Methods
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4.1. Collection of plant samples

The healthy individual plants were selected for studying their endophytic fungal assemblage. Mature, uninfected and intact leaves from individual species were collected and brought to the laboratory in a separate sterile polythene bags and processed within 24 h of collection (Fisher and Petrini, 1987).

4.2. Surface sterilization of leaf samples

Collected plant samples were washed thoroughly in running tap water before sterilization. From each leaf 0.5 cm$^2$ segments of leaf lamina were cut and surface sterilized in 70% ethanol for 5 seconds, sodium hypochlorite (4%) for 50 seconds and rinsed twice in sterile distilled water (Dobranic et al., 1995). Outer tissue of the sterile segments were trimmed and made ready for endophytes isolation.

4.3. Isolation of fungal endophytes

Surface sterilized leaf segments were evenly placed on Petri dishes containing PDA medium added with antibiotic (Chloramphenicol). The Petri plates were sealed and incubated for 28 days in light chamber with 12 h of light and 12 h of dark at 26 ± 1°C (Bills and Polishook, 1992). The plates were monitored regularly for the growth of endophytes from the incubated segments. The hyphae grew out from the segments were sub cultured on PDA and pure fungal strains were maintained at 4°C for further studies. Fungal strains were identified using standard taxonomical keys (Ellis, 1971, 1976; Subramanian 1971; Barnett and Hunter, 1972; Sutton, 1980; Onions et al., 1981; Ellis and Ellis, 1985).
The sterile isolates that could not be assigned to any taxonomic groups were coded based on their morphological characters (Bills and Polishook, 1994).

4.4. Data analysis

4.4.1. Percentage of colonization frequency (CF%) 

The percentage of colonization frequency of endophytes was calculated by following the method of Hata and Futai (1995).

\[
\text{CF} \% = \frac{N_{\text{col}}}{N_t} \times 100
\]

Where, \( N_{\text{col}} \) is the number of segments colonized by each endophyte, \( N_t \) is the total number of segments observed.

4.4.2. Relative frequency (RF %) 

Relative frequency of an endophyte was calculated by following the method of (Photita et al., 2001).

\[
\text{RF} = \frac{\text{Number of isolates of one species}}{\text{Total of isolates}} \times 100
\]

4.4.3. Relative percentage of occurrence of groups of fungi (RPO) 

The relative percentage of occurrence of different groups of endophytic fungi such as hyphomycetes, coelomycetes, ascomycetes, etc. were calculated using the formula,

\[
\text{RPO} = \frac{\text{Total colonization frequency of one group}}{\text{Total colonization frequency for all the groups of fungi}} \times 100
\]
4.4.4. Species diversity indices

Shannon-Wiener species diversity index ($H_s$), Shannon species evenness index ($J'$) were calculated in online version of the Shannon – Weiner calculator (http://www.alyoung.com/labs/biodiversity_calculator.html) to evaluate the species richness of fungal endophytes in each host plant.

$$H_s = -\sum_{i}^{S} P_i \log P_i$$

Where,

$H_s$ = Symbol for the diversity in a sample of $S$ species or kinds

$S$ = number of species in the sample

$P_i$ = relative abundance of $i^{th}$ species or kinds measures = $n/N$

**Simpson's index (D)**

$$D = \frac{\sum n_i (n_i - 1)}{N (N - 1)}$$

Where,

$n$ = the total number of organisms of a particular species

$N$ = the total number of organisms of all species

**Shannon evenness index (J')**

$$J = \frac{H'}{H'^{\text{max}}}$$

$H'$ = is the value obtained from Shannon diversity index

$H'^{\text{max}}$ = Maximum diversity possible
4.5. Cultivation of fungal strains for secondary metabolites production

To screen the bioactive secondary metabolites produced by endophytic fungi, liquid static cultivation method was adopted. Fresh culture of each fungal strain was grown in Erlenmeyer flasks containing 300ml of Czapek-Dox broth (CDB) medium. The cultures were incubated at room temperature for 24 days without shaking. For voluminous production of secondary metabolites each strain was cultivated in 50 (500ml) Erlenmeyer flasks.

