Journal of Advanced Zoology
ISSN: 0253-7214
Volume 43 Issue 01Year 2022 Page 315:326

Molecular Identification, Antioxidant And Anticancer Potential Of Endophytic Fungi From *Moringa Oleifera*

Ashok Kumar[1], Kumud Kant Awasthi[2*]

[1,2*]Department of Life Sciences , Vivekananda Global University, Jaipur, For

***Corresponding Author:** Kumud Kant Awasthi
Email: kumud.awasthi@vgu.ac.in

Article History

Received: 09/09/2022
Revised: 30/09/2022
Accepted: 5/10/2022

Abstract

Endophytic fungi inhabit apparently healthy plant tissues and are prevalent in terrestrial plants, especially root tissues, which harbour a wide assemblage of fungal endophytes. Therefore, this study focused on the isolation and characterisation of endophytic fungi from the leaves of *M. oleifera*. The endophytic fungi were initially sorted according to morphological characteristics and identified using the sequences of the Internal Transcribed Spacer (ITS) having 18-s RNA regions of other fungi. The most common fungal isolates were species of the genus *Acremonium strictum* AF2B-3B (**AccessionNumber:CU737841.2**). This study showed that the leaves of *M. oleifera* harbour a diverse group of endophytic fungi. The most potent fungal isolate, antioxidant, and anticancer activity against MCF-7, 3T3 cancer cell lines. The findings revealed that leaves of *M. oleifera* has the capacity to host a wide variety of fungal endophytes and that secondary metabolites from the endophytic fungus may be a source of alternative naturally occurring antioxidant, and cytotoxic compounds.

Keywords: 18-s RNA sequencing; Antioxidant; Anticancer; *M. oleifera*; *Acremonium strictum*

Introduction

Endophytic fungi interrelate with the host plant through 3 mode of actions , like mutualistic, commensalistic, and pathogenic interactions . In mutualistic symbiosis, both host plants and EFs benefit from each other thus causing evolutionary and environmental progress. These fungi depends on colonizing the host plant tissue resists their metabolism enhancing plant's tolerance to abiotic stresses like heavy metal and drought, augmenting development and reproduction , nutrient attainment. It helps in defence of the host plant from herbivore animals, pests, and pathogenic microorganisms whereas the host plant open the way of shelter and prominent quantity of nutrients to the EFs for their proliferation and life cycle completion.
These PRRs are engaged in triggering the initial layer of plant instinctive immunity. Typically, during the formation of a mutualistic symbiotic association , the signalling pathways that resists the extension of endophytic propagation, such as miRNA-mediated routes engaged in plant defence mechanisms, are concealed (Plett and Martin, 2018). This extracellular recognition via MAMPs/PAMPs or damage-related molecular patterns (DAMPs) has been analysed resulting in initial stages of innate immunity via triggered defences, thus called as pattern-triggered immunity (PTI) (Saijo et al., 2018)
Studies on natural products synthesize by microorganisms colonizing curious or distinct environmental circumstances have created on new possibilities toward searching novel natural products of therapeutic

interest and developing them as key medicines to combat various disorders. Endophytic fungi associated with the plants have been recommended in conventional medicines are of keen interest as these abides in between the intercellular spaces of healthy tissues and creates hardly any disorder while also synthesizing a embarrassment of novel compounds with varieties of pharmacological and biotechnological uses (Strobel and Daisy, 2003.

Initially, with the invention of 1st generation sequencers including amplicon based sequencing for different regions of genomes was being analysed for deciphering microbiome research. However, all-embracing research on root morphology and root exudation in analyzing rhizobiomes can be initiated employing next generation sequencing (NGS). Metagenomics permits detailing of complete genomic knowledge by assembling DNA sequences into genes. It also gives information about novel genes, bio based-products, biomolecules, and interaction between microbial communities. Whereas, transcriptomics utilizes the NGS based knowledge to identify and quantify the presence of the particular RNA molecule in biological samples. Metabolomics, therefore is a process which is utilized to identify and analyze metabolite variations occurring due to overexpression or mutation in a required gene (Chen et al., 2022).

