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Original article

Endophytic fungi of *Tradescantia pallida* mediated targeting of Multi-Drug resistant human pathogens

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ABSTRACT

Antimicrobial resistance (AMR) has emerged as one of the most serious worldwide public health issues of the twenty-first century. The expeditious rise of AMR has urged the development of new, natural effective therapeutic strategies against drug-resistant pathogens. Endophytic fungi, which inhabit distinctive environments like endosymbiotic relationships with plants, are gaining interest as alternative reservoirs for novel compounds that exhibit a broad range of chemical diversity and unique modes of action by releasing a variety of secondary metabolites with antimicrobial properties. The objective of the current research was to isolate and identify endophytic fungal species from leaves of *Tradescantia pallida* and to investigate their antagonistic effects on Multi-Drug-Resistant human pathogens. Endophytic fungus TPL11 and TPL14 showed maximum inhibition in agar plug and agar well diffusion assay. The ethyl acetate crude extract effectively suppressed growth of MRSA (Methicillin-resistant *Staphylococcus aureus*) ATCC 43300,700699 strains and VRE (Vancomycin-resistant *Enterococcus*) with the Inhibition zone of 22 ± 0.05, 23 ± 0.11 and 24 ± 0.11 mm respectively with minimum inhibitory concentration (MIC) of 3.125 μg/mL. Whereas TPL11 fungus revealed antibiosis of 22 ± 0.05 and 21 ± 0.15 mm against MRSA(ATCC 43300,700699) and 24 ± 0.05 mm for VRE with MIC of 6.25,3.125 and 1.56 μg/mL respectively. The MIC (Minimum inhibitory concentration) index further confirmed that both the extracts were bacteriostatic against MRSA and bactericidal against VRE. The isolates TPL11 and TPL14 were identified as *Fusarium oxysporum* and *Nigrospora sphaerica* by 18S rRNA internal transcribed spacer (ITS) sequencing. To our insight, it is the first report to reveal the presence of *F.oxysporum* and *N.sphaerica* in *T.pallida* and their antibacterial activity.

1. Introduction

Antibiotic overuse has aided the outbreaks of drug-resistant bacterial infections, particularly the multiple drug-resistant strains, and a growing percentage of drug-unresponsive infectious disease agents are posing a significant threat to the global healthcare system (Janes et al., 2007). Infections triggered by antibiotic-resistant *S.aureus* represent an important threat to the health of people around the world. Methicillin-resistant *S.aureus*, drug-resistant *Streptococcus pneumonia*, mono to multiple drug-resistant *M. tuberculosis*, and VRE species are the most common Gram-positive pathogens that cause infections (Ratnaweera et al., 2018). Many human disorders have been attributed to *Staphylococci,* including osteomyelitis, periodontitis, endocarditis, chronic wound infection, and implanted device infection (Sadrati et al., 2023). MDR and XDR-TB are developing worldwide problems that are problematic to identify, costly to treat and result in varied outcomes. The organisms *S. aureus, A. baumanii, K. pneumoniae, P. aeruginosa, Enterobacter* sp. and *E. faecium* are the primary pathogens in most US hospitals (Deshmukh et al., 2015). Despite intensive attempts to suppress them, resistant microbial infections remain prevalent, triggering death and disability, continuing to pose a severe danger to human health (Ratnaweera et al., 2018). There are several methods for developing novel antimicrobial chemicals that are biologically active. The most conventional measures in the world of natural products is the research of lesser-known microorganism species and genera (Sadrati et al., 2023).

Medicinal plants play a key role in treating several human diseases, as they are the potential sources of biologically active molecules. The bioactive molecules comprise simple alkaloids, naphthopyrone glucosides, saponins, terpenes, anthraquinones (emodin, chrysophanol and physcion) and steroids (carpesterol, ecdysterone and β-sitosterol). Either

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plants or some microbial consortium that resides asymptomatically within the certain medicinal plant parts synthesizes these active molecules and this microbial community is referred to as endophytes (Yadav et al., 2021). Medicinal plants are the well-known reservoir of endophytic fungi with pharmaceutically important novel metabolites. The structures of the compounds from fungal endophytes are unique with interesting biological properties, thereby offering a wider scope for different clinical applications (Santos et al., 2015). Endophytes are microbial entities that inhabit the host plant tissues at any stage of their life cycle and perform different ecological relationships without causing any without causing any adverse effects on the host organism/or host plant (Silva et al., 2018). Endophytic fungi are the least considered microorganisms, but they have attracted attention owing high biological diversity and aptitude to synthesize new pharmacological substances (Sadrati et al., 2023). They are eminent cherished homes of an extensive variety of bioactive substances with cytotoxic, antimalarial, antimicrobial, antioxidant, antiviral and anticancer actions. About 80 % of endophytic fungi are responsible for biologically active product productions that have herbicidal and antimicrobial properties (Mousa et al., 2021). Fungal endophytes have recently gained impetus, due to their immense potential to produce numerous medicinally important metabolites. Hence, exploring endophytic fungi from different plant species would provide plenty of opportunities to discover new potential bioactive metabolites (Dhayanithy et al., 2019).

Tradescantia pallida (Rose) D. R. Hunt var. purpurea, an ornamental plant from the Commelinaceae family, generally acknowledged as Purple Heart or wandering jew. It is a shade-tolerant low-growing tetraploid plant that can grow well in different soil conditions. It also holds tough resistance to insects and parasites thereby colonizing rapidly in different environments. They are broadly scattered in tropical and subtropical sections and are ordinarily grown as ornamental, hanging, or ground-covering plants (Tan et al., 2014). In all Brazilian states, the genus *Tradescantia* was largely used as an ornament as they can grow and propagate easily and are highly resistant to environmental factors and climatic conditions (Cabral et al., 2022). They can eliminate unstable organic impurities from the air effectively (Tan et al., 2014). Traditionally, *T.pallida* was used as an anti-toxic and anti-inflammatory property and in Taiwanese traditional medicine, it is used to improve blood circulation (Bokhari et al., 2020). The leaves were used as dyes and they acted as analgesics against joint pain and rheumatism. It has been reported that the plant exhibits various biological activities like antioxidant, antimicrobial and anticancer properties (Cabral et al., 2022).

It is also reported to have antagonistic activity against gram-positive and negative bacteria (Kamiya et al., 2019). The chloroform leaf extract obtained from *T.pallida* purpurea has demonstrated significant efficacy against pathogens affecting *Labeo rohita* fish. Additionally, natural colorants such as anthocyanin and annatto derived from *T. pallida* are associated with numerous health advantages (Bokhari et al., 2020). Biosynthesized silver nanoparticles with *T.pallida* extract (TpAgNPs) exhibited cytotoxic activity on the rhabdomyosarcoma cell line. TpAgNPs revealed significant antibacterial activity against *Pseudomonas aeruginosa* with MIC of 64 µg/mL and antifungal activity against *Candida parapsilosis*. Further, the plant extract had effective antioxidant potentials of 77.2 ± 1.0 % and 45.1 ± 0.5 % free radical scavenging activity (Shahzadi et al., 2022). In Malaysia, the Ayta communities of Porac Pampanga utilize this plant to treat sore eyes (Ragragio et al., 2013). *T. pallida* has also been recognized as a viable alternative for in situ mutagenesis testing (Suyama et al., 2002). Ethanol extract showed beneficial hepatoprotective activity against CCl4-induced liver damage in rats (Villanueva-Toledo et al., 2002). Hexane extract obtained from aerial parts of *T. pallida* displayed significant antifungal activity against *Rhizopus stolonifera, Sclerotinia sclerotiorum,* and *Penicillium digitatum*. It also revealed cytotoxic activity against human tumor cell lines such as MCF-7, fibroblasts, glioblastoma and HeLa (Cabral et al., 2022). The aqueous extract of *T. pallida* was reported to exhibit insecticidal properties (Rocha et al., 2022).

