoning assay was laid down. PDB medium was supplemented with the mycelial plugs of the endophyte measuring 5mm each into the 100 ml flask at 28±2ºC and 220 rpm for 10 days.Then centrifugation at 3000rpm was done for 20 minutes to collect the supernatant and the pellet containing the hyphal mass was discarded. In order to conduct the antifungal analysis, the Petri plates with the solidified PDA medium containing 1ml of the endophytic cultural extract was prepared, onto which each of the pathogen was centrally placed. The decline in the growth of the pathogens was a measure to evaluate the antifungal potential of the endophytic extract when compared to the control colony, which was devoid of the endophytic extract.Inhibition of the pathogenic mycelial growth on the treatment plates as compared to the control was assessed using the formula:

Percentage of mycelial growth inhibition = (dc-dt)/dc × 100,

where dc = the average diameter (in mm) of fungal colonies in the control, dt = the average diameter (in mm) of fungal colonies in the treatment groups.

***Disc diffusion assay***

The prepared fungal endophytic extract was extracted and then filtered thrice with 100 ml of aqueous, acetone, and the methanolic solvents. Rotatory vacuum evaporator was used to concentrate the extracts at 45ºC to obtain the resultant concentrations of 25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml which were dissolved in 1 ml of 50% DMSO (kept as a negative control). The discs were flooded with these concentrations onto the agar plate, before which the pathogenic inoculum was spread.Fluconazole (2mg/ml) Pfizer Pharmaceuticals, India served as the positive control. 50µg disks weremadewith 25µl of fluconazole solution (Kirkpatrick *et al.* 1998).

***Broth dilution method***

MIC was evaluated by the broth dilution method following Kalidindi *et al.*(2015) and Wiegand *et al.*(2008). The pathogenic cultural filtrate was initially inoculated into the potato dextrose broth medium of approximately 2ml. To this, the methanolic concentrations (25, 50, 75, 100, 125, 150 and 175 mg/mL) of the mycoendophyte was added each in separate test tubes for about 72 hours at 28±2ºC under dark and repeated thrice to validate the accuracy. To measure the pathogenic inhibition, 10mg/ml of fluconazole was kept as a positive control and the test tube without the pathogenic filtrate was kept as a negative control. The absorbance of each test tube containing different concentrations was measured at 625nm and finally compared with the controls.

***$^{1}$H+$^{13}$C NMR***

Solvent used for analysis was deuterated chloroform ($CDCl_3$). Analytical techniques employed were $^{1}$H NMR and $^{13}$C NMR at the frequency of 400MHz and 100MHz respectively. The spectra were recorded under the standard conditions using Bruker instrumentation.

***GC-MS/MS analysis***

Gas Chromatography with tandem mass spectrometric analysis of the mycoendophyte was done with Rxi-5 capillary column measuring 30 m x 0.25 mm ID x 0.25 mm, which was interfaced with SSQ 7000 quadrupole spectrometer. The oven temperature was set initially at 45 ºC (3 min hold) to 280 ºC at a rate of 5ºC/min with a final hold of 20 min (total rum time: 60 min), and the injector temperature along with the MS transfer line too at 280 ºC. The methanolic extract of the endophyte was injected as 1:15 in a split mode. Helium was used as a carrier gas at the rate of 1 ml/min. Other conditions for the spectrometric analysis include 60 mA filament emission current, 70 eV ionization, along with 200 ºC ion source. The compounds were identified based upon their fragmentation patterns, compared with the NIST library.

***LC-MS***

The endophytic methanolic extracts were centrifuged at 15000 rpm for 20 minutes. The setup consisted of 2 pump systems and 1 injector which were automated in an instrument named QTRAP 4500. Chromatographic technique employed a C-18 column (Agilent Eclipse, 5µm, 15 cm, 4.6 mm i.d.). The mobile phases included two solvents 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B), with the constant flow rate of 500µL/min. The LC conditions in the gradientinvolved 5% B for 12 minutes, increased gradually upto about 65% at time period of 25 minutes, then to 90% at time of 20 minutes, and then back to 5% between the time 20 to 25 minutes. A UPLC-TQD mass spectrometer was included in the studyas positive ion mode for MS analysis, with automatic transitionsamong MS and MS/MS in a data-dependent manner. Other working conditions included the gas temperature of 220 ºC, gas flow at the rate of about 5 Liter/minute, nebulizer pressure at rate of 40 Pascal, and the capillary voltage of 2000V.

