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Research article

Exploring anticancer, antioxidant, and antimicrobial potential of *Aspergillus flavus*, a fungal endophyte isolated from *Dillenia indica* leaf callus

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ABSTRACT

Background: Endophytic fungi represent a compelling assemblage of microorganisms that inhabit plant tissues without inflicting any discernible detriment to the host organism. They foster a symbiotic association with their host plants, frequently conferring advantages such as augmented growth, enhanced resilience to stressors, and safeguarding against pathogens.
Study design: Dillenia indica is a medicinal tree of Dilleniaceae. This study aims to isolate and identify the fungi growing as a contaminant in leaf callus. For the identification, both morphological observation and molecular methods were used. The presence of secondary metabolites in different fungal extracts were observed by FTIR and High-resolution accurate mass spectroscopy (HRAMS) methods. Different biological activities (antioxidant, antibacterial and antitumor) of fungal extracts were assessed.
Methods: For callus initiation, leaf tissues of *Dillenia indica* were inoculated on Murashige and Skoog's medium supplemented with BAP (1mgl-1) and NAA (1mgl-1) plant growth regulators. To raise pure cultures of endophyte, fungal hyphae were isolated from the contaminated cultures and were grown on Potato Dextrose Agar medium. For molecular identification, genomic DNA (gDNA) was isolated from fungal mycelia. Internal transcribed spacers (ITS1 and ITS4) were used to amplify the conserved ITS region of the fungal gDNA. Previously deposited sequences in the Gene bank were used for the identification and making of phylogenetic tree. Antioxidant, antibacterial and anticancer potential of fungal extracts were studied.
Results: The endophyte was identified as *Aspergillus flavus*. FTIR study showed the presence of diverse types of secondary metabolites in fungal extract. A significant presence of phenolics, flavonoids, terpenes, steroids, etc. was observed by High-resolution accurate mass spectroscopy

Abbreviations: 2,4-D, 2,4 – dichlorophenoxyacetic acid; BAP, 6- Benzylaminopurine; KIN, Kinetin; NAA, Naphthalene acetic acid; gDNA, Genomic DNA; BLAST, Basic local alignment search tool; FTIR, Fourier Transform Infrared Spectroscopy; DPPH, 2,2-Diphenyl-1-picrylhydrazyl.

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analysis (HRAMS) of fungal extract. Endophyte extract prepared in chloroform showed both antioxidant (IC_{50} 430.23) and antibacterial (maximum inhibition of *E. coli*:15 ± 0.62 mm) potential compared to other solvents. Cell viability decreased at high concentrations of endophyte extract prepared in chloroform and ethyl acetate solvents. Fungal extract prepared in ethyl acetate showed considerable cytotoxicity and growth inhibition of DL tumor cells.
Conclusion: In the present study, isolated endophyte of *Dillenia indica* showed high occurrence of secondary metabolites. Fungal extracts showed antioxidant, antibacterial and antitumor activities. As, endophytes are remarkable source of active constituents, there is a great need to explore such endophytes. Their extensive studies are required to develop an alternative of plant less production of valuable compounds.

1. Introduction

Plant tissue culture is an efficient technology for the multiplication and conservation of plants. The technology has emerged as a major platform for the biotechnological approach to plant improvement, production of secondary metabolites, and development of genetically engineered plants. Aseptic condition is a major requisite for the maintenance and establishment of in vitro cultures. High nutritional availability in tissue culture medium provides a suitable substrate for microbial contamination. The Presence of endophytes in explant is another major reason for culture contamination. Different interactions exist between plants and fungi such as saprophytic, mutualistic, competitive, parasitic, pathogenic, etc [1,2]. Plants and fungi are non-motile eukaryotes. Both show the presence of diverse metabolic pathways and the compounds thus produced are involved in nutrient circulation, chemical defense, and other activities. Plants directly provide some nutrition to their colonized fungi. Colonized fungi on plants accumulate valuable free nutrients that can enrich free amino acids, non-structured carbohydrates, organic nitrogen-containing compounds, vitamins, and lipids in plants or fungal tissues [3]. Fungal colonization induces the chemical defense process in plants by increasing the levels of terpenoids, alkaloids, fatty acid derivatives, etc. Plants and fungi are rich reservoirs of secondary metabolites. Phytopathogenic fungi produce some special secondary metabolites, such as phytotoxins and phytoalexins. Endophytes are of great therapeutic values due to the presence of diverse types of metabolites [4]. Many fungi show an association with plants as symptomless endophytes. They can develop as pathogens or saprotrophs. Spores of *Aspergillus* species are among the microbial cells with the greatest longevity, highest tolerances to heat, pressure, and chaotropic, and ability to germinate at the lowest water activity. *Aspergillus flavus* produces a broad spectrum of degrading enzymes. It behaves as an opportunistic pathogen by utilizing the nutrition of culture medium [5].

Dillenia indica, commonly known as Elephant Apple, is a medicinal plant and it has been used in various traditional herbal formulations. Taha et al [6] developed a regeneration protocol for *D. indica* by using nodal explants. The plant contains several active constituents such as polyphenols, tannins, alkaloids, steroids, saponins, and flavonoids. Betulin and betulinic acid are two major constituents of plants and both phytochemicals show anti-cancer, anti-diabetic, anti-inflammatory, and other pharmacological

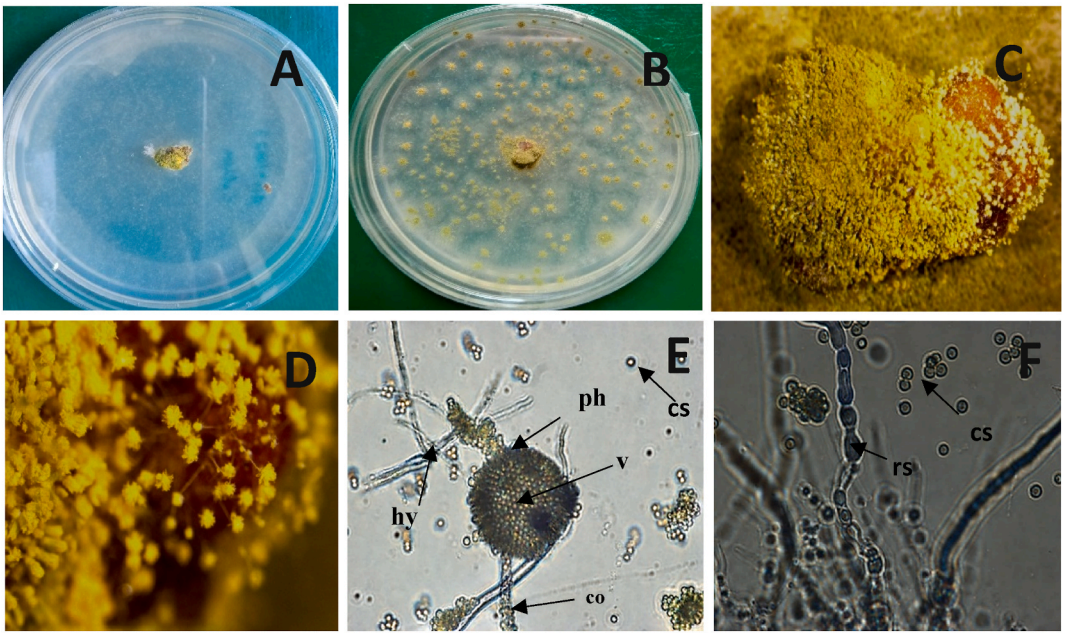

Fig. 1. (A) Inoculation of contaminated callus, (B)–(D) Fungal growth on PDA media, (E) Magnified image of fungi showing different structures: hyphae (hy), conidiophore (co), phialides (ph), vesicle (v), conidiospore (cs), (F) Image showing resting spore (rs) & conidiospore (cs).