Hence, the present study describes the isolation, characterization and screening of endophytic fungi isolated from the ornamental plant, *Tradescantia pallida*. Further, the antimicrobial activity of the fungal extracts will tested against multi-drug resistant human pathogens. In this way, we provide a new resource that efficiently produces antibacterial agents from endophytic fungi that we obtained from *T. pallida*.

2. Materials and methods

2.1. General experimental measures

Potato dextrose agar (PDA), broth (PDB), Muller Hinton agar (MHA), broth (MHB), Tryptic soy agar (TSA) and broth (TSB) were acquired from Hi-Media (Mumbai, India). Glassware was purchased from Borosil and the solvents used for the analysis and experiments were purchased from SRL.

2.2. Procurement of test microorganisms

The resistant bacterial pathogens utilized were Methicillin-resistant *Staphylococcus aureus*-MRSA (ATCC 43300; 700699), *Staphylococcus aureus* (ATCC 25923), *S.aureus* (MTCC 3160), Vancomycin-resistant *Enterococci*-VRE (ATCC 51299) and *Enterococcus fecalis*. The cultures were acquired from American type culture collection (ATCC) and Microbial type culture collection (MTCC; Chandigarh, India). Test pathogens sub-cultured in Tryptic soy agar and Brain heart infusion agar respectively for further use.

2.3. Plant sampling, authentication and isolation of endophytic fungi

To isolate endophytic fungi, a healthy *Tradescantia pallida* plant was collected from the Vellore district (12°56′17.3″N 79°09′24.5″E), Tamilnadu, India in November (2020). The samples were collected aseptically in a clean polyethylene cover and handled within 8 hrs. The specimens were authenticated by the botanist from Dr. P. Jayaraman, (Director), Plant Anatomy Research Center, Chennai. Samplings were cleaned first with distilled H_2O (5 mins) to eradicate adhered soil particles and surface-adhering microorganisms. Then sterilized aseptically by sequential immersion of samples into 70 % ethanol (C_2H_5OH) (1 min), 35 % Sodium hypochlorite (NaOCl) for 30 s followed by double distilled water. The segments were cut into 2 cm long with a sterile scalpel blade and placed on a Potato Dextrose Agar medium supplement with 50 mg/L chloramphenicol to prevent the growth of bacteria. In addition, incubated for 3–7 days at 27 ± 1 °C. The emerging endophytic fungi from the explants were isolated, purified and maintained by continuous subculturing. Explants and last rinsed aliquot were considered as a control measure. The media dish impregnated with 50 Âµl of the last rinsed H_2O with no growth of microbes determines the success ratio of the surface sterilization method (Potshangbam et al., 2022).

2.4. Preservation of endophytic fungal isolates

The pure entities were transferred individually to PDA slants and in a 15 % sterilized glycerol (v/v) solution. The growth was observed for 3 to 7 days and the slants at −4°C and glycerol stock at −20 °C were preserved for further study (Ibrahim et al., 2021).

2.5. Susceptibility test: Primary evaluation through agar plug diffusion technique

The pure cultures derived from *T.pallida* were first screened for antibacterial properties by agar Plug diffusion standard protocol. The agar plugs were made from the 7-day-grown pure fungal cultures. Then the pure culture plugs were positioned onto the Muller Hinton agar medium previously seeded by respective bacterial pathogens using a

sterile cotton swab. The plates were refrigerated overnight at 4 °C to facilitate diffusion of metabolites, followed by 16–18 hrs incubation at 37 °C for the bacterial growth. The potential fungal isolates were selected based on the zone of inhibition observed against the test pathogens for secondary screening (Sadrati et al., 2020).

2.6. Characterization of the potential isolate

2.6.1. Morphological characterization

The fungal isolates were grown on a PDA medium and morphological examinations were carried out after the isolates mycelium occupied the entire plates. Characteristics comprise microscopic and macroscopic characterization. The shape, textures, pigmentation, colony morphology and characteristics of spores were categorized under macroscopic inspections. Microscopic was done by the Lacto phenol cotton blue staining technique and images were taken at 100x (Manganyi et al., 2018).

2.6.2. Scanning electron microscopy (SEM) analysis

The spore surface morphology and hyphae configuration were observed under a Scanning electron microscope. To study the morphology of test bacteria upon treatment with fungal crude extract, the samples were analyzed under SEM (EVO/18 Research, Carl Zeiss) (Santra et al., 2022).

2.6.3. Cultural characterization

The potential isolates were grown on assorted commercially available media such as Potato dextrose agar, Sabouraud Dextrose Agar, Malt extract agar (MEA), Czapek Dox agar (CDA) and Corn meal agar purchased from Hi Media, India for optimization of media for maximal growth and bioactivity.

2.6.4. Phylogenetic investigation

The fungal endophyte that exhibited the greatest antibiosis was classified molecularly with 18S rRNA ITS sequencing. The genomic DNA was isolated in the laboratory. Universal primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) were chosen, PCR amplification was processed with thermal cycling conditions of initial denaturation for 2 mins at 95 °C, followed by 25 cycles denaturation at 95 °C of 30 secs, primer annealing for 30 secs at 55 °C, first extension for 1 min and last extension (72 °C) for 5 mins. ABI PRISM® was used to achieve sequencing outcomes. Results from BLAST were matched with NCBI Genbank to find out the identities of the isolates (Settu et al., 2023).

2.7. Analysis of the antagonistic activity of crude extract

2.7.1. Optimization of ideal culture medium for the production of antibacterial substances

The purified potent 7 days old fungal cultures that were selected in the previous step, were cultivated on PDA, SDA, MEA, CDA, CMA and incubated for 3 weeks at room temperature under a consistent state in 500 mL volume conical flask. Post incubation, culture broth was drained by Whatman filter paper No. 1 (Z240079) to section mycelium and filtrate. The obtained filtrate was extracted with equivalent measurements of multiple polarity solvents such as Hexane, Dichloromethane (DCM), Ethyl acetate (EA) and Butanol with a Borosil separating funnel. The mycelial mat was extracted with methanol. Using a (Model: RE100-Pro rotatory evaporator, the solvent extracts are condensed. Derived metabolites were directly used to assess the in-vitro assays (Supaphon et al., 2013).

2.7.2. Secondary antibiosis screening by Kirby-Bauer method

The inhibitory effect of the obtained extracts from the selected fungus was tested for the antibacterial properties by the Kirby Bauer disc diffusion method (Katoch et al., 2017). The screening was carried out against human pathogenic bacteria. Muller Hinton agar served as the basal medium. Bacterial turbidity was normalized with standard Mc Farland (0.5) before carrying out the microbial assay. Agar dish seeded with pre-prepared inoculum of trial microbes. Subsequently, sterile discs about 6 mm comprising the testing extract of the chosen concentration (25, 50, 75, 100 μg/mL) were placed onto the bacteria-seeded plate. DMSO (5 %) was used as a negative control to detect the effect of solvent. Commercial antibiotics Oxacillin (1mcg) and Vancomycin disc (30 mg) as positive control. Petri dishes were incubated at 37 °C for 16–18 hrs and the inhibition zone was measured. The test was carried out in independent triplicates.

2.7.3. Evaluation of minimum inhibitory and bactericidal concentration

Broth micro-dilution susceptibility assay was executed to find out the MIC and MBC of endophytic fungal extract. In a sterile 2 mL 96 well plate, 100 μL of sterilized MH broth was added. The test samples of the extracts were prepared at 1 mg/ml concentration and 100 μL was added to the first well. Followed by two-fold serial dilutions were made resulting in different concentrations ranging from 50 to 0.78 μg/mL. To the wells, 10 μL of pre-prepared bacterial inoculum was added. The well with MHB alone serves as positive control and the well with bacteria and MHB serves as negative control and reference drug controls. Petri dishes were incubated for 16–18 hrs at 35 ± 2 °C. The lowermost concentration at which no observable bacteria in the wells following incubation was recorded as the MIC value. Further, the concentration showing a complete absence of visible bacterial growth in the MIC assay was determined. About 50 μL was subcultured onto a Muller Hinton agar medium and incubated for 18 to 24 hrs at 37 °C. The lowermost extract concentration showing a full absence of bacterial growth was recorded as the MBC (Valle et al., 2016).