***Statistical analysis***

The averages of three replicates and the standard errors wereassessed for their statistical

differences using ANOVA Turkey HSD post hoc with the SPSS software. The significant statistical differences were based upon the value of pd"0.05.The mean Zone of Inhibition (ZOI) expressed along with the standard error (±) with respect to *R. solani* and*F. oxysporum*in the disc diffusion assay and their significance level has been presented. Values with different superscript letters differ statistically (a, b, c, d). The control, Fluconazole concentration is kept 2 mg/mL. Mean values represented by the same letters (a, b, c) are significantly same.

## RESULTS AND DISCUSSION

### *Phylogenetic analysis and morphological description*

Colony appeared liver brown (8F6) to almost black in colour when cultured on Potato dextrose agar, rapid growth, sporulation within 2 – 3 days of incubation at 26±2ºC in darkness, superficial powdered growth, compact. Conidia observed were globose to sub-globose, smooth as well as echinulated surface, longitudinal striatios seen in some, hyaline, light brown to dark brown coloured, measuring 2.8 – 4.2 µm in diameter (Fig. 1).

The phylogenetic tree constructed by the Neighbor joining (NJ) analysis through Mega XI software of the ITS sequences distinctly resolves the evolutionary tendencies with respect to *Aspergillustubingensis* TJED16 (PX930888), with *Alternaria alternata* considered as outgroup (Fig. 2). Alignment of the isolated species with their closely related thirteen sets of the NCBI sequence data, validate its genomic distinctness, thus ensuring the identification, and the branch support was analyzed by bootstrap analysis with 1000 replicates.DNA based molecular identification of the species has been put forward to eliminate ambiguity (Xanthopoulou *et al.* 2019).

### *Antifungal activity*

Variousreports on fungal endophytes have suggested heir significant antimicrobial potential due to the presence of different classes of bioactive compounds (Rao *et al.* 2015).The endophytic isolate in the present investigation strongly inhibited (80.36% and 85%) two plant pathogenic fungi i.e.*Rhizoctonia solani*(Fig. 3c) and *Fusarium oxysporum*(Fig. 3d) in dual culture assay. In the food poisoning assay, the endophytic cultural filtrate against *R. solani* and *F. oxysporum* has been represented in Fig. 3e & 3f, in comparison to their control colonies depicted in Fig. 3a, 3b. In this technique it was observed that the count of sclerotial bodies dropped significantly upon addition of 1ml culture extract of the endophyte into the agar media in case of *R. solani*, whereas in case of *F. oxysporum* the inhibition of the mycelial growth was noted upto 56.25%. In the disc diffusion assay, methanol, acetone, and aqueous extracts with varying concentrations (25 mg/mL, 50 mg/mL, and 75 mg/mL)wereevaluated against *R. solani*(Fig.4a) and *F. oxysporum*and their zone of inhibition (ZOI)was assessed( Fig. 4 a & b).The maximum ZOI was observed in positive control i.e. fluconazole (16±0.2887 mm) and no inhibition zone around the negative control DMSO. The mean zone of inhibition was calculated in methanolic, acetone, and aqueous extract with increasing concentrations (25 mg/mL, 50 mg/mL, and 75 mg/mL). Various alterations in the extracts and the concentrations influenced the response.Among the extracts, the highest ZOI against *R. solani* was observed in methanolic extract (1.116±0.016) at 75 mg/mL concentration and lowest in acetone extract (0.606±0.006) at 50 mg/mL concentration. However, it was maximum in case of methanolic extract (1.483±0.016) at 75 mg/mL concentration and minimum in acetone extract (0.616±0.033) at 25 mg/mL concentration in case of *F. oxysporum*. The Minimum Inhibitory Concentration of the mycoendophyte was found to be 50 mg/mL (Table 1).

*Aspergillus* species has emerged as an exceptional fungus in terms of producing bioactive compounds (Frisvad 2015), a mycoendophyte possessing an abundant origin of natural products (Hagag *et al.* 2022) especiallythe antifungal metabolites (Kamel *et al.* 2020, Klausmeyer *et al.* 2005).Further, *A. tubingensis* has been known to possess certain biosynthetic and biological activities including antioxidant (Carboué*et al.* 2019), antibacterial (Rodrigues *et al.* 2021), and anticancer (Lin *et al.* 2022). Though *A. tubingensis* has been earlier reported as an