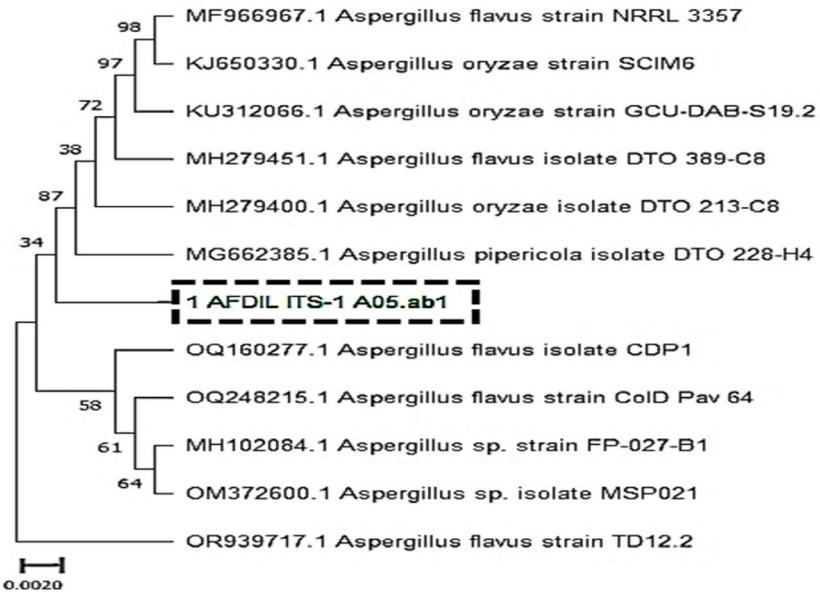

Fig. 2. The phylogenetic tree was constructed by Mega 11 using the neighbor-joining method having bootstraps values of 1000 per runs. The constructed tree was based on the ITS rDNA gene sequence of isolated endophytic fungus and its related species. An optimal tree is shown, with a branch length sum of 0.027. The branch lengths are in the same units as the evolutionary distances used to estimate the phylogenetic tree, and the tree is drawn to scale. The evolutionary distances were calculated by using the Maximum Composite Likelihood technique and are in base substitutions per site unit.

activities. Twenty-five endophytes were isolated from *D. indica* by Prasher and Kumar [7]. In recent years, researchers have turned their attention towards understanding the endophytic microbial communities residing within plants, and *A. flavus* has emerged as a prominent candidate. This study aims to explore the fungal endophyte *Aspergillus flavus* isolated from *D.indica* callus cultures and its potential applications in antimicrobial, antioxidant, and in vitro anticancer activities.

2. Materials and methods

2.1. Chemicals and reagents

Potato dextrose agar (Hi-media, India), NAA (Sigma Aldrich, United State), BAP (Hi-Media, India) Streptomycin (Hi-Media, India), KBr (Hi-Media, India), Nutrient agar media (Hi-Media, India), DPPH (Hi-Media, India), RPMI 1640 Media (Sigma Aldrich, United State), MTT (Hi media, India), Annexin V PE (Biolegend, San Diego, California), Annexin V-FITC/PI (Biolegend, San Diego, California) etc.

2.2. Plant collection and callus induction

The leaves of *D.indica* were collected from the Ayurvedic garden of the Dravyaguna Department, Banaras Hindu University in March (Fig. 1). Plants were identified by Prof. Nawal Kishore Dubey, Department of Botany, Banaras Hindu University, Varanasi (*Accession No. Dillenia 2023/01*) (25.27444376734682, 82.99832720402496). Leaves were washed under running tap water for 15–20 min. For the surface sterilization, leaves were transferred to a conical flask containing 100 ml of distilled water. Further 2–3 drops of tween-20 and 3 % (v/v) sodium hypochlorite were added to the flask and the leaves were rinsed for about 20 min. Water was drained from the flask and leaves were washed thrice with distilled water. Further sterilization of leaves was carried out under laminar flow. After that leaves were sterilized again by using 0.02 % mercuric chloride for 2 min and washed with autoclaved double distilled water twice. Then leaves were rinsed with 70 % alcohol for 30 s and washed thrice with sterile double distilled water. The explants of 1 $\times$ 1 cm^2 size were prepared by using a sterilized scalpel and were dried on autoclaved blotting paper. For callus initiation, Murashige and Skoog's medium supplemented with different growth regulators were taken either alone or in combinations. Different concentrations of 2,4-D were taken and their combinations with kinetin were also tried. Similarly, different concentrations of BAP alone and their combinations with NAA were tried. The cultures were then transferred to the culture room at 25 $\pm$ 2° C with 16/8 h (light/dark) photoperiod for 3–4 weeks. After callus initiation, calli were subcultured either on the medium of the same composition or MS basal medium for their proliferation and differentiation.

2.3. Collection of endophytic fungi isolation and purification

After 2–3 subcultures, several replicates of calli showed the occurrence of a contamination problem. Fungi from such calli were isolated and their pure culture was raised (Fig. 2-A-F) as per the methodology used by Ref. [8]. The fungal hyphae were isolated from the callus inside the laminar air flow chamber and were grown on the potato dextrose agar (PDA) plates containing Streptomycin at a concentration of 250 μg/ml to overcome bacterial contamination. The PDA plates were incubated in REMI Orbital Shaking Incubator (REMI CIS-24 PLUS) at 27 ± 2 °C for about 8–12 days. After that, the mycelia were subcultured on fresh PDA plates. Subculturing was done till the development of pure individual colonies.

2.4. Morphological and molecular identification

To observe morphological characterization, semi-permanent slides of fungal mycelia were prepared according to the methodology used by Ref. [9]. A fungal colony was taken from a PDA plate using a sterile needle and placed on a glass slide. For its staining, a few drops of lacto-phenol cotton blue were added and the slide was examined under a bright field microscope (Nikon Eclipse Ni, H600L, Japan). The Nucleo-pore gDNA fungus/Bacterial micro kit was used to isolate genomic DNA (gDNA) from fungal mycelia for molecular identification. The manufacturer's instructions mentioned on the kit were used for the extraction of DNA, and synthesis of universal primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′). ITS1 and ITS4 were used to amplify the conserved ITS region of fungal gDNA. The composition of the PCR reaction mixture taken was as such: 2 μL of extracted gDNA +1.5 μL of each forward and reverse primer (10 μM concentration) + 2 μL of buffer + 0.5 μL of Taq DNA polymerase (BR Biochem, New Delhi, India) + 0.75 μL of 10 mM deoxynucleotide triphosphates (BR Biochem). Milli-Q water was used to maintain the volume up to 20 μL. The steps of PCR reaction were as such: (i) 5-min initial denaturation phase at 94° C was followed by 35 cycles of 30 s at 94° C, (ii) 40 s of annealing at 60° C, (iii) 1 min of extension at 74° C and (iv) 10-min final extension step at 74° C. After the completion of the PCR cycle, 2 % agarose gel in 1X TAE buffer gel electrophoresis was used to evaluate the PCR results. ITS1 and ITS4 primers were used to sequence the purified PCR product after it had been purified using the Nucleopore Quick PCR Purification Kit in accordance with the manufacturer's instructions.

2.5. Phylogenetic tree construction

The acquired Sanger sequence was queried within the NCBI repository utilizing the Basic Local Alignment Search Tool (BLAST) accessible at https://www.ncbinlmgov/BLAST [10]. The identification of the fungus at the species level was done through a comparative analysis of sequences against those previously deposited in the Gene bank. Utilizing CLUSTAL W, multiple sequence alignments of the Internal Transcribed Spacer (ITS) regions of endophytic fungi were conducted alongside reference taxa. The establishment of evolutionary relationships and construction of a phylogenetic tree encompassing the isolated taxa and their respective species were facilitated by MEGA 11 software employing the neighbor-joining method [11]. The Internal Transcribed Spacer (ITS) consensus sequences were employed to configure the phylogenetic tree with 1000 replications bootstrap [12].