2.7.4. MIC index of the extracts

To validate the effectiveness and the outcome of whether ethyl acetate extract is bactericidal or bacteriostatic, MIC index was assessed by dividing MBC by MIC value. If the index is lesser than or equal to 4, then it is considered bactericidal and bacteriostatic if it is greater than 4 (Sadrati et al., 2020).

2.7.5. Synergistic activity of the extracts

Interactive inhibition of synergy between the fungal extract and antibiotics was measured using the chequerboard method following the protocol of Omokhua et al., 2019. A synergistic combination was performed using antibiotics and the fungal extract to which the bacterial strains were resistant. Antibiotics and crude concentration began at their MIC and were serially diluted at two-fold rates. Combination effect was estimated by valuing the FICI of individual combinations and the average values were measured.

Fungal extract FIC = MIC of fungal extract in combination with antibiotic/ Fungal extract MIC alone.

Antibiotic FIC = MIC of antibiotics combined with fungal extract/ antibiotics MIC only

FICI = Fungal extract FIC + Antibiotic FIC

FICI ≤ 0.5 was used to define synergy. A FICI between 0.5 and –4 specifies the absence of interaction between agents. FIC greater than 4 designates that there is an antagonistic reaction between agents.

2.8. Mycochemical screening

The phytochemical analysis of ethyl acetate crude extract was carried out using a standard protocol (Sharma et al., 2016). The test was done to determine the existence of secondary metabolites such as phenols, alkaloids, steroids, terpenoids and tannins in the fungal extract.

2.9. Gas chromatography and mass spectrometry analysis

Perkin Elmer Clarus 680 with MS Clarus 600 (electron ionization)

fitted out with elite-5MS capillary medium was carried out to assess the crude extracts obtained from *F.oxysporum* and *N.sphaerica*. The crude diluted with the same solvent was studied. The oven's initial temperature was 55° for 3 mins and the ramp program was 10 mins to 300 °C for 6 mins. Helium with a 1 mL/min continuous flow rate is used as carrier gas. The mass transfer line and temperature are fixed at 240 °C. Turbo version 5.4.2 software was used for analysis. About 25 min were required to complete GC. On comparing the component's spectrum with the known spectrum database saved in the NIST (National Institute of Standards and Technology) library, the constituent's structures were determined. Area peak percentage (%) analysis was done to determine the measurable percentage of assessed biological compounds present in the fungal crude extract at the respective retention time. (Settu et al., 2023).

2.10. FTIR analysis of fungal extract

The dried fungal extracts were subjected to FTIR instrumentation study by IR Affinity model, Shimadzu FT-IR. At 4000–400/cm range, the chemical constituent's functional groups were recorded. The chemical bonds of a molecule are determined using the infrared absorption spectrum. According to the annotated spectrum, chemical bonds present in the extract take up a certain light wavelength (Segaran et al., 2020).

2.11. Statistical assessment

Results are stated as a mean of triplicate determination ± standard deviation for experiments done *in vitro*. Statistically, the data were calculated by Version 9.5.1 GraphPad Prism software.

3. Results

3.1. Ethno-medicinal investigation of selected plant

In the current investigation, the ornamental plant *Transcantia pallida* was investigated for fungal endophytes and has tremendous importance in traditional medicine despite being one of the ornamental plants. Previous research has shown that plant extracts have anti-inflammatory, antimicrobial and antioxidant effects. As a result, the major purpose of this research was to find an effective endophytic fungus with antibacterial action.

3.2. Isolation of endophytic fungi from Transcantia pallida

In the present study, fourteen (n = 14) morphologically distinct fungi were isolated from 14 mature leaves obtained from the same plant. Among the four different growth media used, PDA media was found to be excellent for the development and yield of fungus.

3.3. Identification of potent endophytic fungi

Based on the bioactivity, the most potent endophytic fungi TPL11 and TPL14 isolated from the surface-sterilized leaves were identified by the morphological appearance on solid medium and microstructure variation under SEM using CLSI guidelines (Fig. 1). The TPL11 isolate was white, velvety, raised in the center and brownish yellow on reverse whereas TPL14 isolate was white and cottony. Further confirmed by molecular technique and identified successfully as *Fusarium oxysporum* and *Nigrospora sphaerica* by 18S rRNA ITS gene sequencing and was

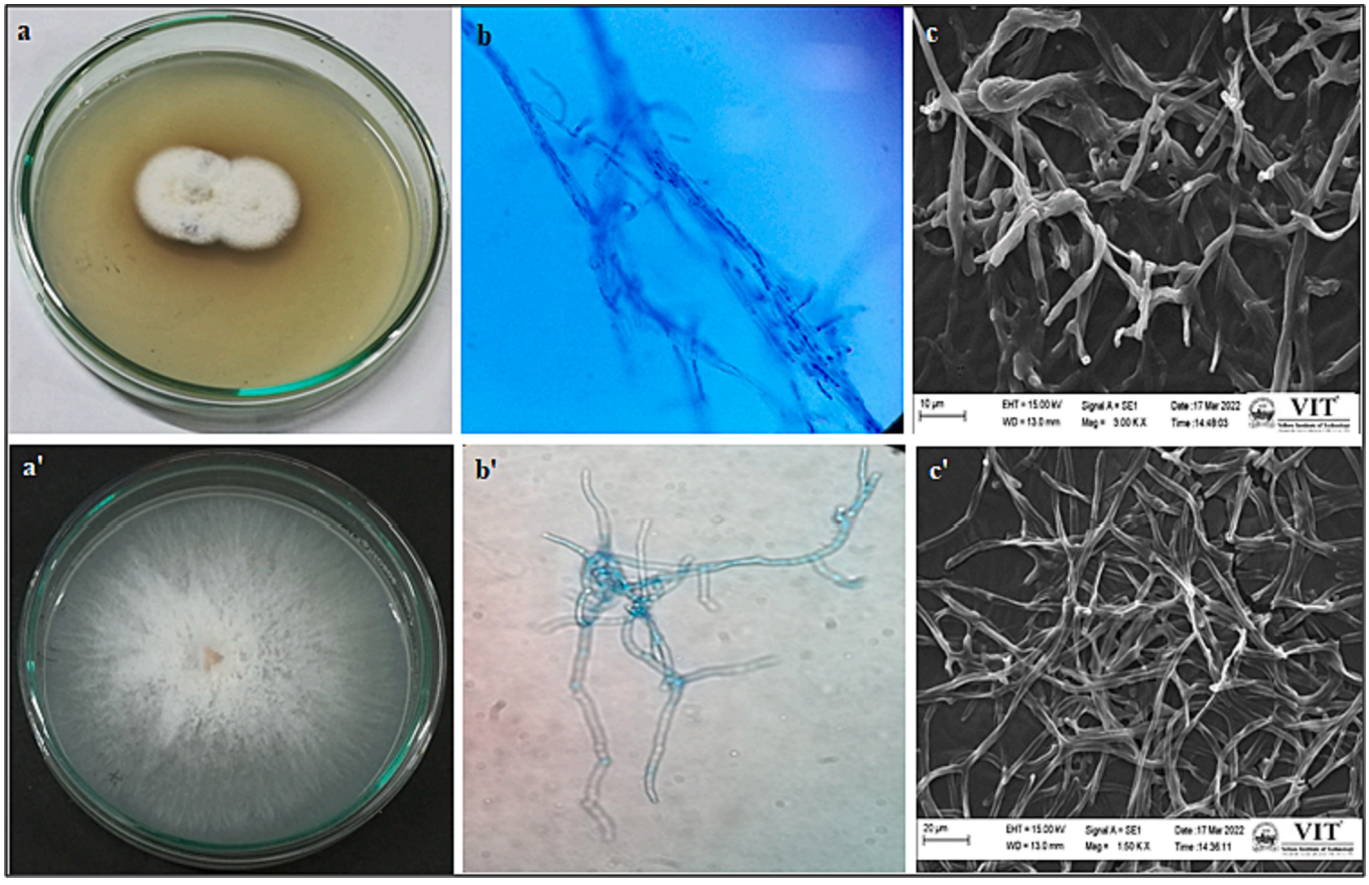

Fig. 1. Characterization and identification of endophytic fungus from *T. pallida*: (a) Morphology of TPL11 on PDA medium, (b) LCB staining at 100x magnification, (c) SEM Spore morphology at 3.00KX of TPL11 isolate; (a') Morphology of TPL14 on PDA medium, (b') LCB staining at 100x magnification, (c') SEM Spore morphology at 1.50KX of TPL14 isolate.

confirmed through the neighbor-joining tree (Fig. 2). Further deposited in GenBank accession numbers (ON076556) and (ON248210).