**Table 1:** Effect of different extracts of mycoendophyte against mycelial growth of *R.solani* and *F.oxysporum*in petri plates

| Treatment | 25 mg/mL | 50 mg/mL | 75 mg/mL |
|---|---|---|---|
| Flu (+control)- RS | $15.833^{a}$±0.441, | $15.833^{a}$±0.1667, $15.833^{a}$±0.441 | $15.66^{a}$±0.333, $16^{a}$±0.2887 |
| Flu (+control)-RS | $15.833^{a}$±0.1667 | | |
| ME- RS | $0^{b}$, | $0.733^{b}$±0.034, $1.333^{b}$±0.033 | $1.116^{b}$±0.016, $1.483^{b}$±0.016 |
| ME- FO | $1.066^{b}$±0.033 | | |
| AE-RS | $0^{b}$, | $0.606^{b}$±0.006, $0.783^{b}$±0.016 | $0.883^{b}$±0.016, $1.066^{b}$±0.033 |
| AE-FO | $0.616^{b}$±0.033 | | |
| AqE-RS | $0^{c}$, | $0.733^{c}$±0.016, $1.066^{c}$±0.033 | $0.866^{c}$±0.016, $1.216^{c}$±0.016 |
| AqE-FO | $0.883^{c}$±0.016 | | |
| DMSO(-control) | $0^{c}$ | $0^{d}$ | $0^{d}$ |

Mean Zone of Inhibition (ZOI) expressed with the standard error (±) pertaining to *R. solani*( RS) followed by *F. oxysporum* (FO)in the disc diffusion assay and the significance level (ANOVA, $p < 0.05$, Turkey HSD post hoc). Values in the respective columns with different superscript letters differ statistically (a, b, c, d). Flu: Fluconazole (2 mg/mL). Mean values followed by the same letters (a, b, c) are not significantly different.

**Table 2:** List of the compounds identified in the endophytic extract through GC-MS/MS.

| Chemical formula | Retention time (min) | Height | Area | Area % |
|---|---|---|---|---|
| $C_{16}H_{50}O_7Si_8$ | 10.081 | 161737 | 330840 | 47.24 |
| $C_{32}H_{66}O_5Si_4$ | 11.686 | 22233 | 43683 | 6.24 |
| $C_{42}H_{64}O_2$ | 40.504 | 43525 | 552921 | 78.95 |
| $C_{20}H_{24}Cl_3NO_3Si$ | 42.087 | 31236 | 26528 | 3.79 |
| $C_{14}H_{16}Cl_3NO_4Si$ | 42.265 | 147285 | 407236 | 58.15 |
| $C_{22}H_{31}NO_3Si$ | 43.757 | 38918 | 90279 | 12.89 |
| $C_{16}H_{15}N_3S$ | 44.539 | 34468 | 17852 | 2.55 |
| $C_{22}H_{33}NO_3Si_2$ | 45.045 | 80665 | 203340 | 29.04 |
| $C_{12}H_{38}O_5Si_6$ | 47.686 | 56041 | 23877 | 3.41 |
| $C_{15}H_{19}NO_4S_2$ | 48.429 | 43420 | 29811 | 4.26 |
| $C_{20}H_{25}NO_4$ | 48.776 | 63377 | 61164 | 8.73 |
| $C_{19}H_{20}BrNO_5$ | 50.201 | 71154 | 97841 | 13.97 |
| $C_{21}H_{26}BrNO_4$ | 54.844 | 94595 | 700310 | 100.00 |
| $C_{14}H_{25}NO_2Si_2$ | 55.590 | 152041 | 541355 | 77.30 |
| $C_{18}H_{20}N_2O_4$ | 55.963 | 54348 | 36039 | 5.15 |
| $C_{19}H_{27}N_3O_2Si_2$ | 60.078 | 54007 | 59957 | 8.56 |
| $C_{20}H_{24}Cl_3NO_3Si$ | 64.718 | 61114 | 56896 | 8.12 |
| $C_{18}H_{20}N_2O_4$ | 73.530 | 64935 | 42660 | 6.09 |