4.6. Optimization of cultivation conditions to enhance bioactivity

The fermentation conditions were optimized for each strain to enhance the bioactivity of their secondary metabolites. The conditions such as incubation period (7,11, 15, 20, 24 days), pH (4.5, 5.5, 6.5, 7.5, 8.5) and nutrient composition (Sucrose, NaNO₃, MgSO₄, FeSO₄, K₂HPO₄, KCl) of CDB media were optimized by altering one factor at a time and maintaining other elements constant.

4.7. Extraction of secondary metabolite and mycelia from culture media

After 24 days of fermentation the cultures were filtered to collect mycelia and broth separately. Mycelia were washed thoroughly with sterile distilled water and dried in oven at 40°C. The dried mycelia were homogenized using pestle and mortar followed by extraction with ethyl acetate. The culture broths were filtered through whatman filter paper and the metabolites were extracted thrice with an equal volume of ethyl acetate. The crude extracts were concentrated under reduced pressure at 40°C and stored at –20°C until further studies.
4.8. Antibacterial assay

Antibacterial activity of the endophytic fungal extracts was determined by Kirby-Bauer disk diffusion assay (James, 1973).

**Inoculum preparation:** Pure culture of the test strains maintained in the laboratory at 4°C were transferred to freshly prepared nutrient broth and incubated at 37°C until it reaches 0.5 McFarland standards (1x10^8 CFU/ml).

**Disc diffusion method:** Muller- Hinton agar served as the basal medium to carry out the assay. Sterilized media plates were seeded with bacterial suspension using sterile swab. Sterile disks (6mm) were loaded with fungal extracts at a desired concentration and were placed onto the bacteria seeded plates. Dimethyl sulfoxide (DMSO) was used as negative control and antibiotics discs as positive control. The plates were incubated at 37°C for 24 h. Diameter of the zone of inhibition around each disk was measured and interpreted.

4.9. Molecular identification

**DNA extraction**

Total genomic DNA of the fungus was extracted using InstaGene TM Matrix (Catalog # 732-6030) genomic DNA isolation kit, following the instructions of the manufacturer. The endophytic fungi were grown in 10ml of potato dextrose broth at 28°C for 5 days and the mycelim were harvested by centrifugation at 10,000 – 12,000 rpm for 1 minute. The pellet was resuspended in 1ml of sterile double distilled water and centrifuged at 10,000 – 12,000 rpm for 1 minute. The supernatant was discarded and 200µl of InstaGene matrix was added to the pellet. This mixture was mixed at moderate speed in magnetic stirrer and incubated at 56°C for 15–30 minutes. Later the mixture was vortexed at high
speed for 10 seconds and placed in 100°C heat block for 8 minutes. Again the mixture was vortexed at high speed for 10 seconds and spun at 10,000 – 12,000 rpm for 2-3 minutes. The supernatant was used for PCR reaction (Polymerase chain reaction for DNA amplification). The ITS region of the ribosomal DNA was amplified in Peltierthermal cycler (MJ. Research PTC- 225) with universal primers ITS-1 (fwd primer: 5’-CCGTTAGGTGAACCTGCGC-3’) and ITS-4 (rev primer: 5’-TCCTCCGCTTATTGATATGC-3’) (White et al., 1990). PCR was performed with 25µl reaction mixture containing 1µl of isolate DNA, 1µl of each primer, 1µl of deoxyribonucleotide triphosphate, 11µl of water and 5µl of Taq polymerase with buffer. The thermal cycling program was as follows: 35 cycles of denaturation for 45s at 94°C, 60s annealing at 55°C and 60s extension at 72°C. Genomic DNA of E. coli and water was used as the positive and negative control respectively instead of template DNA in the amplification process.

**Purification of the PCR products:**

The unincorporated PCR primers and dNTPs were removed from the amplified PCR product by Montage PCR cleanup kit (Millipore). The purified PCR products were electrophoresed in 1% (W/V) agarose gel for 2 h. Nighthawk gel documentation system 9 pdi inc., USA, was used to capture image after staining with ethidium bromide.