2.6. Fermentation and extraction of crude extract

Pure individual colonies from the plates were fermented in PDA broth for three weeks in six conical flasks (250 ml) supplemented with streptomycin (250 μg/mL). After every week the solvents were filtered through Whatman filter paper (No. 1) to prevent any contamination followed by extraction in three different solvents, viz., Methanol (Me) chloroform (CH) and ethyl acetate (EA), by using a separating funnel in different flasks labeled as Me1, CH1, and EA1 respectively for the 1st-week ferment extract in methanol, chloroform, and ethyl acetate. Likewise, 2nd and 3rd week ferments were extracted and labeled as Me2, CH2, EA2 and Me3, CH3, EA3 respectively.

2.7. Fourier-transform infrared spectroscopy (FTIR) evaluation

Fourier transform infrared (FTIR) spectroscopy is widely used as a tool to understand key cellular compositions [13] as infrared spectra provide detailed information about various cellular components such as proteins, polysaccharides, and lipids [14]. The extracts (filtered) were evaluated in the Bruker Alpha II FTIR (PerkinElmer) instrument. Further analysis was conducted using FTIR spectroscopy to identify the functional groups associated with the different fungal extracts. The spectral data was collected in the range of 400–4000 cm^{-1} utilizing the attenuated total reflectance (ATR) technique with an IR spectrophotometer.

2.8. Identification of compounds through High-Resolution Accurate Mass Spectrometry system (HRAMS)

High- Resolution Accurate Mass Spectrometry (HRAMS), a powerful analytical technique was used for the identification and quantification of compounds in various samples. The technique relies on the measurement of accurate masses of ions with high resolution, providing detailed information about the elemental composition of the compounds. In mass spectrometry, the sample becomes ionized, meaning that molecules are converted into ions. Ions are accelerated into the mass analyzer, where they are separated based on their mass-to-charge ratio (*m/z*). Positive and negative modes were analyzed to detect the maximum number of compounds [15]. For HRAMS, all samples were dissolved in HRMS grade methanol and filtered through 0.02 μm pore size syringe filter paper. Then, the

presence of compounds in the samples was observed by HRAMS.

2.9. Antioxidant activity

To assess antioxidant potential, the free radical scavenging method by DPPH was used as per the procedure followed by Ref. [16] with some modifications.0.02 mg DPPH was taken and dissolved in 100 ml methanol. Triplicate reading was taken for each concentration (50, 100, 200, 400, 600, 800, and 950 μg/ml) of fungal extract. For each replicate of different concentrations, 300 μL of fungal extract was taken in the test tube and 3 ml of DPPH was added. The mixture was kept in the dark for 15 min at room temperature. The absorbance was recorded at 517 nm in a UV–Vis-spectrophotometer. A Methanolic solution of DPPH (3 ml) and methanol (300 μL) was used as a blank.

The antioxidant activity of the isolated extracts was evaluated using DPPH free radical scavenging test. For each extract, IC_{50} values were calculated using Graph Pad Prism 8.0.2 software, and the antioxidant test data were analyzed for statistical significance using the one-way ANOVA method. The percent inhibition were calculated by the formula mentioned bellow.

Percent inhibition = [(Absorbance of Control–Absorbance of sample)/ Absorbance of Control]*100

2.10. Antibacterial activity

For the antibacterial assay, 30 mL of nutrient agar media were poured into sterilized petri plates and allowed to solidify. A bacterial colony was grown by adding 20 μL of freshly prepared bacterial broth culture. The process was the same as followed by Gupta et al. [16] with slight modifications. Specifically, 6 mm diameter discs were placed on the plates, on which 10 μL of extracts were applied. The antibacterial activity of the extracts was compared with streptomycin (positive control), while DMSO served as the negative control. Following inoculation, the plates were incubated at 37 °C for 24 h, and the diameter of the zone of inhibition was measured in millimeters.

2.11. Other activities: cytotoxicity, cell viability, cell growth inhibition and apoptosis

2.11.1. Cell culture

Murine Dalton's lymphoma (DL) cells were taken to study cytotoxicity, cell viability, and cell growth inhibition assays of fungal extracts. Roswell Park Memorial Institute (RPMI) i.e. RPMI 1640 (Invitrogen, Carlsbad, CA) medium was used for growing DL cells as suggested for mammalian cell culture by Moore et al. (1967). RPMI 1640 supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT) + 100 U/ml penicillin +100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) was used as a complete medium for the maintenance of DL cells. DL cells were also grown as semisolid tumors in the peritoneum of BALB/c mice following serial transplant in order to maintain the tumorigenicity potential.

2.11.2. Cytotoxic assay

The cytotoxic effects of the extracts Me3, CH3, and EA3 against DL cells were measured by using CytoTox 96 cytotoxicity assay kit, (Promega, USA) as used by Ref. [17]. Tumor target cells (5 × 103) were co-cultured in the presence of increasing concentrations of the indicated formulations in a 96-well culture dish. The cells were incubated for 18 h at 37° C, 5 % CO_2. Specific lysis (percentage of cytotoxicity) was calculated by under mentioned formula:

$$\%\ \text{Cytotoxicity} = \frac{(\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous})}{(\text{Target Maximum} - \text{Target Spontaneous})} \times 100$$

2.11.3. Cell viability assay

Effect of Me3, CH3, and EA3 on DL tumour cell viability was estimated by a colorimetric XTT assay. XTT colorimetric assay is based on the reduction of tetrazolium salt (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate or XTT to an orange formazan dye by metabolically active cells (Altman, 1976). Tumour cells were seeded (5 × 103 cells/well) in a 96-well culture dish and incubated for 18 h at 37° C, 5 % CO_2. In a plate reader (Synergy HT, BioTek, USA) the OD was recorded at 450 nm. The proportion of viable cells was computed using the formula below [17].

$$\%\ \text{Cell Viability} = \frac{\text{Experimental OD}_{450}}{\text{Control OD}_{450}} \times 100$$

2.11.4. Cell growth inhibition assay

MTT assay was used to check the growth inhibitory potential of different extracts (Me3, CH3 & EA3) against DL cells by the methodology of [18]. The MTT reagent (3–4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a mono-tetrazolium salt. Metabolically active cells reduce MTT to a violet-blue water-insoluble molecule called formazan. Water-insoluble formazan (5 mg) was dissolved in 1 ml Dimethylsulfoxide (DMSO). Optical densities of homogenized MTT-formazan solution were taken by a plate reader at 570 nm. Tumor target cells (5 × 103 cells/well) were transferred to a 96-well culture dish and were treated with serial