**Table 3 :** List of compounds found though LC-MS of the mycoendophyte.

| Compound Name | Retention time | Area | m/z ratio | Area % |
|---|---|---|---|---|
| Enrofloxacin-D5 | 2.83 | 63447025 | 365.3646 | 3.01 |
| Norfentanyl | 3.01 | 281026562 | 233.4444 | 13.32 |
| Fenpropidin | 3.12 | 131421912 | 274.5790 | 6.23 |
| Trihexyphenidyl | 3.20 | 198191249 | 302.7022 | 9.39 |
| Quinine | 3.64 | 93738809 | 325.4393 | 4.44 |
| Salvinorin B | 3.92 | 92579461 | 391.7007 | 4.39 |
| Ziprasidone | 4.20 | 269206117 | 413.5704 | 12.76 |
| Deepoxy-deoxynivalenol | 4.82 | 151260831 | 281.4000 | 7.17 |
| Tulathromycin | 4.89 | 72538057 | 577.7759 | 3.44 |
| Spinetoram B | 6.10 | 116722594 | 760.7639 | 5.53 |
| Vincamine | 6.44 | 137459449 | 355.5009 | 6.51 |
| Acebutolol | 6.59 | 210940908 | 337.5600 | 10 |
| Dimefuron | 7.24 | 235076358 | 339.6000 | 11.51 |
| Ajmaline | 7.54 | 68352325 | 327.6274 | 3.24 |
| Norclozapine | 7.81 | 244455312 | 313.5600 | 11.59 |

endophyte from variety of hosts (Huang *et al.* 2011, Ma *et al.* 2016, Meghnous*et al.* 2022, Nisa *et al.* 2020, Yu *et al.* 2021) and explored for its antifungal activity (Ramadan *et al.* 2023, Wu *et al.* 2022), yet its potential against *Fusarium oxysporum*and *Rhizoctonia solani* from *Dalbergia sissoo* has not been known till date. In order to bridge this research gap, the present study marks the pioneer report aiming towards the isolation of *A. tubingensis*, a mycoendophyteharboring within the leaves of *Dalbergia sissoo*, and evaluation of its antifungal potential against two fungal phytopathogens, *Fusarium oxysporum* and *Rhizoctonia solani*.

### *$^{1}$H+$^{13}$C NMR analysis*

The NMR analysis of the methanolic endophytic extract has been shown in Fig. 5. The $^{1}$H-NMR spectrum of the crude extract showed multiple resonances spanning from 0.8 to 5.5 ppm, characteristic of a complex mixture of fungal metabolites. Key H resonances were:ä 0.83 – 1.00 ppm suggested strong multiplets corresponding to terminal methyl (–CH*f* ) groups, indicating long aliphatic chains, ä 1.20 – 1.65 ppm represented broad and intense signals attributed to methylene (–CH, –) protons, typical of fatty acid or lipid-derived structures, ä 2.00 – 2.40 ppm depicted signals assigned to allylic and carbonyl-adjacent methylene protons, suggesting unsaturated lipid components, ä ~2.79 ppm indicated protons adjacent to heteroatoms or conjugated systems, ä 3.6 – 4.1 ppm signified weak signals attributable to oxygenated methylene/methine protons (–CH–O / –CH, –O),suggesting ester or alcohol functionalities, ä 5.35 – 5.40 ppm represented distinct olefinic proton signals confirming the presence of C=C unsaturation, above 6.5 ppm reported no prominent aromatic proton envelope observed, indicating minimal aromatic or phenolic content.

The $^{13}$C-NMR spectrum further supported the lipid-rich nature of the extract. Key carbon resonances: ä 14.0 – 14.1 ppm suggested terminal methyl carbons (–CH*f* ) of aliphatic chains, ä 22.5 – 34.0 ppm represented multiple