**Sequencing of the PCR product:**

Single-pass sequencing was performed on each template using combinations of ITS-1/ITS-4 primers. The fluorescent–labeled fragments were purified from the unincorporated terminators with an ethanol precipitation
protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). After sequencing the data was aligned, edited and analyzed. The nucleotide sequence was compared with sequence details of other organisms in NCBI using BLAST program. Identification was based on the maximum similarity of the nucleotide sequence. The nucleotide sequence obtained for two fungal strains have been submitted in the GenBank and accession numbers have been assigned.

**Estimation of genetic distance of the isolates:**

The sequences determined were aligned by using the MEGA program (version 5.0) (Tamura et al., 2011) with grouping by the neighbor-joining (NJ) method (Saitou and Nei, 1987), using a p-distance matrix for nucleotides with the pair-wise gap deletion option adopted with 10,000 bootstrap repetitions.

4.10. Thin layer chromatography

TLC is a simple and quick procedure to find out the number of components present in a crude extract. The ethyl acetate extracts of the fungus were spotted over aluminium - baked TLC plates (60 F<sub>254</sub> - Merck) of size 3 X 9 cm (width and length respectively). The spotted TLC plates were developed with combination of eluents, chloroform: ethyl acetate: methanol in the ratio of 6:3:1. The mobile phase separated the components into various range of R<sub>F</sub> values. The developed chromatograms were visualized under UV light and Iodine.


The agar overlay method was followed to detect the antimicrobial compounds present in the crude extract. The developed chromatogram was kept
for evaporation and placed on sterile Muller Hinton agar medium seeded with 1x10^8 CFU/ml of *Staphylococcus aureus* – MRSA 21, *Vibrio parahaemolyticus* MTCC 451 and *Enterococcus faecalis* ATCC 29212 and the plates were incubated at 37°C for 24 h. After incubation the plates were checked for formation of clearing zone on the adjacent sides of the TLC plate.

4.12. Separation of active compounds by silica gel column chromatography

**Requirements:**

Glass column

Stationary phase – Silica gel G (60-120 mesh)

Admixture – Silica gel (100-200 mesh)

Mobile phase – Petroleum ether, Hexane, Chloroform, Ethylacetate, Acetone and Methanol

**Preparation of extract:**

The ethylacetate extract of the fermented culture media was evaporated to dryness, weighed and pulverized. It was used for the separation of active compounds.

**Column preparation:**

The column preparation included adsorption of the extract, charging and saturation of the column. The extract selected for fractionation was adsorbed on stationary phase in the ratio of 1:3. Glass column with appropriate dimension was selected based on the quantity of sample to be separated. Selected column was rinsed with solvent to remove impurities. A cotton pad was placed firmly at the bottom to prevent the flow of stationary phase. The silica gel in the ratio of (1:7)
of the admixture was used to make the stationary phase as it ensures complete separation of the compounds. The column was charged with stationary phase by wet packing method. The solvent was eluted till surface level of stationary phase and the adsorbed extract was charged into the column. Second layer of cotton pad was placed above the admixture to prevent messing of the stationary phase while adding eluting solvents from the top. The charged column was left for 4 h for complete saturation, removal of air bubbles and to make the bed static. The charged column was eluted with various individual solvents (Petroleum ether, Hexane, Chloroform, Ethyl acetate, acetone and methanol) and combination of the solvents in increasing gradients of polarity.