In strong living cell, the emergence of free radicals is dispassionate by the antioxidative resistance mechanism though , oxidative stress is initiated when stability is preoccupied towards production of free radicals results in reduction in antioxidant level. The oxidation of lipid, DNA, protein, carbohydrate, and different biomolecules through ROS leads in DNA mutation and may damage target cells or tissues, and which finally cause apoptosis and death. Cancer protection by chemotherapies is routed on free radical mechanism which has proved boon as prominent efficient in initiating important fundamental benefit for public health, and has been recommended by various scientific researchers as a vital tool for resisting , delaying, or reverting the mode of action of tumors (Khan et al., 2016).

The alarming rate of cancer can be controlled with promising novel bioactive agents from endophytic flora of fungi which are non toxic and in expensive and biocompatible, have fewer toxicity issues, and are less resistant in contrast to traditional anti-cancer agents. These phytochemicals are substitutes ways to chemotherapeutic drugs and probable treatment for cancer treatment. These bioactive agents possess anti-cancer efficacy and are considered as safe drugs to combat different types of tumours. Due to their high existence they can be recommended as therapeutic agents to combat cancer development progression (Hridoy et al., 2022). Endophytic fungi have gained extremely urge from scientific community as they are rich deposits of secondary metabolites. Endophytic fungi can be cultured in the fermentation media and uninterrupted extraction of secondary metabolites can be made from it. Endophytes are the symbiotic group of microbes , usually fungi and bacteria that accumulate into the different tissues of their respective host. Endophytic fungi are key classes among all microorganisms due to their multipurpose therapeutic effects

Materials and Methods

Molecular identification of isolated fungal isolates by ITS sequence :

Molecular identification of fungal strains was completed by DNA amplification and sequencing of the internal transcribed spacer (ITS) region using the molecular biological protocol (Crouch et al., 2005). A segment of fungal hyphae (0.5–1.0 cm2) was collected from the Petri dish and lyophilized in an Eppendorf tube (2 mL) (Eppendorf, Germany). Further samples were pulverized and the infection was disrupted. DNA was extracted according to DNeasy Plant Mini Package (QIAgen, USA). Then the isolated DNA was amplified by polymerase chain reaction (PCR). The PCR was performed using the Master Mix Kit of Hot Star Taq (QIAgen, USA). As primers, ITS 1 (with TCCGTAGGTGAACCTGCGG base sequences) (In vitrogen, USA) were mixed with the Hot Star Taq Master Mix Kit and DNA template with a total volume of 50 µL. (BioRad, USA). The amplified fungal DNA (PCR product) was then submitted by a to Amnion biosciences, Bangalore for sequencing and the base sequence was compared using BLAST Algorithm with publicly accessible databases, including GenBank

Antioxidant assays

Total Antioxidant Assay (Phospho molybedate assay):
(Stock solution of 1mg/ml)

This assay was performed as described by Guha *et al.*, (2010) with slight modifications. The antioxidant potential of sample in this activity was measured by colour change because of reduction of Mo (VI) to Mo (V) and formation of phosphate-Mo (V) complex.

In this assay, the sample was taken in different concentrations and was dissolved in DMSO and distilled

water. The reaction mixture was prepared using ammonium molybdate, sodium monobasic, and concentrated sulphuric acid. To the various sample concentrations (100,200 and 300µl) with distil water, reaction mixture about 3.3ml was added. All the sample tubes and blank were incubated for about one hour in water bath at temperature of 95^0 C in water bath and absorbance was taken at 695nm.
Blank contains 1ml of distilled water and 3.3ml reaction mixture.

DPPH radical scavenging activity (Stock solution of 1mg/ml)
Antioxidant activity or free radical scavenging activity of selected fungal isolate was measured by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) assay using Prieto *et al.*, (1999) with slight modifications. In this method hydrogen or electron donation ability of fungal isolate or sample is measured from bleaching of purple coloured methanol solution of DPPH which serves as stable reagent.
To different concentrations of sample extracts in DMSO and distil water, 1mL of 0.2mM DPPH was added and incubated in dark for 30min sat room temperature and absorbance was taken at 517nm.
Inhibition of DPPH in percentage (I %) was calculated as:

$$I\% = (Ab-As)/Ab*100$$

Where, Ab=absorbance of control reaction i.e. with all reagents except test compound and As=absorbance of sample. Ascorbic acid was used as positive control.