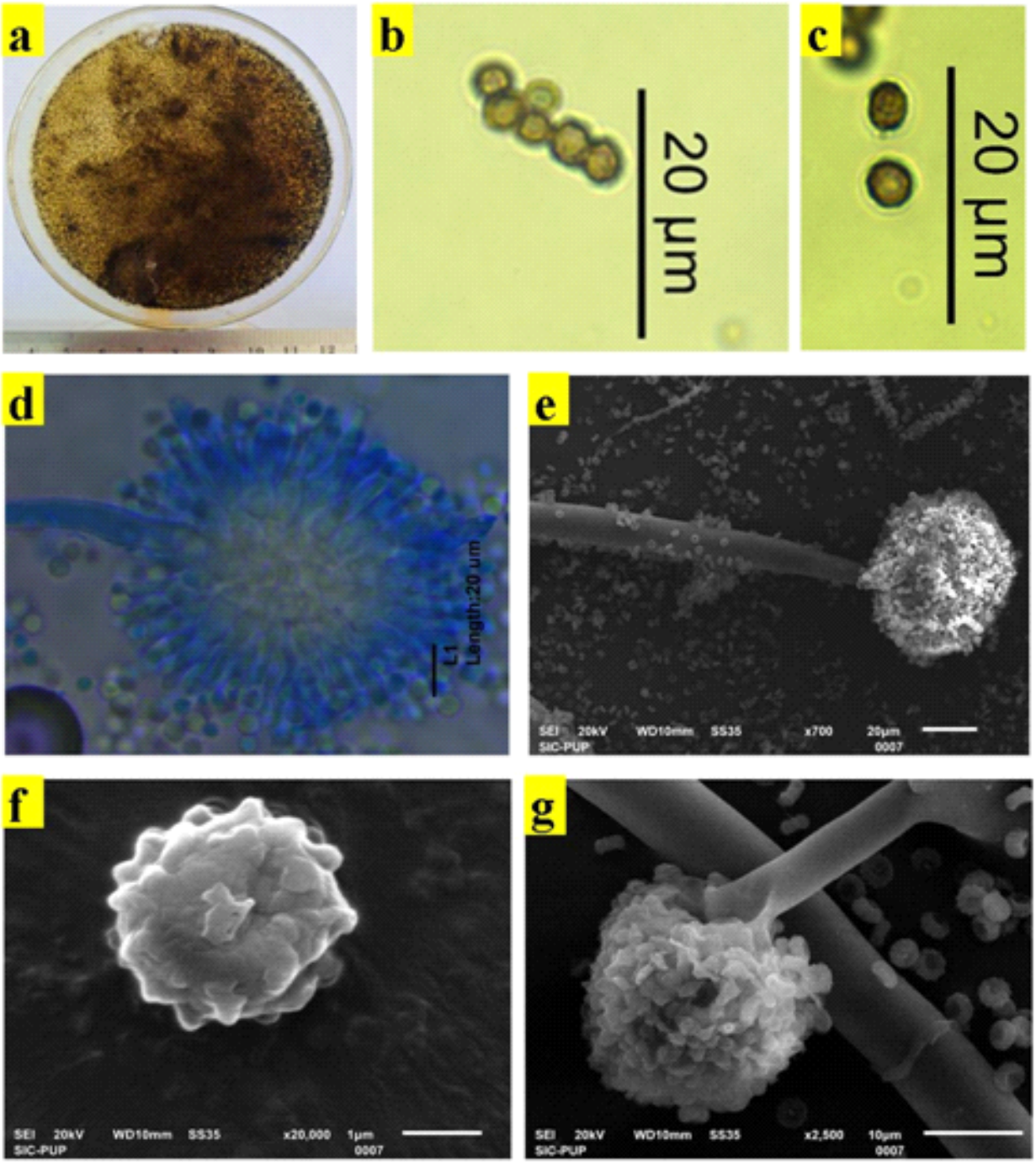


**Fig. 1:** *Aspergillus tubingensis*: a. Colony on PDA, b-c. Microphotograph of conidia, d. Photomicrograph of conidiophore, e-g. SEM analysis showing conidiophore (e,g) and conidia (f).

methylene carbons (–CH, –) characteristic of long saturated hydrocarbon chains, ä 62.1 ppm revealed oxygenated carbon, consistent with alcohol or ester functional groups, ä 127.9 – 130.2 ppm reported olefinic carbons (C=C) confirming unsaturated lipid or sterol-like structures, and >160 ppm depicted no strong signals observed, indicating the absence of dominant free carbonyl-containing aromatic acids or aldehydes.

Based on combined $^{1}$H- and $^{13}$C-NMR analysis, the crude extract is characterized by the predominance of long-chain aliphatic metabolites, presence of unsaturated fatty acids / lipid esters, likely occurrence of sterol-type antifungal metabolites like ergosterol which are common to *Aspergillus* species (Alcazar-Fuoli and Mellado 2013), minor oxygenated functionalities (esters/

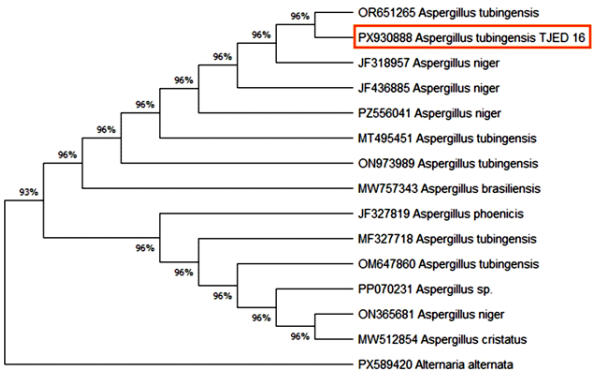


**Fig 2:** Phylogenetic relationship among *Aspergillus tubingensis* and related taxa through ITS rRNA sequences, and *Alternaria alternata* taken as an outgroupusing neighbor joining method in the form of condensed tree).