3.4. Identification of optimal growth media and antibacterial potential of F.oxysporum and N.sphaerica

The antibacterial potential of the most active endophytic fungal extracts obtained from strain *F.oxysporum* (TPL11) and *N.sphaerica* (TPL14) was examined using different growth media in liquid fermentation. The results obtained revealed that only the PDB medium displayed a varied range of growth inhibition against the tested pathogens at 100 µg/mL concentration as verified by a recorded zone of inhibition (Table 1). The delicate activity was witnessed in PDB medium followed by SDB and MEA.

3.5. Inhibitory effect of endophytic fungi on resistant pathogen

3.5.1. Screening for potential isolates

The results obtained revealed that the tested isolates showed a varying range of antibacterial effects against the tested bacterial pathogens. Among the 14 fungal isolates, TPL11 and TPL14 displayed significant antagonistic activity against the test pathogens (Fig. 3) by the agar plug diffusion method (primary screening) followed, it was confirmed with the disc diffusion technique (secondary screening) with reference to CLSI (2021) recommendations. These findings revealed that *F.oxysporum* and *N.sphaerica* isolated from those associated with *T.pallida* have a range of notable disease-suppressing properties.

3.5.2. Antibiotic sensitivity assay

The studied endophytic fungal samples confirmed varying levels of inhibition against the tested pathogen Table 2, Fig. 4. The results display clearly that the tested strain was susceptible with a substantial variance ($P < 0.05$) in mean inhibitory zone diameter on different solvent crude extracts. *F.oxysporum* ethyl acetate extract exhibited maximum activity at a concentration of 100 (µg/mL) against MRSA-ATCC 43,300 and VRE with ZOI of 22 ± 0.05 and 24 ± 0.05 mm respectively. Against *S.auerus* ATCC 25923 the ZOI was 19 ± 0.15 mm. Ethyl acetate extract of *N. sphaerica* showed significant activity against MRSA-ATCC 43,300 and ATCC 700699 with inhibition zones of 22 ± 0.05 and 23 ± 0.11 mm. Whereas it showed ZOI of 24 ± 0.11 and 19 ± 0.2 mm against VRE and *E.fecalis* which is also maximum in activity to positive control Oxacillin (7 ± 0.05 mm). DCM and Hexane exhibited minimum activity whereas butanol crude extract of both fungi exhibited an inhibition zone, similar to that of Oxacillin (7 mm). No zone was observed in negative control. The positive control had no zone of inhibition around the disc showing resistance against MRSA (ATCC 700699) and VRE. This indicates that the ethyl acetate extract of both the fungus was more efficiently suppressing the growth of resistant pathogens with varying potency than the commercially available Oxacillin antibiotic. Further, the negative control (DMSO) has not shown any ZOI, indicating the solvent used to dissolve the extracts does not affect the test pathogens.

3.5.3. Bacteriostatic and bactericidal effects of fungal extracts

The extracts attained on the PDB filtrate were exposed to a microdilution trial to decide the MIC and MBC. The ethyl acetate extract of *N. sphaerica* was more active against MRSA (ATCC 43300, 700699) with the MIC of 3.125 and 3.125 µg/mL compared to extract of *F.oxysporum* (6.25 and 3.125 µg/mL) followed by *S.auerus* and VRE. As shown in Table 3, the MBC values ranged from 6.25 µg/mL to 25 µg/mL. Although all extracts demonstrated antibacterial activity, results of the disc

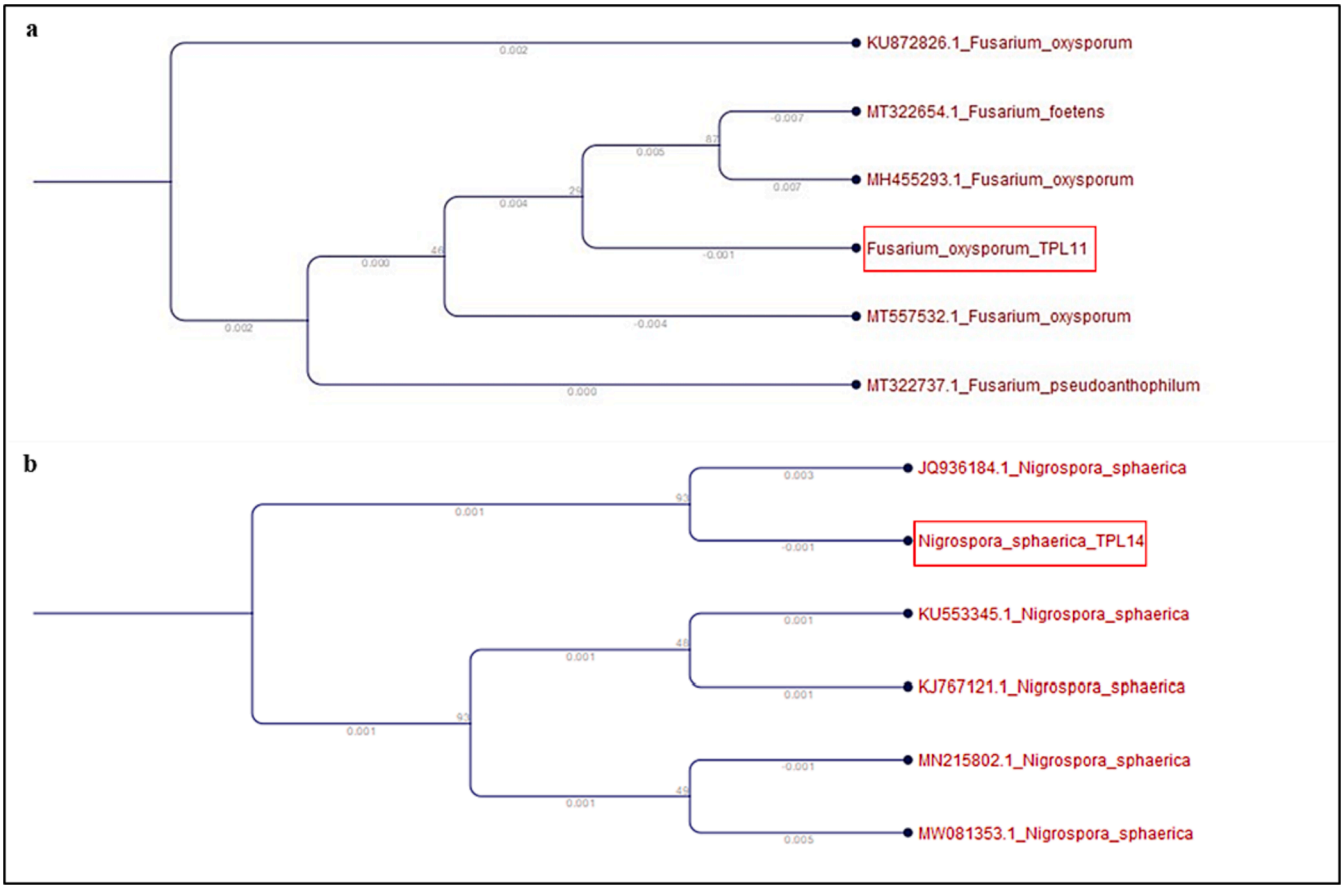

Fig. 2. Phylogenetic tree derived from the neighbor-joining analysis of endophytic fungus: (a) TPL11 identified as *Fusarium oxysporum*; (b) TPL14 identified as *Nigrospora sphaerica* isolated from the leaf region of *T. pallida*.