Reducing Power Activity (Stock solution of 10mg/ml)
The reducing potential of the fungal extract was determined using reducing power assay also called as Ferric reducing power by method given by Oyaizu (1986) with some modifications. The reductive power of the sample was decided on the basis of absorbance of reaction mixture, more is the absorbance value of the mixture more is the reductive potential.
The fungal extract (1ml) at different concentrations (100,200 and 300µl) in triplicates was mixed with 2.5ml phosphate buffer (0.2M, pH6.6) and 2.5ml of 1% potassium ferrocyanide $K_3Fe(CN)_6$.The mixture was incubated at 50^0C for about 20 minutes. Trichloroacetic acid (10%) was prepared and 2.5ml volume was added to the mixture and mixed well. From the mixture 2.5ml was collected and 0.5ml ferric chloride $FeCl_3$ (0.1%) was added and mixed well. Blank had all reagents except fungal extract.
Absorbance was taken at 700nm.

Anti-cancerous activity of Selected fungal extract:
MTT assay was performed for anticancer activity using MCF7 cancerous cell lines at Centre for biological sciences, Pondicherry (Boyom et al., 2014)

Reagent Preparation
MTT Solution
MTT was dissolved in Dulbecco's Phosphate Buffered Saline, pH=7.4 (DPBS) to 5 mg/ml. This MTT solution was filter-sterilized through a 0.2 µM filter into a sterile, light protected container. The MTT solution was stored at 4°C for frequent use or at -20°C for long term storage and was protected from light.

Solubilization Solution
All the work was done in a ventilated fume hood and selection of appropriate solvent resistant container was done. Firstly, 40% (vol/vol) dimethylformamide (DMF) was prepared in 2% (vol/vol) glacial acetic acid. To this 16% (wt/vol) sodium dodecyl sulfate (SDS) was added and dissolved, at pH = 4.7. The solution was stored at room temperature to avoid precipitation of SDS. If a precipitate forms, warm to 37°C and mix to solubilise SDS.

MTT Assay Protocol
All the cells and test compounds were prepared in 96-well plates containing a final volume of 100 µl/well. Incubation for desired period of exposure was carried out and 10 µl MTT solution was added per well to achieve a final concentration of 0.45 mg/ml. Incubation was done for 1 to 4 hours at 37°C. To this, 100 µl Solubilisation solutions were added to dissolve formazan crystals to each well and were mixed thoroughly for complete solubilisation.
Absorbance was taken at 570 nm.

Dual AO/ EB fluorescent staining protocol :
This dual staining was carried to check apoptotic or necrotic nature of the cells.

The cells were treated with test compound at IC_{50} concentration and incubated for 24 hours in CO_2 incubator at 37°C. The cells were removed by trypsination and collected by centrifugation including the non adherent cells. The cell pellet was resuspended in medium and cell suspensions (25 µl) were transferred to glass slides. Dual fluorescent staining solution (1µl) containing 100 µg/ml AO and 100 µg/ml EB (AO/EB, Sigma) was added to each suspension and then covered with a cover slip. The morphology of apoptotic cells was examined and counts the cells within 20 min using a fluorescent microscope.
Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin

TOXICITY STUDY:

The toxicity studies were carried out in 3T3 fibroblast cell lines using MTT assay at Centre for biological sciences, Pondicherry (Carrie et al., 2014)

Reagent Preparation
MTT Solution
MTT was dissolved in Dulbecco's Phosphate Buffered Saline, pH=7.4 (DPBS) to 5 mg/ml. This MTT solution was filter-sterilized through a 0.2 µM filter into a sterile, light protected container. The MTT solution was stored at 4°C for frequent use or at -20°C for long term storage and was protected from light.