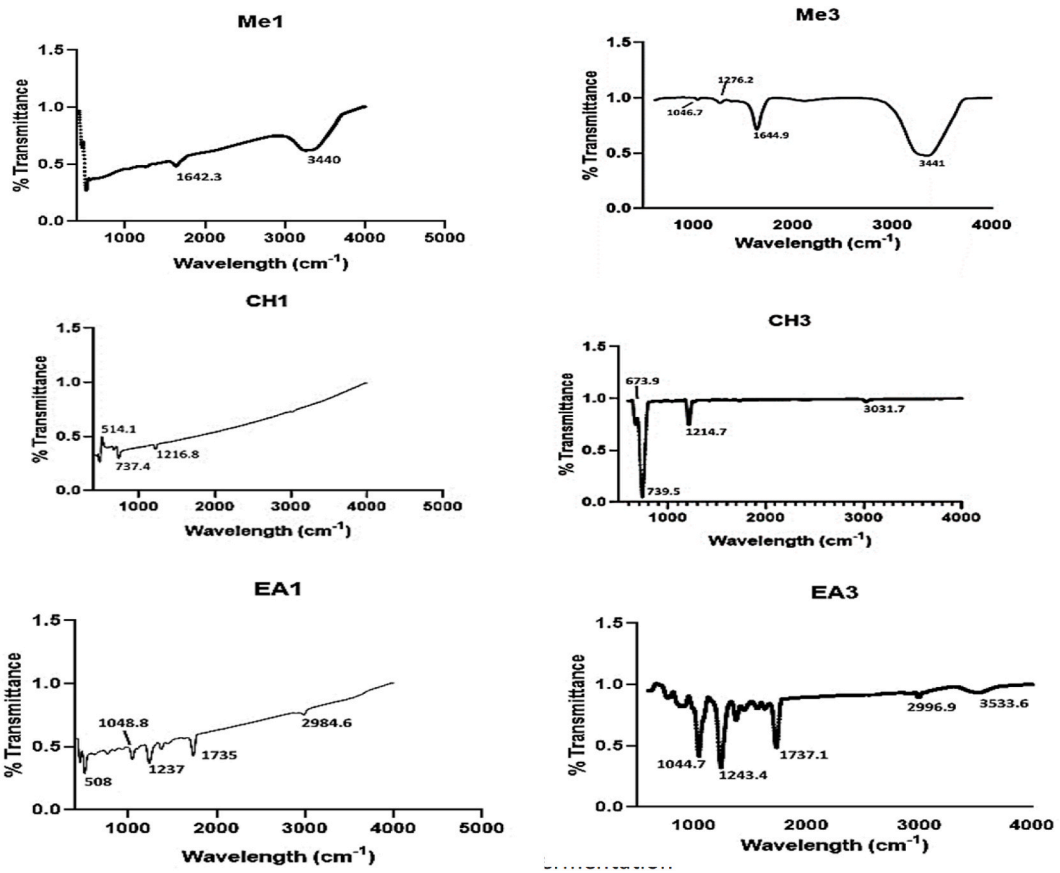

Fig. 3. FTIR peaks of different fungal extracts: 1-week fermented (Me1, CH1, EA1) and 3- week fermented (Me3, CH3, and EA3).

concentrations of various extracts. Following incubation at 37° C, 5 % CO_2, for 48 h, the proliferation of the tumor cells was observed by MTT assay using a CellTiter 96 kit (Promega, USA). Optical densities of homogenized MTT-formazan solution were taken by a plate reader (BioTek, USA) (Hira, 2014) at 570 nm. Percent inhibition of the tumor cells was calculated using the under-mentioned formula:

$$\% \text{ Growth Inhibition} = \left[1 - \frac{\text{Experimental OD}_{570}}{\text{Target OD}_{570}}\right] \times 100$$

The Experimental OD indicates the values of the tumor cells in the presence of the extracts and the Target OD indicates the corresponding values of untreated tumor cells cultured in medium only.

2.11.5. Apoptosis study

For the evaluation of apoptotic cell death, DL tumor cells were treated with different extracts (50 μg/ml) for 12 h. Untreated cells were used as a positive control. The cells were washed in PBS (Phosphate-buffered saline) and stained with PE (phosphoerythrin) conjugated Annexin V for 30 min. These cells were washed in the Annexin buffer. PE-conjugated Annexin V-positive cells were visualized under a fluorescence microscope (EVOS FL Cell Imaging System equipped with Plan Fluor, 40 × , NA 0.75 objective, Life Technologies) as described earlier by Ref. [19].

2.12. Statistical analysis

Unpaired student's t-test or one way ANOVA followed by Tukey's post hoc test was performed while comparing between the groups. Each experiment was performed in triplicate and the data were presented as mean ± SD (standard deviation). Differences were considered significant for 'p' values < 0.01–0.05 (*), $p < 0.001$–0.01 (**), $p < 0.0001$–0.001 (***) and $p < 0.0001$ (****). ns = non-significant.

3. Results and discussion

Endophytes have emerged as a novel source of promising therapeutic agents. Their potential has been exploited for industrial,

Table 1
Different peak positions with their functional groups (A) Me1, (B) CH1, and (C) EA1 in 1 week fermented extract.

Me1 (A)			CH1 (B)		EA1 (C)	
Peak	X (cm^{-1})	Functional Group	X (cm^{-1})	Functional Group	X (cm^{-1})	Functional Group
1.	1642.3	C=C stretching vibrations (presence of alkenes)	737.4	N-H wag stretching vibration (presence of primary, secondary amines)	508	C-Cl stretching vibration (presence of halogen compounds)
2.	3440	O-H stretching vibration (presence of alcohols, phenols)	1216.8	C-N stretching vibration (presence of aliphatic amines)	1048.8	C-N stretching vibration (presence of aliphatic amine)
3.			3031.7	O-H stretching vibration (presence of carboxylic acids), C-H stretching vibration (presence of alkenes)	1237	C-N stretching vibration (presence of aliphatic amine)
4.					1735	C=O stretching vibration (presence of ester fatty acid groups)
5.					2984.6	C-H stretching vibration (presence of alkenes)

Table 2
Different peak positions with their functional groups (A) Me3, (B) CH3, and (C) EA3 in 3 weeks fermented extract.

Me3 (A)			CH3 (B)		EA3 (C)	
Peak	X (cm^{-1})	Functional Group	X (cm^{-1})	Functional Group	X (cm^{-1})	Functional Group
1.	1046.7	C−N stretching vibration (presence of aliphatic amines)	673.9	C-Br stretching vibration (presence of alkyl halides)	1044.7	C-N stretching vibration (presence of aliphatic amines)
2.	1276.2	C-O stretching vibration (presence of carboxylic acid)	739.5	N-H wag stretching vibration (presence of primary, secondary amines)	1243.4	C-O stretching vibration (presence of alcohols, carboxylic acids, esters, ethers)
3.	1644.9	C=O stretching vibrations (presence of carbonyl group, amide)	1214.7	C-O stretching vibration (presence of alcohols, carboxylic acids, esters, ethers)	1737.1	C=O stretching vibration (presence of carbonyl compounds)
4.	3441	O-H stretching vibration (presence of alcohols, phenols)	3031.7	O-H stretching vibration (presence of carboxylic acid), C-H stretching vibration (presence of alkenes)	2996.9	C-H stretching vibration (presence of aliphatic compounds)
5.					3533.6	O-H stretching vibration (presence of carboxyl, hydroxyl compounds)

agricultural, and medicinal uses. Endophytes of medicinal plants show similar biosynthetic capabilities as their host plants [20,21]. Taxol production at an industrial scale has been possible from endophytic fungi of Taxus [22]. Endophytes also produced other high-value compounds such as podophyllotoxin, camptothecin, vinblastine, and vincristine [32]. The present study showed floccose surface formation by the isolated fungal colonies. Initially, the color of the colony was white which later turned to yellow-greenish (Fig. 1). The hyphae were hyaline with a smooth texture, unbranched, aseptate, and the range of their diameter was observed from 250 μm to 450 μm. Dense mycelial growth of fungi took place on the PDA medium (Fig. 1A–D). Conidial spores were visible in microscopic observation of the colony (Fig. 1E). Rough textured conidiophores arise from the hyphae of the fungi. These conidiophores bear vesicles on which uni- or biseriate phialides were arranged. Conidial spores were formed on the phialides. Resting spores were also seen (Fig. 1F). Similar morphological characteristics of *Aspergillus flavus* were observed by Arifah et al. [23]. Fungal endophytes, isolated from the callus of *D. indica* leaves were identified as *Aspergillus flavus* by ITS sequencing. ITS sequences were compared with the sequences of the GenBank database to identify the isolated fungi at the molecular level based on the highly conserved Internal Transcribe Spacer (ITS) region. PCR product of amplified ITS region yielded 638 bp sequences. The sequence after BLAST analysis showed 98 % similarity with *Aspergillus flavus*. Finally, Sequences of closely related species were aligned using ClustalW, and a phylogenetic tree was drawn using Mega 11 software (Fig. 2). Phenotypic identification is an important tool for fungi, but based on phenotypic characters is not reliable [24]. Internal transcribed spacer region of fungi is widely used for more accurate identification of the fungi [25,26]. The phylogenetic tree showed the evolutionary relationship of the endophyte with other fungi. FTIR analysis of fungal extract showed the presence of several active constituents in different solvents (Fig. 3). After a 3-week fermentation of fungal extract, more active constituents were observed. Most of the biosynthetic gene clusters of fungi are not expressed under normal growth conditions [27]. So, scientists are now trying to activate cryptic biosynthetic genes by different methods such as genetic manipulation of transcription and translation, use of elicitors, metabolism remodeling, and co-cultivation [28]. The fermentation process of fungi can activate only some of the enzymes, which may witness the presence of some more secondary metabolites. Optimization of culture conditions of the fermentation is required to maximize the production of secondary metabolites [29]. In the present study, only a few peaks increased in the FTIR assay after three weeks of fermentation. Two FTIR peaks were observed for methyl extract of 1-week

Table 3
HRAMS Data showing Compounds in Different Fungal Extracts.