Table 1
Antibacterial significance inhibition of microbes with ethyl acetate extract obtained from *F.oxysporum* and *N.sphaerica* isolated from *T.pallida* cultivated in different growth media.

Culture growth medium	Concentration 100 (µg/mL); ZOI (mm)											
	MRSA (ATCC 43300)		**MRSA (ATCC700699)**		***S.aureus* (ATCC 25923)**		***S.aureus* (MTCC 3160)**		**VRE (ATCC 51299)**		***E.fecalis***	
	TPL11	TPL14	TPL11	TPL14	TPL11	TPL14	TPL11	TPL14	TPL11	TPL14	TPL11	TPL14
Potato Dextrose Broth	22	22	21	3	22	20	15	18.5	24	24	20	19
Czapek Dox Broth	16.2	–	14	–	19	15	12.6	14	–	–	18	16
Sabouraud Dextrose Broth	18	17	15	13	17.5	15.7	18	13.6	19	18	21.5	19
Corn Meal Agar	–	12	–	14	15.2	17	15	12.5	18	–	14	–
Malt extract agar	15	12	15	17	18	14	21	12	19	18	15.8	13

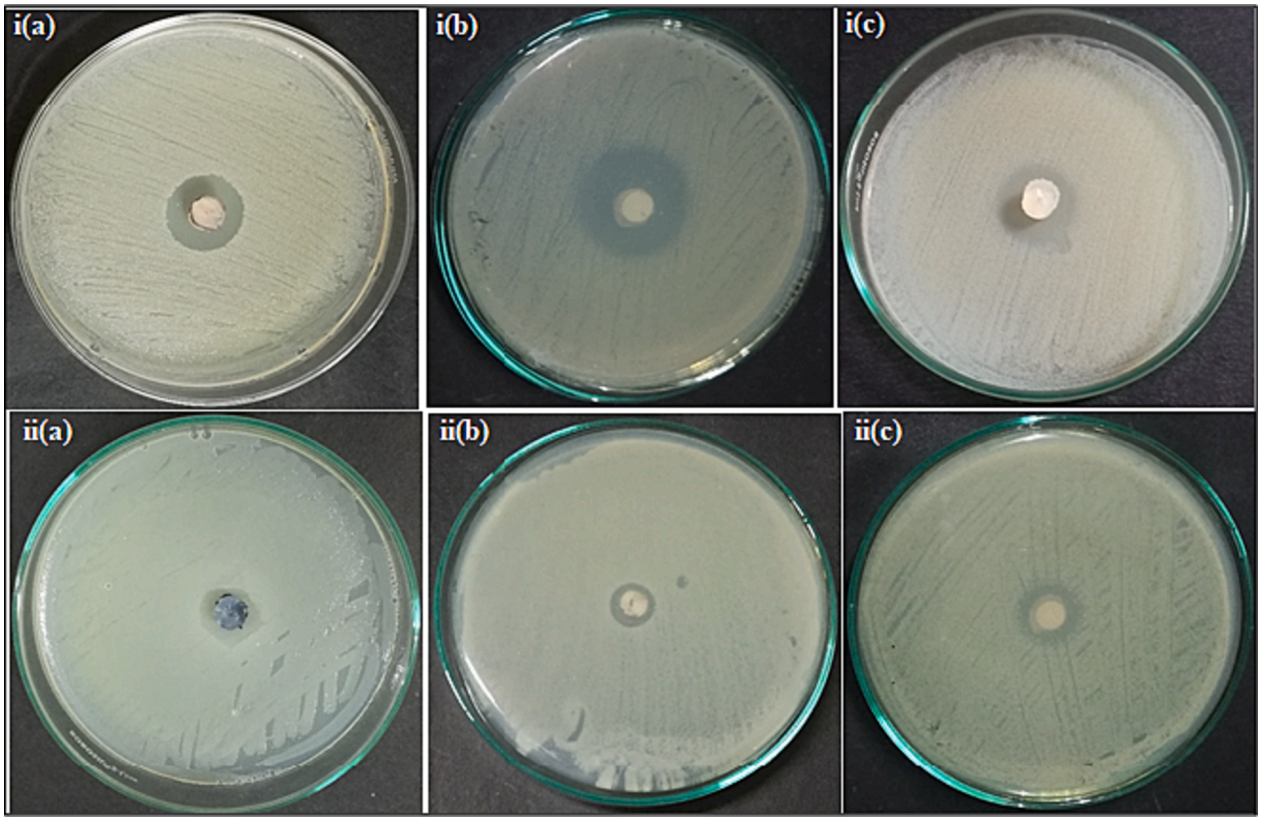

Fig. 3. Inhibition of resistant pathogens by endophytic fungus *F.oxysporum* against i(a) ATCC 43300, i(b) ATCC 700699, i(c) ATCC 51299 and endophytic fungus *N. sphaerica* against ii(a) ATCC 43300, ii(b) ATCC 700699, ii(c) ATCC 51299 strain by Agar plug diffusion method.

diffusion, MIC and MBC values suggest that the ethyl acetate extract provided the highest antagonistic activity against the tested strains followed by DCM, hexane and butanol. The least potent was butanol extract. All the extracts were bactericidal for all the tested resistant strains. The MIC verified for individual antibiotics was also shown in Table 4.

3.5.4. Checkerboard assays

The combination of ethyl acetate crude with a variety of classes of antibiotics was evaluated against the bacterial pathogens. The fractional inhibitory concentration index (FICI) exploration by the checkerboard assay exhibited values below 0.5 (FICI $\leq$ 0.5), which indicates a synergistic effect. As shown in Table 4, it was observed that against MRSA and VRE strains there is a significant decrease in drug MICs when the ethyl acetate crude extract is merged with the antibiotics reaching a 2–3 times fold reduction. For all combinations tested, ethyl acetate crude extract of *F.oxysporum* combined with Vancomycin declined the MIC value among all the tested combinations from 6.25 to 0.19 µg/mL and from 1.56 to 0.19 µg/mL against MRSA (ATCC 43300) and VRE respectively with FICI of 0.1 indicates synergisim. Whereas, *N.sphaerica* ethyl acetate was highly active when it was combined with Vancomycin resulting in MIC from 3.12 to 0.39 µg/mL against MRSA (ATCC 43300) with FICI of 0.2 indicating synergistic.

3.6. Mycochemical screening

Phytochemical analysis of ethyl acetate extracts exposed the existence of phenols, alkaloids, tannin, steroids and terpenoids. Entirely these classes of composites have been stated as antimicrobial antibacterial activity of endophytic fungus.

3.7. Fungal extract analysis by GC–MS

The potent endophytic fungi TPL11 and TPL14 ethyl acetate crude were exposed to GC–MS to identify volatile compounds, alcohols, long-chain hydrocarbons, ketones, etc. Chemical complexes were recognized

Table 2
Anti- Multiple Drug Resistant (MDR) bacterial activity of ethyl acetate crude extracts.