Procedure:

The charged column was first eluted with 100% petroleum ether and the polarity of the mobile phase was gradually increased by hexane, ethyl acetate, acetone and methanol for *Chaetomium globosum* extract, whereas for the *Nigrospora sphaerica* extract the first elute was 100% petroleum ether followed by chloroform and methanol. The solvents eluted from the column were collected as 50ml individual fractions in separate containers. Each fraction was concentrated and spotted on a TLC plate. The TLC was developed with appropriate mobile phase and visualized under long UV (365nm) or in Iodine chamber. Based on the TLC profile similar fractions were pooled together and the fractions with more than one compound were again subjected to small sized column chromatography.
4.13. Bacterial samples preparation for field emission scanning electron microscope (FESEM) (Elizabeth et al., 2012)

The bacterial strains were inoculated with bioactive fractions and incubated at 37°C for 16 h. The treated cells were centrifuged at 6000 rpm for 6 min at 4°C. The pellets were gently washed twice with PBS (0.1 M, pH 6.8) and fixed with 2.5% glutaraldehyde in PBS at 4°C for 5 h. After fixation, the samples were again washed twice to remove fixing agent. The samples were dehydrated ones with 25, 50, 75% ethanol series for 5 minutes and then with 100% ethanol by three times for 10 min. The dehydrated samples were kept overnight in a desiccator and thereafter gold coated by spurttering. The samples were then analyzed by FESEM (model: SUPRA: CARL ZEISS 55).

4.14. MTT assay (Mosmann, 1983)

Human lung cancer cell line (A549) cultured in DMEM medium supplemented with 1% fetal bovine serum and 1% antibiotic (streptomycin) was used to determine cytotoxicity of the crude extract and individual fractions. The cytotoxicity was assessed based on the MTT- dye reduction by viable cells.

**Principle:**

The assay is based on the reduction of soluble tetrazolium salt to insoluble formazan crystals by metabolically active cells. The succinate dehydrogenase enzyme produced by the mitochondria of the live cells is able to convert yellow colored tetrazolium salt to purple colored formazan crystals. The cells are lysed and dissolved in DMSO solution. The purple color developed was read at 570nm in an ELISA reader.
Reagents:

MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide): 0.5mg MTT/ml of serum-free DMEM medium.

Phosphate Buffer Saline (PBS) pH 7.4: As described under cell culture reagents

Solubilizing solution: Dimethyl sulfoxide (DMSO)

Procedure:

The cells were seeded in 96 well plates at a concentration of $1 \times 10^5$ cells/well. After 24 h, cells were washed twice with 100µl of serum-free medium and starved for 60m at 37°C. After starvation, cells were treated with different concentrations of test compound (10-200µg/ml) for 24 h. After the treatment period, the medium was aspirated and serum free medium containing MTT (0.5mg/ml) was added and incubated for 4 h at 37°C in a CO₂ incubator. After incubation MTT containing medium was discarded and the cells were washed with PBS (200µl). The insoluble formazan crystals were dissolved by adding 100µl of DMSO. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software. The 50% inhibitory concentration value ($IC_{50}$) of the test compound was identified for treated cell line.

4.15. LDH assay

Principle:

Lactate dehydrogenase enzyme catalyzes the reduction of pyruvate to lactate in the presence of reduced nicotinamide adenine nucleotide (NADH) at pH 7.5. The rate of decrease in absorbance resulting from the oxidation of NADH to
NAD\(^+\) was monitored at 340nm, which is proportional to the activity of LDH present in the sample.

**Procedure:**

1.0 ml of the reaction mixtures and 20µl of sample/control are pipetted into cuvette and mixed gently. Cuvette was inserted into the cell holder and stopwatch is started. Exactly after 1, 2 and 3 minutes absorbance was read and recorded. The difference between the absorbance was calculated based on that the average change in absorbance per minute were calculated (ΔA/min).

**4.16. DNA fragmentation assay**

**Principle:**

Apoptosis is the significant mechanism responsible for cell death due to toxicity. DNA laddering is the key assay used to detect apoptosis. The enzyme responsible for apoptotic DNA fragmentation is the Caspase-Actiated Dnase (CAD). CAD is normally inhibited by another protein, the Inhibitor of Caspase Activated DNase (ICAD). During apoptosis the apoptotic effector Caspase (Caspase-3) cleaves ICAD and cause CAD to become activated. This endogenous endonucleases cleave chromatin DNA into internucleosomal fragments, roughly 50 base pairs and multiples of it (50, 100, 150 etc.).