S.No	CH3	EA3	Me3
1.	Apronalide	Brassylic acid	Aceglutamide
2.	Benzodepa	Clobetasone	Butabarbital
3.	Clavulanic acid	Deterryle stearate	Cianidanol
4.	Doxofylline	Etilevodopa	Diacetylphloroglucinol
5.	Eglumetad	Fraxetin	Eptazocine
6.	Fosfosal	Gentisic acid	Fusarenone x
7.	Guvacine	Guanadrel	Guanadrel
8.	Jervine	Homaline	Hodgkinsine
9.	Hypusine	Iclaprim	Ibuprofen
10.	Itaconic acid	Karwinaphthol b	Kynurenine
11.	Lactide	Mitomycin	Midodrine
12.	Moxaverine	Neurosporaxanthin	Nefazodone
13.	Nifurimide	Pinoxodan	Oxetacaine
14.	Osalmid	Quinoxalin	Pirbuterol
15.	Propofol	Resveratrol	Quinolinic acid
16.	rolodine	Ryania	Resveratrol
17.	Tricetamide	Benzoyl glucuronide	Sibutramine
18.	rutaevin	Shanzhiside	Thiouric acid
19.	Sancycline	Terephthalic acid	Threonylserine
20.	Sotalol	Adlerol	Valclavam
21.	Tretinoin	Altretamine	Xylitol
22.	Salidroside	Xylitol	Zearalenone
23.	Santene	Yangonin	
24.	valganciclovir	Ursolic acid	
25.	Vanillin	frescolat ML	
26.	Veralipride	Veliparib	
27.	Vorinostat		
28.	Vulgaxanthin-II		
29.	Xaliproden		
30.	Zanoterone		
31.	Zearalenone		

fermented fungal isolates indicating the presence of alkenes, alcohols, and phenols, whereas four peaks of FTIR were observed for 3-week fermented methanolic fungal extract showing the presence of more compounds such as aliphatic amines, carboxylic acids, carbonyl compounds, amide, alcohols and phenols (Tables 1 and 2). Similarly, a fungal extract prepared in chloroform solvent showed only three peaks (primary, secondary amines, carboxylic acids, etc) in 1-week fermented extract, while four peaks were seen in 3-week fermented extract indicating the presence of alkyl halides, primary & secondary amines, alcohols, carboxylic acids, esters, ethers, etc. Extract prepared in Ethyl acetate showed five peaks in FTIR analysis in both 1- and 3-week fermented extracts. Ethyl acetate extracts of fungi showed a large number of compounds: after 1 week of fermentation (halogen compounds, aliphatic amines, esters, fatty acids, and alkenes and after 3-week fermentation (aliphatic amines, alcohols, carboxylic acids, esters, ethers, carbonyl compounds, aliphatic compounds, carboxyl and hydroxyl compounds). HRAMS data shows the presence of a large number of secondary metabolites in all three solvents (Fig. 3, Table 3). Methyl extract of fungal extract (Me3) showed the presence of flavonoids, phenols, barbiturates, steroids, tryptophan, dicarboxylic acid, indole, etc. Most of the compounds are of therapeutic value. Two mycotoxins were also present in methyl extract of fungi: fusarenone and zearalenone. Chloroform fungal extract also showed rich content of pharmaceutically active compounds such as alkaloids, terpenes, azridine, barbiturates, amino acids, ester, glucoside, phenolic compounds, benzamide, etc. In several studies, ethyl acetate is considered a selective and effective solvent for the isolation of secondary metabolites especially phenols from fungal extracts. The antioxidant activities of Me3, CH3, and EA3 extracts were assessed against the synthetically produced DPPH free radicals. The findings indicated that the extracts effectively inhibited the DPPH free radicals, as evidenced by an IC_{50} value of 547.11, 430.23, and 712.23 μg/mL for Me3, CH3, and EA3, respectively (Fig. 4). The spectrophotometric analysis at a wavelength of 517 nm revealed a decline in the absorbance as the concentration of extracts increased, signifying the scavenging of free radicals. CH3 extract showed more antioxidant potential than Me3 and EA3 extracts. A large number of active constituents extracted by CH3 may be responsible for the significant antioxidant activity [30]. The antibacterial activities of the Me3, CH3, and EA3 extracts were assessed against pathogenic bacteria that affect humans (Fig. 5, Table 4). The CH3 extract displayed activity against all the bacteria that were selected. *E. coli* exhibited the maximum zone of inhibition (with a zone of inhibition measuring 15 ± 0.62 mm), followed by *Staphylococcus aureus* (with a zone of inhibition measuring 14.4 ± 0.8 mm), and *Enterococcus faecalis* (with a zone of inhibition measuring 14.3 ± 0.75 mm). On the other hand, the Me3 and EA3 extract showed no activity against selected bacteria. In the present study, CH3 extract showed the presence of some important antibacterial compounds (clavulanic acid, itaconic acid, lactide, sancycline, vanillin, vulgaxanthin, etc.), which might be responsible for the antibacterial function of fungal extract. Due to the high biosynthetic capabilities of fungi, wide ranges of secondary metabolites of many fungi show antibacterial, antifungal, and antiviral activities [33]. Mouse models are frequently used in biomedical research due to genetic and physiologic similarities of mice with humans, small size, reliable breeding, and short life span [31]. DL tumor cells are a murine lymphoma aggressive and highly metastatic. Treatment of DL cells with the increasing concentration of the compounds Me3, CH3, and EA3 showed a variable degree of effect concerning the loss of