Extracts	Endophytic Fungi	Concentrations (μg/mL)	MRSA (ATCC 43300)	MRSA (ATCC 700699)	*S.aureus* (ATCC 25923)	*S.aureus* (MTCC 3160)	VRE (ATCC 51299)	*E.fecalis*
Ethyl acetate	***F.oxysporum***	25	6 ± 0.15	–	17 ± 0.1	8 ± 0.4	–	11 ± 0.5
		50	13 ± 0.12	15 ± 0.1	18.5 ± 0.2	10 ± 0.25	17 ± 0.12	13 ± 0.1
		75	16 ± 0.15	19 ± 0.05	20 ± 0.1	12 ± 0.2	21 ± 0.1	17 ± 0.1
		100	22 ± 0.05	21 ± 0.15	22 ± 0.06	15 ± 0.5	24 ± 0.05	20 ± 0.05
	N.sphaerica	25	14 ± 0.11	–	15 ± 0.5	12 ± 0.1	–	–
		50	18 ± 0.05	19 ± 0.05	17 ± 0.1	14 ± 0.25	18 ± 0.05	14.5 ± 0.5
		75	20 ± 0.1	20 ± 0.05	18.2 ± 0.15	17 ± 0.4	21 ± 0.11	17 ± 0.2
		100	22 ± 0.05	23 ± 0.11	20 ± 0.06	18.5 ± 0.5	24 ± 0.11	19 ± 0.2
Dichloromethane	***F.oxysporum***	25	–	–	14 ± 0.2	7 ± 0.5	–	7 ± 0.5
		50	10 ± 0.2	–	17 ± 0.2	10 ± 0.25	–	12 ± 0.1
		75	11.5 ± 0.5	17 ± 0.05	19 ± 0.15	12.5 ± 0.5	15 ± 0.05	18 ± 0.1
		100	13 ± 0.01	19 ± 0.02	21 ± 0.07	19 ± 0.1	18 ± 0.2	20 ± 0.05
	N.sphaerica	25	–	–	12 ± 0.2	7 ± 0.5	–	10 ± 0.2
		50	14 ± 0.03	–	15 ± 0.2	10 ± 0.25	17 ± 0.05	12.5 ± 0.5
		75	16 ± 0.01	14.6 ± 0.05	19 ± 0.15	12.5 ± 0.5	18 ± 0.1	16 ± 0.2
		100	18.5 ± 0.02	17 ± 0.12	22 ± 0.07	15.9 ± 0.1	20 ± 0.1	17.2 ± 0.2

*Values are reported as Mean ± SD, n = 3 inhibition percentage; significant differences ($p < 0.05$) from the control; '-' denotes no antibacterial activity.

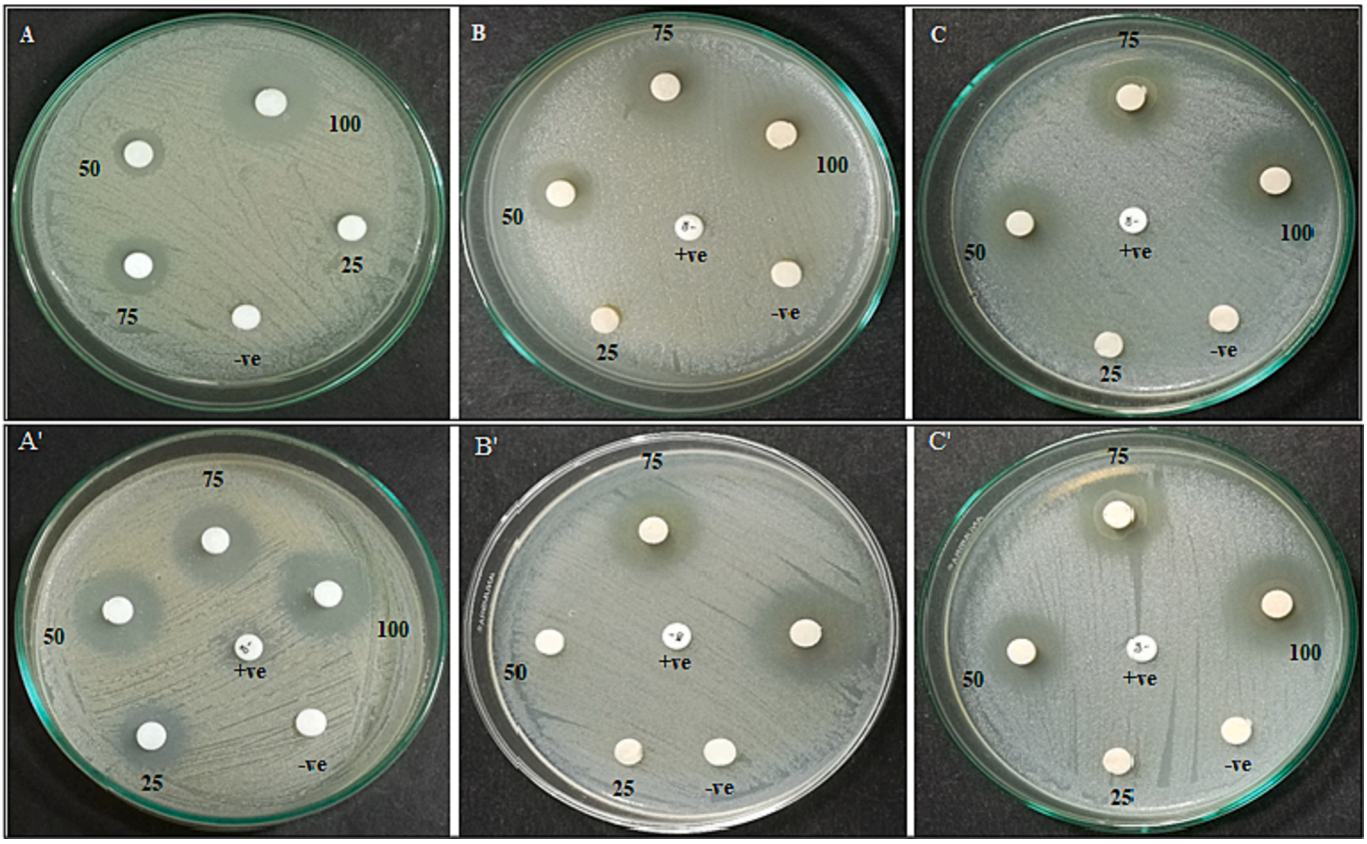

Fig. 4. Anti-Multiple Drug Resistant (MDR) bacterial activity of *F.oxysporum* (TPL11) against (A) MRSA ATCC 43300, (B) MRSA ATCC 700699 and (C) VRE ATCC 51299; *N.sphaerica* (TPL14) against (A') MRSA ATCC 43300, (B') MRSA ATCC 700699 and (C') VRE ATCC 51299 by disc diffusion method at different concentrations (25, 50, 75, 100 μg/mL) after 16–18 hrs incubation.

based on molecular mass, structures and formula and retention time by comparing mass spectra with the MS spectral database (NIST library). The peak range represented a quantifiable proportion of predicted chemicals in the extract. The chromatogram and area percentage analysis obtained are shown in Figs. 5 & 6. The major compound present in TPL11 extract were heptacosanoic acid, methyl ester (RT 18.99, 2.9 %), pterin-6-Carboxylic acid (RT 19.34, 3.9 %), paredrine tms (RT 28.03, 5.4 %). Where, 1,2-benzenedicarboxylic acid, butyl 2-ethylhexyle (RT 19.41, 12.4 %), 8-heptadecene (RT 19.76, 14.5 %), 1,2-benzenedicarboxylic acid, butyl octyl ester (RT 20.34, 2.2 %), 1-docosene (RT

Table 3
Minimum Bacteriostatic and Bactericidal Concentration of ethyl acetate extract.

Bacterial Pathogens	Concentration (µg/mL)					
	MIC		**MBC**		**MIC Index**	
	F.oxysporum	***N.sphaerica***	***F.oxysporum***	***N.sphaerica***	***F.oxysporum***	***N.sphaerica***
MRSA (ATCC 43300)	6.25	3.125	25	12.5	4	4
MRSA (ATCC 700699)	3.125	3.125	12.5	12.5	4	4
S.aureus (ATCC 25923)	6.25	0.78	25	6.25	4	8
S.aureus (MTCC 3160)	3.125	6.25	12.5	12.5	4	2
VRE (ATCC 56299)	1.56	3.125	12.5	25	8	8
E.faecalis	3.125	3.125	25	25	8	8

MIC index $\leq$ 4 is bactericidal and $>$ 4 is bacteriostatic.