**Reagents:**

- DMEM medium
- 0.5M EDTA
- 1M Tris HCl (pH 8.0)
- Triton X-100
- Phenol
- Chloroform
- RNase
**Procedure:**

In 24 wells plate A549 (2 x 10⁵) cells were incubated for 24 h (in triplicate) with different concentrations of samples (10, 25, 50 and 100µg/ml for sample and freshly prepared DMEM medium. Then the cells were centrifuged at low spin and resuspended with 0.5ml PBS. 55µl of lysis buffer (40ml of 0.5 M EDTA 5ml of 1M TrisHCl buffer pH 8.0, 5ml of 100% Triton X-100 50ml of H₂O) was added and incubated for 20 min at (4°C). Samples were again centrifuged at 12,000g for 30 minutes at 4°C. Samples were transferred to new 1.5ml eppendorf tubes and extracted the supernatant with 1:1 mixture of phenol:chloroform (gentle agitation for 5 min followed by centrifugation) and precipitate in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. Spin down, decant, and resuspend the precipitate in 30µl of deionized water-RNase solution (0.4ml water + 5µl of RNase) and 5µl of loading buffer for 30 minutes at 37°C. Prepared sample and 2µl of DNA ladder (marker on the outer lanes) was run in 1.2% gel at 5V for 5min before increasing to 100V. After the dye front reach 3/4ᵗʰ of the gel, observe the image of DNA shearing in 312nm UV illuminator.

**4.17. Determination of antioxidant activity by FRAP assay**

**Principle:**

The reducing power was determined according to the method of Oyaizu (1986). This assay is based on the reduction of colourless Fe³⁺ tripyridyltriazine (ferric complex) to blue-coloured Fe²⁺ tripyridyltriazine (ferrous complex) by the electrons donated by antioxidants at low pH. The reduction was measured by change in absorbance at 593 nm.
Reagents:

Acetate buffer (300mM, pH 3.6)
TPTZ (2,4,6-tri (2-pyridyl)-s-triazine) 10mM in 40mM HCl
Hydrochloric acid (HCl) 40mM
Ferric chloride 20mM

Procedure:

The working reagent was prepared by mixing acetate buffer, TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) and ferric chloride in the ratio of 10:1:1. 100µl of samples (mg/ml) were added to 3ml of reagent. The reaction mixture was incubated in a water bath for 30 min at 37°C. After incubation the absorbance of the samples was measured at 593nm. The absorbance of sample and blank was noted and their difference was used to calculate the FRAP value.

4.18. Determination of antioxidant activity by DPPH scavenging assay

DPPH radical scavenging activity of extract was determined according to the method reported by Blois (1958). An aliquot of 0.5ml of sample solution in methanol was mixed with 2.5ml of 0.5mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. The absorbance was measured at 517nm using UV spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the formula.

\[
\% \text{ of inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100
\]
4.19. Caspase activation assay

Principle:

Caspases are an intracellular protease enzyme that gets activated during the cascade of events associated with apoptosis. Caspase mediated apoptosis has significant contribution in preventing development of tumor. In apoptosis the sequential activation of the caspases is a critical event involved in the execution phase in cell death. Caspases activities were determined by chromogenic assays using caspases activation kits following the manufacturer’s protocol (Calbiochem, Merck).

Procedure:

A549 cells grown to 70-80% confluences in 12-well plates were incubated for 12 h and 24 h with various concentrations of samples with 10% FBS – DMEM medium. After treating with test compounds, the cells were lysed using lysis buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM DTT, 100mM EDTA). The Lysates were centrifuged at 10,000 rpm for 1 min. The supernatants (cytosolic extract) were collected and protein concentration was determined by the Lowry’s method (Lowry, 1951) using BSA as a standard. 100µg protein (cellular extracts) was diluted in 50µl cell lysis buffer for each assay. Cellular extracts were incubated in 96 well plates with 5µl of the suitable substrates for 2 h at 37°C. Based on the amount of free p-nitroaniline (pNA) moiety released due to hydrolysis of the specific chromogenic substrates like p-nitroanilide (pNA), 4-methyl-coumaryl-7-amide (MCA)- Leu-Glu-His-Asp-p-nitroanilide and MCAAsp-Glu-Val-Asp-p-nitroanilide by caspase-3, caspase-9 and caspase-8 respectively (Wang et al., 2005; Kumar, 2007). The caspase activity was measured at 405nm in microtiter plate. Chromogenic units were converted to percentage by comparing the untreated control cells generated free
pNA. Caspase activity was calculated as fold increase over control samples. Camptothecin 1µg/ml concentration was used as a positive control.