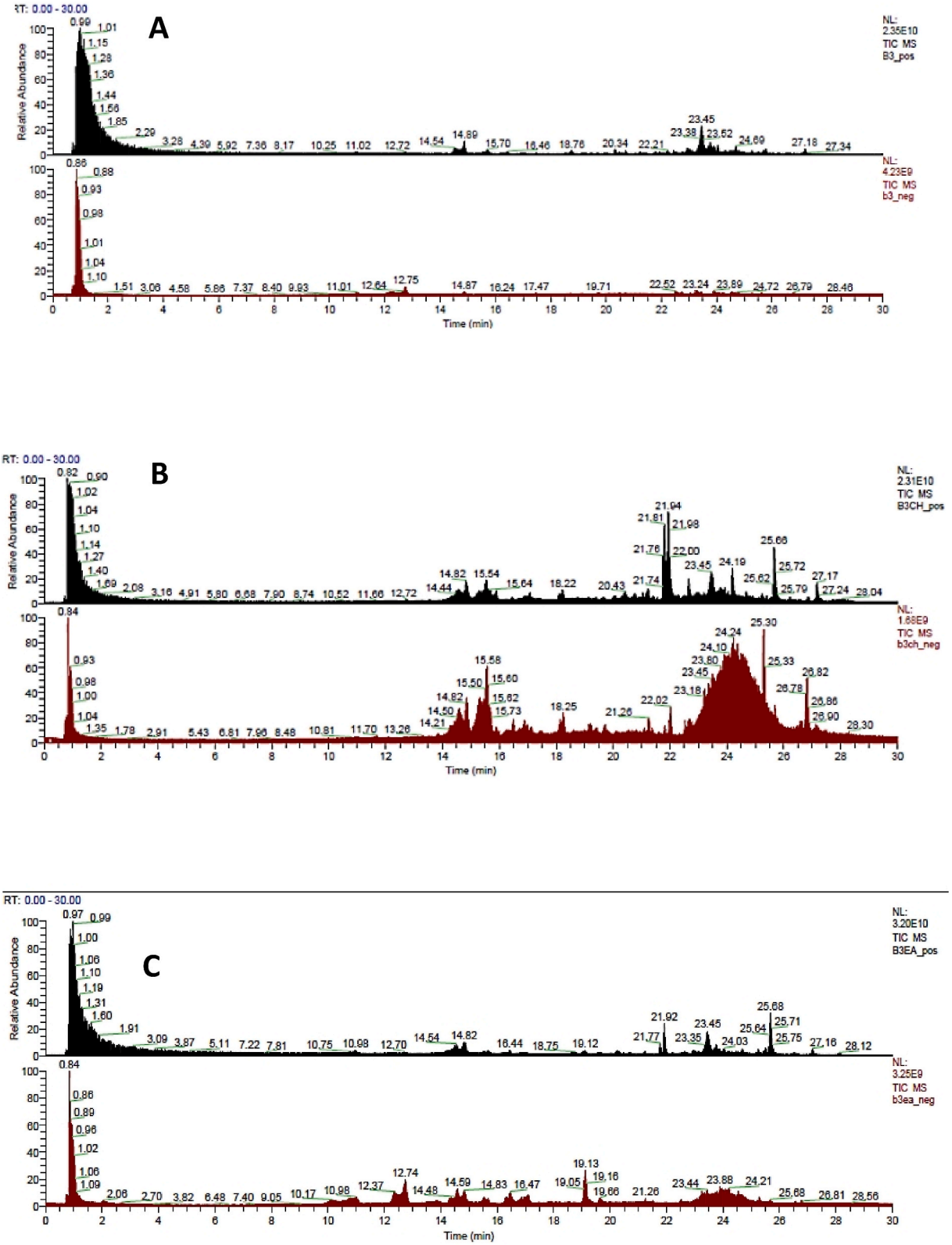

Fig. 4. HRAMS analysis of different fungal extracts.

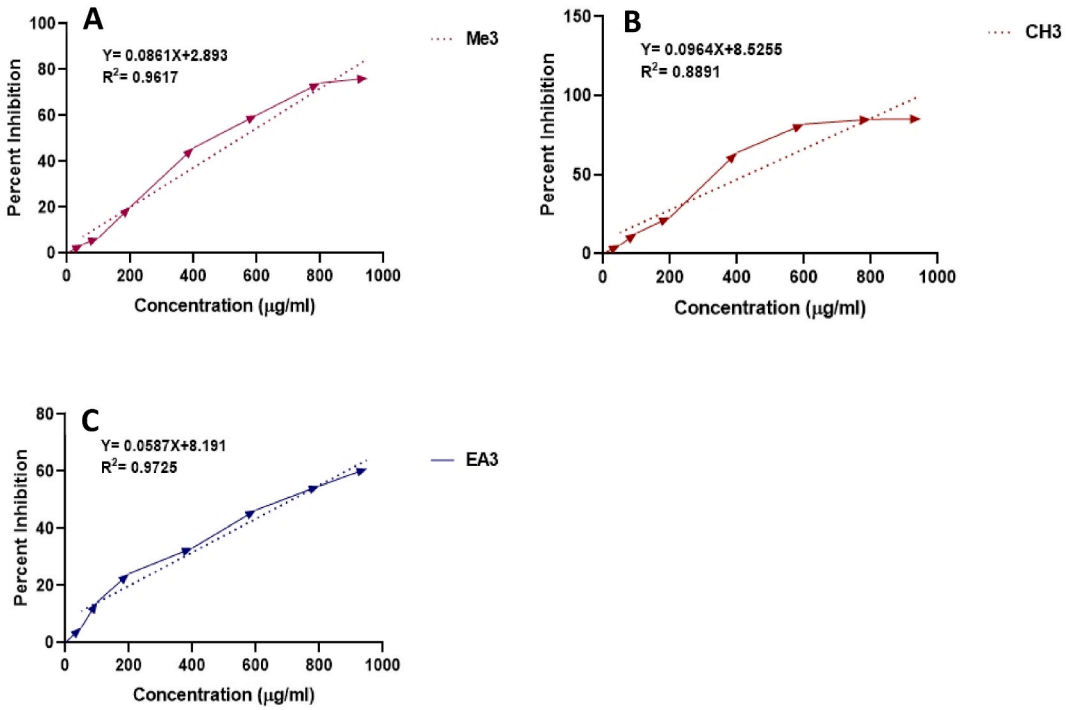

Fig. 5. (A–C) Free radical scavenging activity of fungal extracts.

Table 4
Antibacterial activity of different extracts in terms of inhibition zone (mm).

	Me3 (A)	CH3 (B)	EA3 (C)	DMSO (D)	Streptomycin (E)
Escherichia coli (Ec)	00.00	15 ± 0.6	00.00	00.00	15.03 ± 0.46
Staphylococcus aureus (SA)	00.00	14.4 ± 0.8	00.00	00.00	15.83 ± 0.32
Enterococcus faecium (Ef)	00.00	14.1 ± 0.7	00.00	00.00	00.00

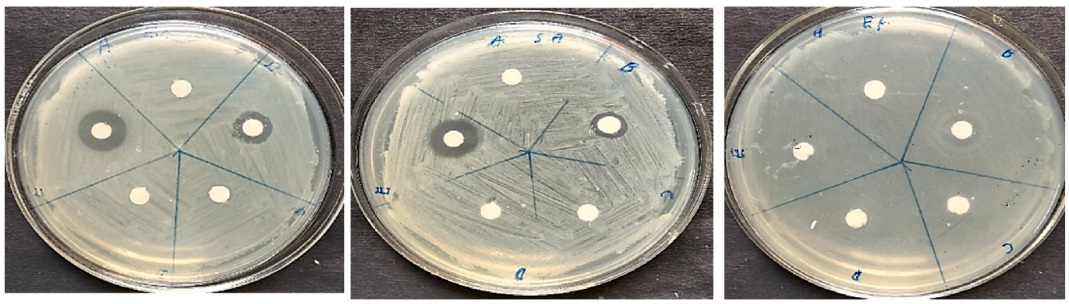

Fig. 6. Antibacterial activity of different Fugal extracts: A- *Escherichia coli (Ec)*, B- *Staphylococcus aureus* (SA), C- *Enterococcus faecium* (EF).

cell viability following 18 h of incubation as judged by MTT assay. DL cells did not show susceptibility to Me3 and there was no significant loss in cell viability. In contrast, CH3 and EA3 showed a loss in cell viability only at the highest concentration tested ($p < 0.001$) (Fig. 6A). Among the extracts, ME3 and CH3 were observed more tolerant to DL tumor cells than EA3. Comparative analysis of the cytotoxicity at concentrations of 100 µg, indicated that EA3 is more cytotoxic to DL tumor cells than ME3 or CH3 (Fig. 6B). The effect of different concentrations of the extracts was seen on the proliferation of DL cells. The inhibition of tumor cell growth was observed by EA3 in a concentration-dependent manner (Fig. 6C). Both ME3 and CH3 were tolerant to the tumor cells and did not restrict the proliferation of the cells. Since EA3 only showed some degree of antitumor effect against DL tumor cells we wanted to know whether EA3 induces apoptosis in DL cells following 12 h of treatment. Data indicate that EA3 induces marginal or very low apoptosis in DL cells although uptake of the compound in DL tumor cells was evident (Figs. 7 and 8). EA3 showed the presence of several