mentioned inTable2. The area percentage reveals the chromatographic area corresponding to the compound quantity present in the extract and detected through quadrupole mass spectrometry. Among the compounds, Papaveroline, 2'-bromo2-methyl-, tetramethyl(ester) exhibited the highest area followed by Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy whose individual chromatogram along with the mass spectral signatures has been shown in Fig. 8& Fig. 9 respectively. Fig. 7 represents the structural details of the compounds detected.

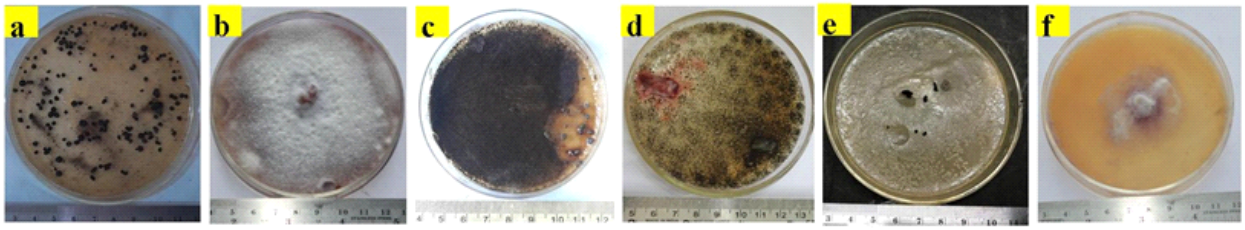


**Fig 3:** Plates representing the control cultures of the pathogens (a,b), dual cultures with the endophyte (c,d), and food poisoning assays (g,f)with respect to*R. solani* and *F. oxysporum* respectively.

alcohols), and negligible aromatic, alkaloidal, or sugar-based constituents.

The NMR spectral analysis of the *A. tubingensis* crude extract confirms that the extract is predominantly composed of lipid-based secondary metabolites, including saturated and unsaturated fatty acid derivatives and sterol-like compounds. Olefinic signals indicate the presence of unsaturation, while oxygenated carbons suggest ester or alcohol functionalities. Aromatic and highly polar metabolites are present only in trace amounts. The overall chemical profile is consistent with typical fungal lipid and sterol metabolite production.

### *GC-MS/MS interpretation*

Qualitative as well the quantitative validations including the chemical, volatile and the investigation of thermally stable compounds could be interpreted well through GC-MS (Derya *et al.* 2020; Mehmet *et al.* 2016). The evaluation in this study led to the identification of eighteen distinct compounds with their distinct retention times as depicted in Fig.6 and their respective details

### *LC-MS of the methanolic extract*

The mass fragmentation patterns, retention time, mass to charge ratios, and the area percentages of the individual fifteen compounds detected through LC-MS has been enlisted in (**Table. 3)** and the chromatogram presented (Fig. 10). In terms of their quantitative analysis, the compound named Norfentanyl (Fig.11a) followed by Ziprasidone (Fig.11b) showed their presence. Compounds such as quinines detected in the present study have been earlier reported to possess antibiotic properties (Gunatilaka 2006) andEnrofloxacin having antifungal potential (Soliam*et al.* 2015).

*A. tubingensis* has been considered as a suitable substitute in the fermentation industry for the exploitation of certain metabolites, enzymes, and the bioactive compounds (Xu *et al.* 2020), but the compounds from this species being an endophyte isolated from *D. sissoo*still remained unexplored.Moreover, this species is known to produce toxins (Mikušová*et al.*2020; Mogensen *et al.* 2010) that had led to the damage of many crops including raisins, peanut, apple, corn,