4.20. Apoptotic gene expression

RNA isolation:

Total RNA from the cell line was isolated using TRIZOL (Sigma, India) by following manufacturer's instructions. The sample and TRIZOL was pipetted repeatedly for complete mixing, the mixture was incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes then 0.25ml chloroform was added and centrifuged at 12,000 x g for 15 min at 4°C. Top aqueous phase layer was mixed with 5mg of RNase-free glycogen and 0.5ml of isopropyl alcohol was added to precipitate nucleic acids and kept for 15 min at room temperature and washed with 75% ethanol (in DEPC-treated water, Invitrogen USA) and centrifuged at 12,000 rpm for 10 min at 4°C. Pellet was resuspended in RNase free water and DNase I (Invitrogen) treatment was carried out. RT-PCR was performed in triplicate for isolated RNA with prime RT premix @ GeneBio (Biobase) cDNA kit, Korea according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA).

RT-PCR reaction:

RT premix and sample was incubated at 45°C for 45 min, followed by 72 °C for 10 min in Agilent amplicon system (AGILENT Biosystems). 1µl of the prepared cDNA was amplified in 20µl of reaction buffer for 40 cycles of denaturation (96°C for 30s), annealing (56°C for 30s) and extension (72°C for 30s) using suitable primers. Real-time PCR was performed by monitoring the
increase in intensity of fluorescence emitted by SYBR Green dye with Stratagene Agilent, according to manufacturer’s instructions. All measurements were performed in triplicate. RT-PCR data were represented as Ct (cycle threshold) vale. Ct value of the target sequence is directly proportional to the absolute concentration of the threshold value for reference genes. The relative expression levels of target genes were plotted as fold change compared to control and determined by a relative quantification algorithm ($2^{-\Delta \Delta ct}$ method) (Livak, 2001). The factor X by which the amount of the changed gene can be calculated with the formula: where $\Delta ct = (Ct$ of target gene) – (Ct of b-actin); $\Delta \Delta ct = \Delta ct$ of test – $\Delta ct$ of Control.

**PRIMERS:**

**Bcl-2**
Fwd: 5′-CATGCTGGGGCCGTACAG-3′
Rev: 5′-GAACCGGCACCTGCACAC-3′

**p53:**
Fwd: 5′-AGGGTTAGTTTACAATCAGC-3′
Rev: 5′-GGTAGGTGCAAATGCC-3′

**β-actin**
Fwd: 5′-TTCTACAATGAGCTGCGTGTG-3′
Rev: 5′-GGGTTGTTAGGGTCTCAAA-3′

**4.21. Statistical analysis**

The results are obtained in triplicates. Mean and standard deviation of the results were analyzed using GraphPad Prism 6.0h.

**4.22. Physical measurements**

FT-IR spectra were obtained on a PerkinElmer Spectrum 100 FT-IR spectrophotometer as KBr pellet in the frequency range of 400–4000 cm$^{-1}$. 
The $^1$H and $^{13}$C-$^1$H NMR spectra were recorded in DMSO-d$_6$ solution on Bruker Avance 500 spectrometer at 400.13 ($^1$H) and 125.35 ($^{13}$C) MHz using TMS as internal standard. The $^1$H-$^1$H COSY, $^1$H-$^{13}$C HSQC and $^1$H-$^{13}$C HMBC spectra were obtained by using the standard Bruker pulse programs. EI–MS spectra were obtained on a JEOL GCMATE II GC-MS spectrometer.