Table 4
Synergistic antibacterial effect of ethyl acetate fungal crude extract of *F.oxysporum* and *N.sphaerica* with conventional antibiotics against human pathogens.

Bacterial strains	Antibiotics	MIC (µg/mL)				FICI	Interpretation
		Extract		**Antibiotic**			
		Alone	**Combination**	**Alone**	**Combination**		
F.oxysporum							
MRSA (ATCC 43300)	Oxacillin	6.25	3.12	50	3.12	0.1	Synergistic
	Tetracycline		3.12	25	6.25	0.7	Indifference
	Chloramphenicol		0.78	25	12.5	0.6	Indifference
	Vancomycin		0.19	6.25	0.78	0.1	Synergistic
MRSA (ATCC 700699)	Oxacillin	3.12	1.56	50	3.12	0.5	Synergistic
	Tetracycline		1.56	25	0.78	0.5	Synergistic
	Chloramphenicol		1.56	25	1.56	0.5	Synergistic
	Vancomycin		0.39	6.25	1.56	0.3	Synergistic
VRE (ATCC 51299)	Oxacillin	1.56	0.78	25	0.39	0.5	Synergistic
	Tetracycline		0.78	25	3.12	0.6	Indifference
	Chloramphenicol		0.78	50	0.78	0.5	Synergistic
	Vancomycin		0.19	50	1.56	0.1	Synergistic
N.sphaerica							
MRSA (ATCC 43300)	Oxacillin	3.12	1.56	50	3.12	0.5	Synergistic
	Tetracycline		1.56	25	6.25	0.7	Indifference
	Chloramphenicol		1.56	25	12.5	1	Indifference
	Vancomycin		0.39	6.25	0.78	0.2	Synergistic
MRSA (ATCC 700699)	Oxacillin	3.12	0.78	50	3.12	0.3	Synergistic
	Tetracycline		1.56	25	0.78	0.5	Synergistic
	Chloramphenicol		1.56	25	1.56	0.5	Synergistic
	Vancomycin		0.78	6.25	1.56	0.4	Synergistic
VRE (ATCC 51299)	Oxacillin	3.12	0.78	25	3.12	0.3	Synergistic
	Tetracycline		1.56	50	0.78	0.5	Synergistic
	Chloramphenicol		1.56	50	1.56	0.5	Synergistic
	Vancomycin		0.78	12.5	0.39	0.2	Synergistic

FICI $\leq$ 0.5: synergy, FICI $>$ 0.5 to 4: indifference, FICI $>$ 4: antagonism.

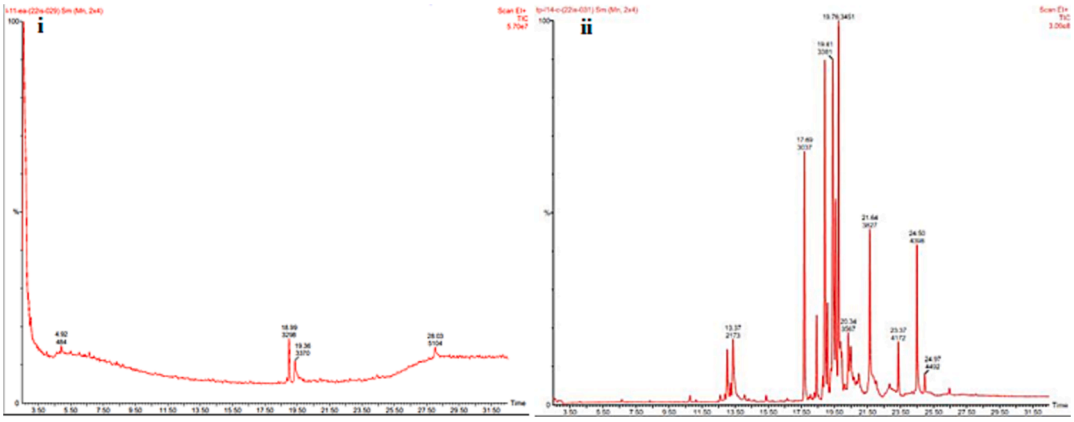

Fig. 5. Gas Chromatography-Mass Spectroscopy ethyl acetate crude extract (i) Chromatogram profile of endophytic fungus *F.oxysporum* TPL11 and (ii) Chromatogram profile of endophytic fungus *N.sphaerica* TPL14.

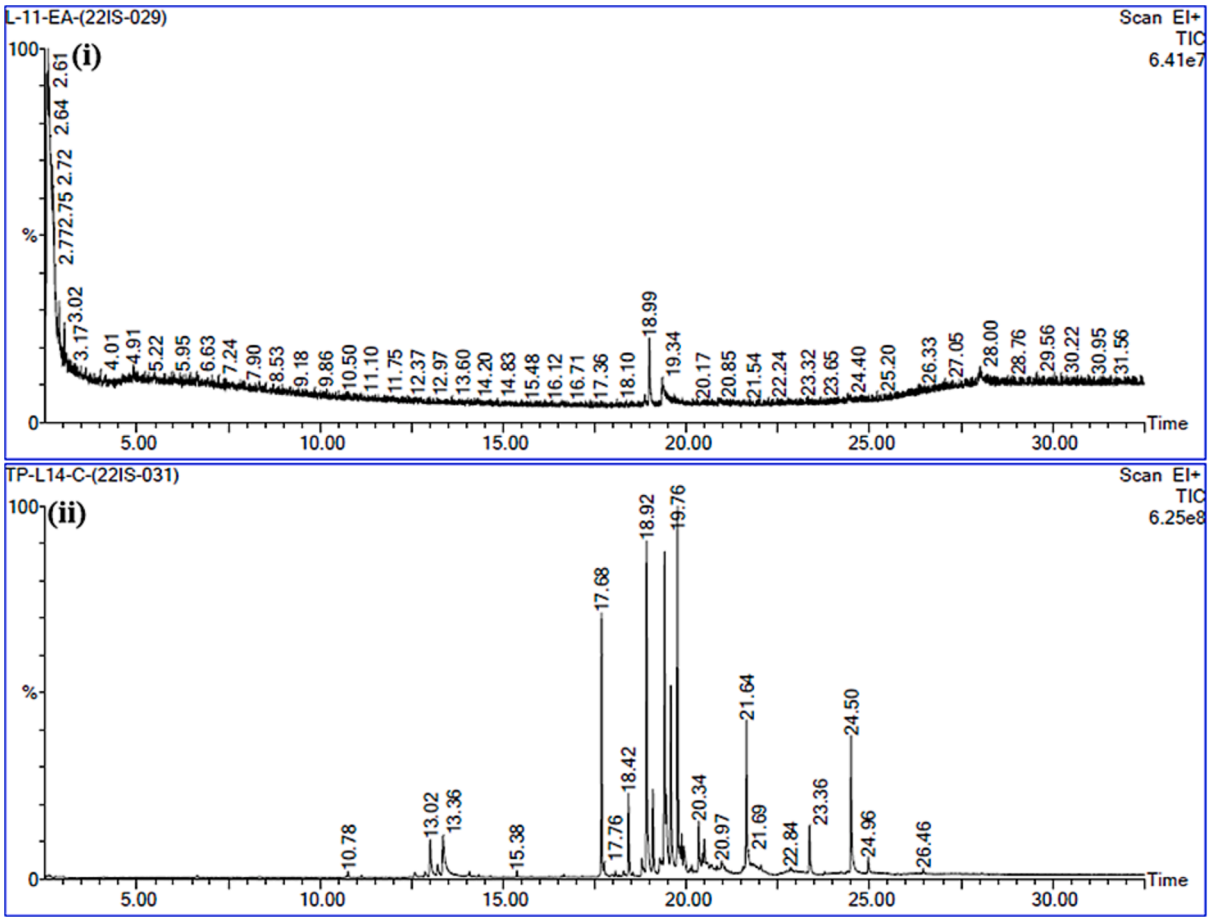

Fig. 6. Area percentage analysis (i) chemical constituents present in ethyl acetate extract of *F.oxysporum* TPL11 and (ii) chemical constituents present in ethyl acetate extract of *N.sphaerica* TPL14.