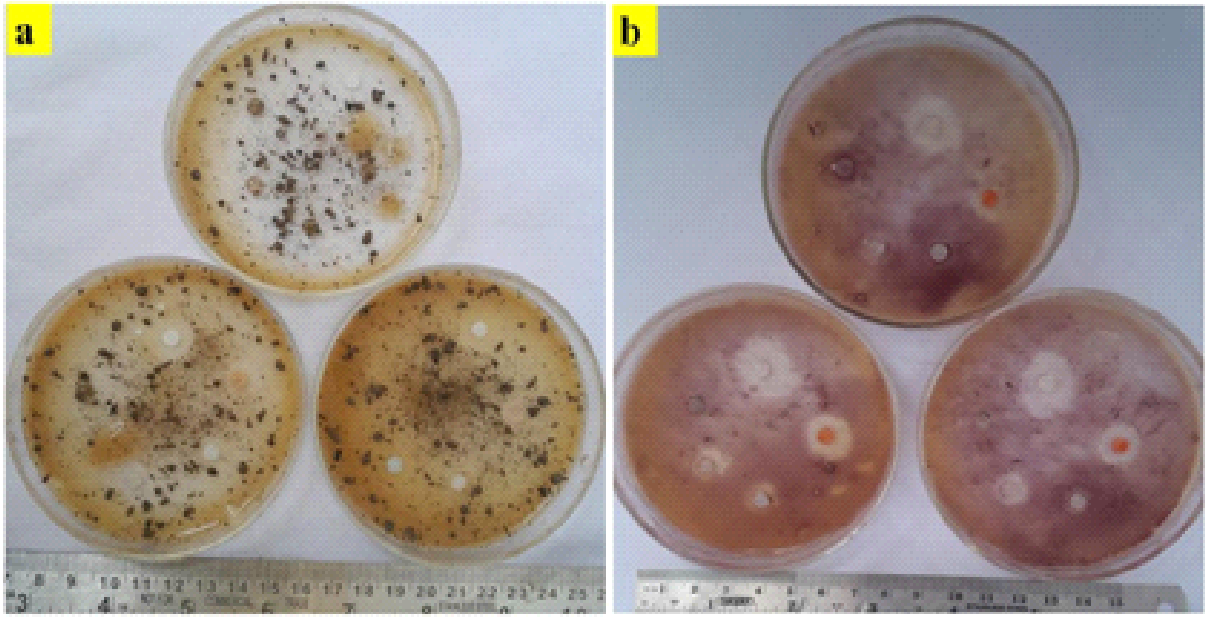


**Fig 4 :**Disc diffusion assay showing the antifungal activity of the extracts (25 mg/mL, 50 mg/mL, 75 mg/mL) of *A. tubingensis* against *R. solani*(a) and *F. oxysporum* (b) with fluconazole at the top centre of each plate kept as standard (2 mg/mL) followed by acetone, methanol, aqueous extracts, and then DMSO in the clockwise direction.

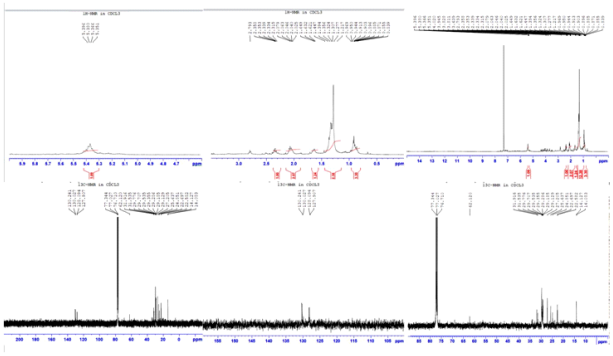

**Fig 5:** $^{1}$H-NMR (top row)and $^{13}$C-NMR (bottom row) spectral chromatogram of methanolic crude extract of *A. tubingensis*.

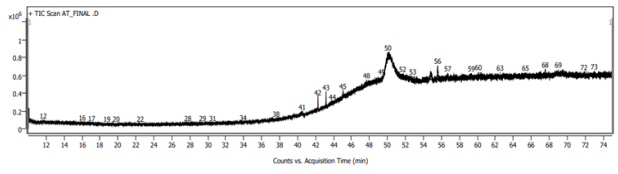

**Fig 6:** GC-MS/MS chromatogram of methanolic extract of *A. tubingensis*.

mango, and meat products (Perrone *et al.* 2007) and some literature suggesting their antimicrobial activity (Lu *et al.* 2014; Song *et al.* 2004) has been reported, buttheir potential as an endophyte and that too from *Dalbergia* remainedunexplored. For this purpose, the current study has been put forward.