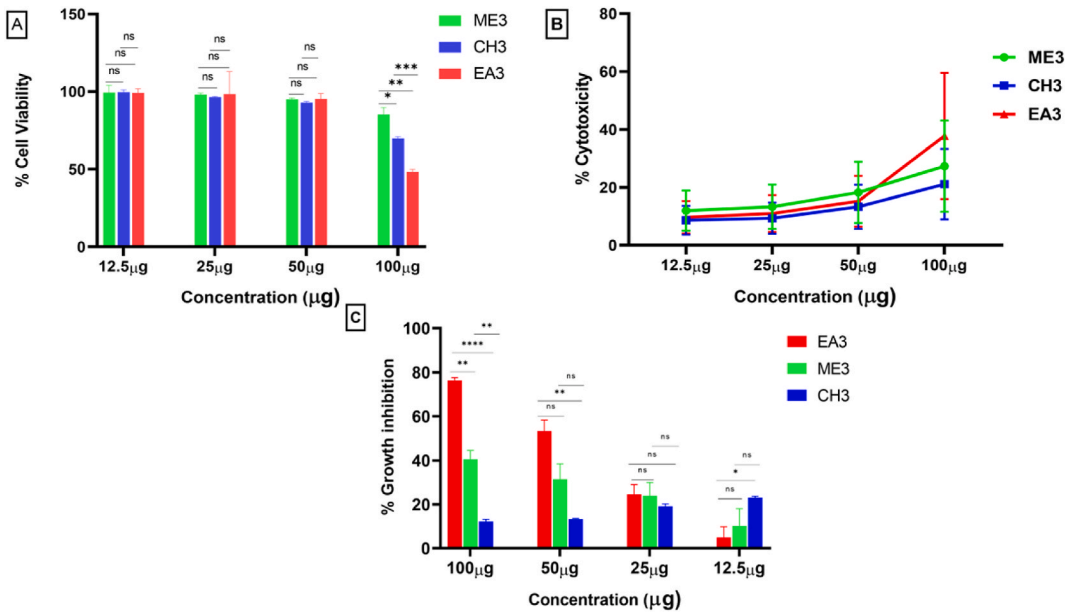

Fig. 7. Antitumor effect of ME3, CH3, and EA3 against DL tumor cells. Concentration-dependent studies on viability (A), cytotoxicity (B), and long-term growth inhibition (C) of DL tumor cells following treatment with indicated compounds. The experiment was performed twice with triplicate determination. Data presented from one experiment with triplicate determination.

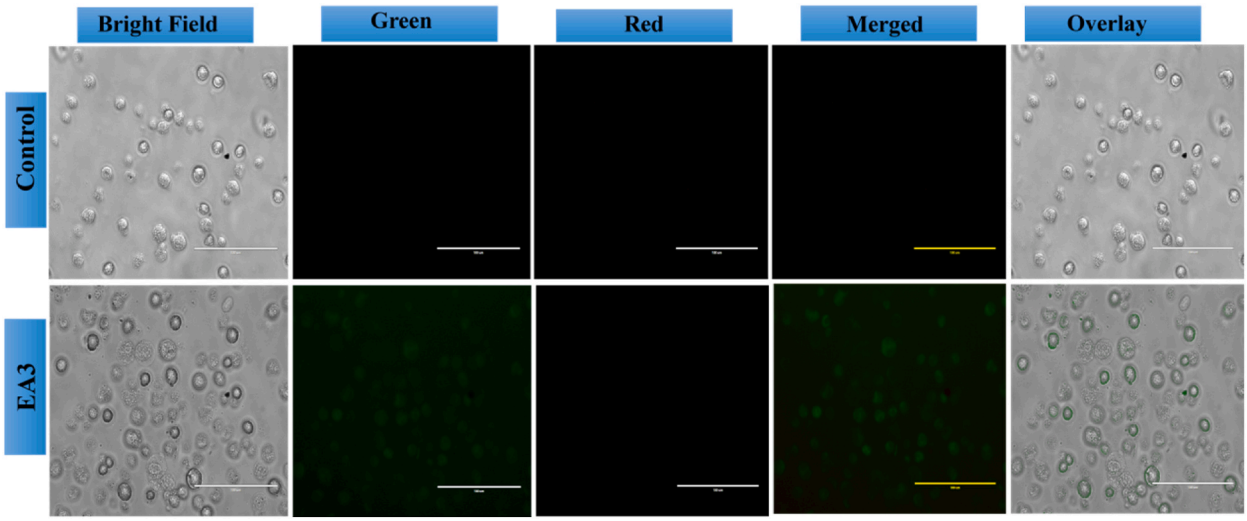

Fig. 8. Temporal uptake and apoptosis in DL tumor cells. DL tumor cells were treated with EA3 (50 μg) for 12 h at 37 °C, 5%CO_2. The cells were washed and treated with PE-conjugated Annexin-V for 30 min. The images were captured in EVOS-FL.

antitumor compounds (Brassylic acid, fraxetin, gentisic acid, homalium, karwinaphthol b, mitimycin, quinoxaline, reserveratrol, adlerol, altretamine, ursolic acid, veliprab. Endophytic fungi are a rich source of antitumor compounds. So, the present study on the endophyte of *Dillenia indica* callus culture shows a wider application of the endophyte *Aspergillus flavus*.

4. Conclusions

Endophytes of medicinal plants display different biological activities and their wider applications have drawn the attention of several researchers. In the present work, the appearance of the endophyte occurred as a contaminant, but they show the presence of diverse types of phytochemicals just like the host plant. It helps in understanding the plant-microbe interactions and biosynthetic background of secondary metabolites. Identifying endophytes and their potential as an antioxidant, antibacterial, and antitumor gives a novel and efficient way of plant-less production of high-value secondary metabolites. Endophyte extract prepared in chloroform

showed both antioxidant (IC_{50} 430.23) and antibacterial (15 ± 0.62 mm zone of inhibition for *E. coli*, slightly higher than the antibiotic Streptomycin) potential. Ethyl acetate extract of endophyte showed cytotoxicity, growth inhibition and reduction in cell viability of DL tumor cells. There is a great need to explore such endophytes not only from medicinal plants but also from non-medicinal plants.

CRediT authorship contribution statement

Ashish Gupta: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Brajesh Chandra Pandey:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Mohd Yaseen:** Methodology, Writing – review & editing. **Renu Kushwaha:** Formal analysis. **Madhavenda Shukla:** Formal analysis, Methodology. **Pratima Chaudhary:** Formal analysis, Methodology, Writing – review & editing. **Partha Pratim Manna:** Formal analysis, Resources. **Aparna Singh:** Resources. **Ida Tiwari:** Formal analysis, Resources, Supervision. **Gopal Nath:** Resources. **Nishi Kumari:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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 research article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42142.