21.64, 6.7 %) were the major compound present in the TPL14 crude extracts These compounds were known to exhibit a major role in the anti-pathogenic activity. Area peak percentage (%) analysis represents a measurable % of estimated biological compounds ie., it determines an approximate concentration of an analyte present in the fungal crude extract at the respective retention time (RT).

3.8. Spectrum analysis

F.oxysporum FTIR revealed different peaks corresponding to various functional groups. A peak at 3334.68 cm^{-1} specifies the existence of an alcohol (O–H stretch). The peak at 2972.79 and 2930.62 cm^{-1} exposed the existence of the alkanes group (C–H stretch), the peak at 1751.54 cm^{-1} relates to the carboxylic acid group (C = O stretch). The peaks 1598.14 and 1450.10 cm^{-1} were alkane (C–H stretch). Peak 1055.62 and 1008.60 cm^{-1} was the existence of esters (C-O stretch). Peak 887.12 and 815.10 cm^{-1} correspond to alkene stretch (C–H). A peak at 521.16 cm^{-1} was due to the halide (C–H stretch) presence. Whereas, *N. sphaerica* revealed peaks at 3295.81 (alcohol), 2957.31 (alkanes), 2917.32 (alkanes), 2856.01 (alkanes), 1590.01 cm^{-1} (alkane), 1459.99 cm^{-1} (alkane), 1377.30 cm^{-1} (alkane), 1316.68 cm^{-1} (alkane), 1267.26 cm^{-1} (alkane), 1074.54 (esters), 1032.70 cm^{-1} (esters), 590.25 cm^{-1} (halide).

4. Discussion

Endophytes are now recognized to be capable of manufacturing chemicals, antibiotics, and a variety of other biotechnologically interesting compounds, in addition to supporting plant protection. Endophytic microbe's ability to create a variety of secondary metabolites makes them an intriguing source in the hunt for novel antimicrobial agents, and their use in the food and cosmetics industries can contribute to a variety of biotechnological applications (Silva et al., 2022). Endophytic fungi have been identified as a source of new secondary metabolites with biologically beneficial attributes. Numerous investigations have been undertaken to assess the antibacterial potential of fungal extracts (Avinash et al., 2015).

Previously a study reported that phomalactone produced from *N. sphaerica* of *Adiantum philippense* showed an efficient inhibitory impact against *E. coli* and *Xanthomonas campestris* in a disc diffusion investigation, with a considerable MIC at 150 g/ml concentration (Ramesha et al., 2020). After 12 h of treatment with ethyl acetate extract, *N. sphaerica* cultivated from old leaves of *Swietenia macrophylla* showed a spectacular growth-killing profile on MRSA, resulting in cell membrane rupture and cell death (Ibrahim et al., 2015). A novel chemical, nigronapthaphenyl, was discovered by bioactive *N. sphaerica* ethyl acetate extract of *Bruguiera gymnorrhyza* plant and showed significant antagonistic effects against *B.cereus* TISTR 688 with MIC of 2 µg/mL and *B. subtilis* TISTR 088 with MIC of 4 µg/mL. It also showed anticancer activity against the HCT 116 colon cancer cell line, with an IC50 of 9.62 ± 0.5 µM. The chemical was also anti-inflammatory and anti-diabetic (Ukwatta et al., 2019). Lactones, pyrones, nigrosporolides, diterpenes, nigrosporins, nigrosporolides, diketopiperazines, and epoxydons have all been new antimicrobial secondary substances reported to be harvested by *N. sphaerica* (Chutulo et al., 2020).

Similarly, a study reported that *F.oxysporum* from *Psidium guava* leaves inhibited the tested bacteria in an extensive spectrum of zones ranging from 6.67 to 0.58 mm to 22–1 mm, with MIC values ranging

from 0.156 to 5.0 mg/ml and MBC values ranging from 0.625 to 10.0 mg/ml (Chutulo et al., 2020). With ZOI ranging from 17 to 9 mm, the culture supernatant of *F.oxysporum* 01 from *Catharanthus roseus* displayed a considerable antibacterial impact on *S. aureus*, *V.parahaemolyticus*, *P.aeruginosa, E.coli*, and *Serratia marcescens*. It also showed antioxidant and anticancer properties in Hep-G2 and MCF-7 cells. (Tu et al., 2021). The chemicals obtained from an ethyl acetate extract of *Fusarium* sp. (internal strain 3042) were colletochlorin B, 4,5-dihydrodechloroascochlorin, llicicolin B, colletorin B, ascochlorin, and 4,5-dihydroascochlorin. Against *Chlorella fusca, F.oxysporum, Ustilago violacea*, the compound colletochlorin, llicicolin B and Colletorin B demonstrated antimicrobial action. Similarly, 4,5-dihydroascochlorin showed an antibacterial on *Bacillus megaterium* growth and 4,5-dihydrodechloroascochlorin showed very strong antifungal activity towards *Eurotium repens* growth (Hussain et al., 2015). The strain BH-3 derived from *Lilium lancifolium* had an inhibitor impact against *Leuc. Mesenteroides* (Liu et al., 2012). Ethyl acetate extract of *F. oxyporum* was isolated from the bark of *Cinnamomum kanehirae*, resulting in the identification of two novel compounds, a new oxysporidinone analog and a new 3-hydroxyl-2-piperidinone derivative and known compounds, fusarinolic acid, gibepyrone D, fusaruside, beauvericin, (−)-4,6′-anhydrooxysporidinone, cerevisterol, (2S,2′R,3R,3′E,4E,8E)-1-O-D-glucopyranosyl-2-N-(2′-hydroxy-3′-octadecenoyl)-3-hydroxy-9-methyl-4,8-sphingadienine. Beauvericin displayed anticancer impact on human cell line A549, PANC-1 and C-3 by MTT method. Further, it demonstrated a substantial antibacterial effect on MRSA with MIC of 3.125 μg/mL and *B. subtilis* (ATCC 66333) (Wang et al., 2011).

Inhibitors were found for the first time in crude extracts of endophytic fungus obtained from *T. pallida*, establishing the framework for future investigation. More studies into the bioactive chemicals of the extracts should be conducted to gain a better understanding of the potential of natural inhibitors. According to the study's findings, the compounds detected in the extracts can be used as antibacterial agents to treat a variety of microbial illnesses. However, more study is needed to analyze all of the costs and benefits of hiding fungal endophytes in a range of environmental settings to increase the use and proficiency of endophytes in pharmaceutics.

5. Conclusion

In conclusion, our findings revealed various endophytic fungi from different tissues of the *T.pallida* plant. This approach is the foremost report of *F.oxysporum* and *N. sphaerica* from the leaves of *T.pallida* and their antibacterial investigation. Agar plug diffusion, disc diffusion and MIC displayed a broad range of activity. Thereby it indicates both the fungal endophytes from the plant serve as potent producers of antibacterial secondary metabolites. It is also evident that they can serve as potential antibacterial mediators against a wide range of clinically important bacterial pathogens. More studies into the bioactive chemicals of the extracts should be conducted to gain a superior understanding of the potential activities of natural inhibitors. The chemicals found in the extracts, according to the study's findings, can be employed in therapeutic remedies to protect plants and eukaryotic models. Additional experiments on the isolation of pure compounds from the ethyl acetate crude extract may generate new novel natural bioactive compounds.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further reading

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