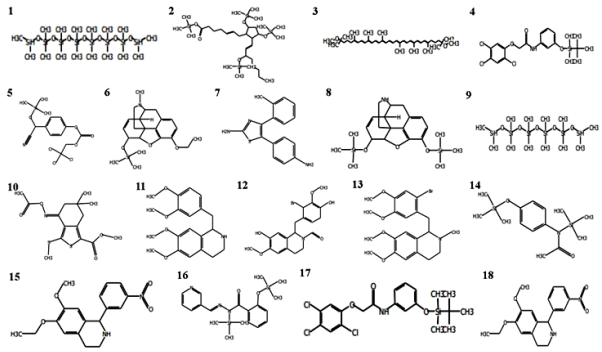

**Fig 7:** Structural representation of the compounds mentioned in Table 2

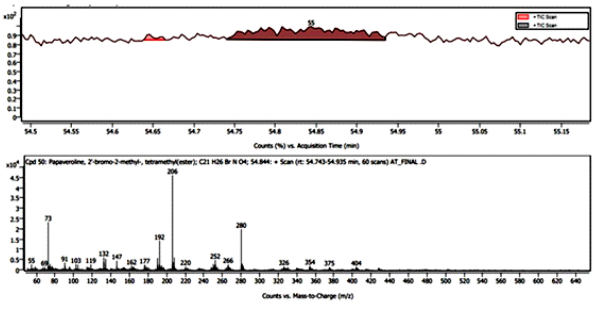

**Fig. 8 :**Chromatogram along with the spectra of Papaveroline, 2'-bromo2-methyl-,tetramethyl(ester).

## CONCLUSON

Endophytic fungi interact with their host species and produce numerous metabolites with

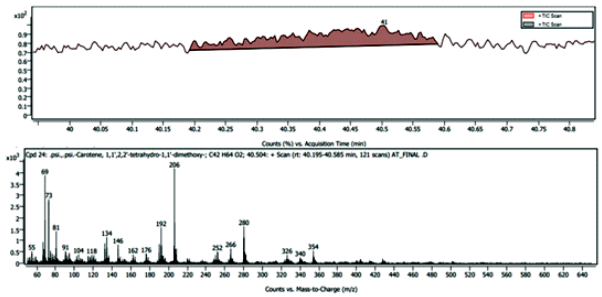

**Fig 9 :** Chromatogram and spectral analysis of the compound, Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy

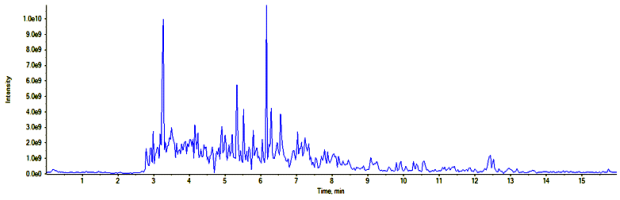

**Fig. 10:** LC-MS chromatogram of the methanolic endophytic extract.

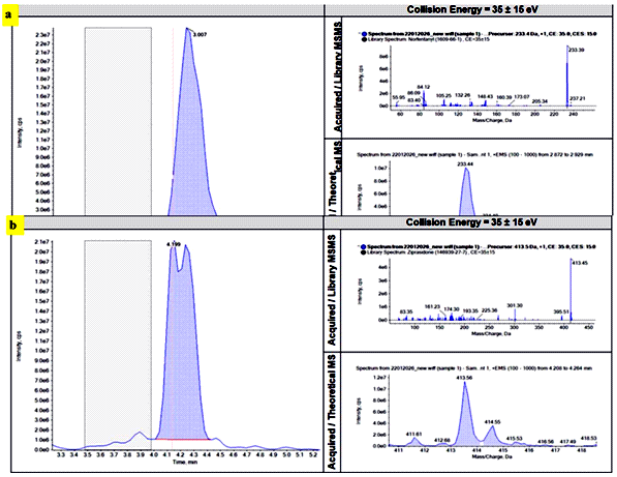


**Fig. 11:** Mass spectrometric characterization along with the fragmentation patterns of the compounds,Norfentanyl (a) and Ziprasidone (b).

promising agricultural, industrial, and pharmaceutical applications. They exhibit a crosstalk within the microbiome for better resistance and adaptation, benefitting the host. In this work, the focus is on investigating the compounds present in the crude endophytic *A. tubingensis* extract along with their antifungal potentialagainst *R. solani* and *F. oxysporum*, thus paving the way for therapeutical applications in the near future. In order totrapits potential applications, it is necessary to isolate and then characterize these identified compounds, for the discovery of novel antimicrobial drugs. The findings will not only underscores its value in the natural product research but also will open the avenues for future work involving the practises such as large-scale isolation, the bioassay-guided fractionation, and mechanistic studies.

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## DECLARATION

Conflict of Interest. The authors declare they have no conflict of interest.

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