References

[1] J. Ljunggren, F. Borrero-Echeverry, A. Chakraborty, T.U. Lindblom, E. Hedenström, M. Karlsson, P. Witzgall, M. Bengtsson, Yeast volatomes differentially affect larval feeding in an insect herbivore, Appl. Environ. Microbiol. 85 (21) (2019) e01761, https://doi.org/10.1128/AEM.01761-19, 19.
[2] Y. Xu, L. Xu, H. He, Chemicals mediate the interaction between plant associated fungi and insects, J. Systemat. Evol. 61 (3) (2023) 506–517, https://doi.org/10.1111/jse.12956.
[3] S.C. Watkinson, Mutualistic symbiosis between fungi and autotrophs, in: The Fungi, Academic Press, 2016, pp. 205–243, https://doi.org/10.1016/B978-0-12-382034-1.00007-4.
[4] M. Ebadi, S. Mollaei, H. Tahmouresi, P. Farnia, Molecular identification of endophytic fungi isolated from *Tragopogon graminifolius* and investigation of their biological activities, Biomedical and Biotechnology Research Journal (BBRJ) 8 (2) (2024) 251–255.
[5] H.L. Mehl, P.J. Cotty, Influence of the host contact sequence on the outcome of competition among *Aspergillus flavus* isolates during host tissue invasion, Appl. Environ. Microbiol. 77 (5) (2011) 1691–1697, https://doi.org/10.1128/AEM.02240-10.
[6] L. Taha, E.A. Ibrahim, N.M. Youssef, A.I. El-sayed, In- vitro culture development and secondary metabolites of *Dillenia indica* tree, Am. J. Plant Physiol. 13 (1) (2018) 44–52, 10:3923/ajpp.2018.44.
[7] I. Prasher, V. Kumar, Diversity of endophytic fungi associated with *Dillenia indica* L, an ethnomedicinal plant, Current Research in Environmental Applied Mycology 11 (1) (2021) 532–559, https://doi.org/10.5943/cream/11/1/35.
[8] S. Garyali, A. Kumar, M.S. Reddy, Taxol production by an endophytic fungus, *Fusarium redolens*, isolated from Himalayan yew, J. Microbiol. Biotechnol. 23 (10) (2013) 1372–1380. https://kiss.kstudy.com/Detail/Ar?key=3169440.
[9] H. Nisa, A.N. Kamili, I.A. Nawchoo, M.S. Bhat, R. Nazir, Isolation and identification of endophytic fungi from *Artemisia scoparia* (Asteraceae), Theor. Appl. Sci. 10 (1) (2018) 83–88.
[10] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2.
[11] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (4) (1987) 406–425, https://doi.org/10.1093/oxfordjournals.molbev.a040454.
[12] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (12) (2013) 2725–2729, https://doi.org/10.1093/molbev/mst197.
[13] X. Wang, X. Chen, Z. Qi, X. Liu, W. Li, S. Wang, A study of *Ganoderma lucidum* spores by FTIR microspectroscopy, Spectrochim. Acta Mol. Biomol. Spectrosc. 91 (2012) 285–289, https://doi.org/10.1016/j.saa.2012.02.004.
[14] D. Helm, D. Naumann, Identification of some bacterial cell components by FT-IR spectroscopy, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 126 (1) (1995) 75–79, https://doi.org/10.1111/j.1574-6968.1995.tb07393.x.
[15] H.A. Alhadrami, A.M. Sayed, A.O. El-Gendy, Y.I. Shamikh, Y. Gaber, W. Bakeer, N.H. Sheirf, E.Z. Attia, G.M. Shaban, B.A. Khalifa, C.J. Ngwa, A metabolomic approach to target antimalarial metabolites in the *Artemisia annua* fungal endophytes, Sci. Rep. 11 (1) (2021) 2770, https://doi.org/10.1038/s41598-021-82201-8.
[16] A. Gupta, B.C. Pandey, J. Verma, I. Tiwari, A.N. Sahu, R.K. Manhas, N. Kumari, Biosynthesis of silver nanoparticle from flower extract of *Dillenia indica* and its efficacy as antibacterial and antioxidant, Microb. Pathog. 193 (2024) 106779, https://doi.org/10.1016/j.micpath.2024.106779.
[17] S.K. Hira, K. Ramesh, U. Gupta, K. Mitra, N. Misra, B. Ray, P.P. Manna, Methotrexate-loaded four-arm star amphiphilic block copolymer elicits CD8+ T cell response against a highly aggressive and metastatic experimental lymphoma, ACS Appl. Mater. Interfaces 7 (36) (2015) 20021–20033, https://doi.org/10.1021/acsami.5b04905.

[18] S.K. Hira, A.K. Mishra, B. Ray, P.P. Manna, Targeted delivery of doxorubicin-loaded poly (ε-caprolactone)-b-poly (N-vinylpyrrolidone) micelles enhances antitumor effect in lymphoma, PLoS One 9 (4) (2014) e94309, https://doi.org/10.1371/journal.pone.0094309.
[19] D. RoyMahapatra, R. Singh, U.H. Sk, P.P. Manna, Engineered artesunate-naphthalimide hybrid dual drug for synergistic multimodal therapy against experimental murine lymphoma, Mol. Pharm. 21 (3) (2024) 1090–1107, https://doi.org/10.1021/acs.molpharmaceut.3c00632.
[20] S. Kaul, S. Gupta, M. Ahmed, M.K. Dhar, Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites, Phytochemistry Rev. 11 (2012) 487–505, https://doi.org/10.1007/s11101-012-9260-6.
[21] A.A. Idris, A.H. Nour, M.M. Ali, I.Y. Erwa, O.A.O. Ishag, A.H. Nour, Physicochemical properties and fatty acid composition of *Ocimum basilicum* L seed oil, Asian Journal of Physical and Chemical Sciences 8 (2020) 1–12, https://doi.org/10.9734/AJOPACS/2020/v8i130104.
[22] S. Kusari, S. Singh, C. Jayabaskaran, Rethinking production of Taxol (paclitaxel) using endophyte biotechnology, Trends Biotechnol. 32 (6) (2014) 304–311, https://doi.org/10.1016/j.tibtech.2014.03.009.
[23] F. Arifah, L.Q. Aini, A. Muhibuddin, Molecular and morphological characterization of fungi isolated from nutmeg (*Myristica fragrans*) in North Sulawesi, Indonesia, Biodiversitas Journal of Biological Diversity 24 (1) (2023), https://doi.org/10.13057/biodiv/d240151.
[24] R. Lucking, M.C. Aime, B. Robbertse, A.N. Miller, H.A. Ariyawansa, T. Aoki, G. Cardinali, P.W. Crous, I.S. Druzhimina, D.M. Geiser, D.L. Hawksworth, K. D. Hyde, L. Irinyi, R. Jeewon, P.R. Johnston, P.M. Kirk, E. Malosso, T.W. May, W. Meyer, M. Opik, V. Robert, M. Stadler, M. Thines, D. Vu, A.M. Yurkov, N. Zhang, C.L. Schoch, Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding, IMA Fungus 11 (2020) 14, https://doi.org/10.1186/s43008-020-00033-z.
[25] M. Ebadi, F. Ahmadi, H. Tahmouresi, M. Pazhang, S. Mollaei, Investigation the biological activities and the metabolite profiles of endophytic fungi isolated from *Gundelia tournefortii* L, Sci. Rep. 14 (1) (2024) 6810.
[26] N.D. Fajarningsih, Internal Transcribed Spacer (ITS) as DNA barcoding to identify fungal species: a review, Squalen Bulletin of Marine and Fisheries Post harvest and Biotechnology 11 (2) (2016) 37–44, https://doi.org/10.15578/squalen.213.
[27] M.F. Grau, R. Entwistle, Y.M. Chiang, M. Ahuja, C.E. Oakley, T. Akashi, C.C. Wang, R.B. Todd, B.R. Oakley, Hybrid transcription factor engineering activates the silent secondary metabolite gene cluster for (+)-asperlin in *Aspergillus nidulans*, ACS Chem. Biol. 13 (11) (2018) 3193–3205, https://doi.org/10.1021/acschembio.8b00679.
[28] K. Ochi, Insights into microbial cryptic gene activation and strain improvement: principle, application and technical aspects, The Journal of Antibiotics 70 (1) (2017) 25–40, 10:1038/ja.2016.8.
[29] A.V. Rusu, M. Trif, J.M. Rocha, Microbial secondary metabolites via fermentation approaches for dietary supplementation formulations, Molecules 28 (2023) 6020, https://doi.org/10.3390/molecules28166020.
[30] A. Zeb, Concept, mechanism, and applications of phenolic antioxidants in foods, J. Food Biochem. 44 (9) (2020) e13394, https://doi.org/10.1111/jfbc.13394.
[31] R. Kohnken, P. Porcu, A. Mishra, Overview of the use of murine models in leukemia and lymphoma research, Front. Oncol. 7 (2017) 22, https://doi.org/10.3389/fonc.2017.00022.
[32] C.M. Madhusudhan, R.T. Bharathi, S.H. Prakash, Isolation and purification of bioactive metabolites from fungal endophytes–a review, Current Biochemical Engineering 2 (2) (2015) 111–117, https://doi.org/10.2174/2212711902999150706114209.
[33] K. Sułkowska-Ziaja, M. Trepa, A. Olechowska-Jarząb, P. Nowak, M. Ziaja, K. Kała, B. Muszyńska, Natural compounds of fungal origin with antimicrobial activity potential cosmetics applications, Pharmaceuticals 16 (9) (2023) 1200, https://doi.org/10.3390/ph16091200.