Solubilization Solution
All the work was done in a ventilated fume hood and selection of appropriate solvent resistant container was done. Firstly, 40% (vol/vol) dimethylformamide (DMF) was prepared in 2% (vol/vol) glacial acetic acid. To this 16% (wt/vol) sodium dodecyl sulfate (SDS) was added and dissolved, at pH of 4.7.

MTT Assay
All the cells and test compounds were prepared in 96-well plates containing a final volume of 100 µl/well. Incubation for desired period of exposure was carried out and 10 µl MTT solution was added per well to achieve a final concentration of 0.45 mg/ml. Incubation was done for 1 to 4 hours at 37°C. To this, 100 µl Solubilisation solutions were added to dissolve formazan crystals to each well and were mixed thoroughly for complete solubilisation.
Absorbance was taken at 570 nm.

Results and Discussion

Isolate PD 20 was identified by sequencing ITS region rDNA. The sequence of the endophytic fungal isolate was compared to the data available in NCBI using BLAST search to estimate the phylogenetic relationship **(Fig. 1)**
The culture, which was labeled as S2 showed similarity with *Acremonium strictum* AF2B-3B (**AccessionNumber:CU737841.2**)based on nucleotide homology and Phylogenetic analysis.
Information about other close homolog's for the microbe can be found from the Alignment.

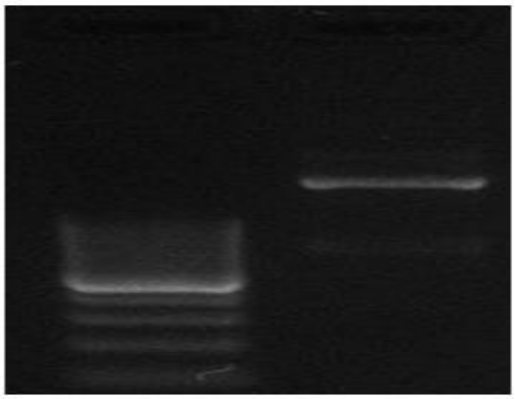


Fig. 1.2% Agarose gel showing single 1500 bp of *18 S-rDNA* amplicon.

Lane 1: 100 bp DNA ladder Lane 2: *18S-rDNA* amplicon

1. R1_8F_S018548_A11_095.abi: Data obtained with Forward primer
2. R1_1492R_S018548_B11_093.abi: Data obtained with Reverse primer

S2_8F_S018548_A11_095.seq (774 bp)

CCGTTCTGGGTTATAAATAGTAGATCGCTCTATCATGCAGTCGAGCGAACAGACAGAGAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGCAACCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCTATGTCACCTCATG
GTGACATACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGA
CGATGCGTAGCCAACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAGGAAGGTTTTCGGATCGTAAAACTCGTTTGTAAGGGAAGAACAAGTAC
AGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAAC

S2_1492R_S018548_B11_093.seq (783 bp)

GGGATTAACTGTCTTTTATTAAACACACCTCCACCTTCGGCGGCTGGCTCAAAAGGTTACCTCACCGACTCGGGTGTTACAAACTCTCGT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACAAGCGATTCCGGCTTCATGTAGGC
GAGTTGCAGCCTACAATCCGAACTGAGAACGACTTTATGGGATTAGCTCCCTCTCGCGAGTTGGCAACCGTTTGTATCGTCCATTGTAGC
ACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCC
CAACTAAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCA
CCACCTGTCACCGTTGTCCCCAAAGGGAAAACTATATTTTTACGGTGGCCAACGAGATGTCAAGACGTGGTGGTGCTCTGCGCGTTGCTG
CAAAATACACCACGCGCTCCACCGCGTGTGCGGGCCCCCGTCTATTCTTTTGATTTTCACTCGTGCCACCGCACTCCCCGCGCAGAGTGC
TAAGTGTGTTATCTGCAACACTAAGGGGCGGACACCCCCTCACTTTTAGCACTCAGCTTTCAGCGCGTGCACCACCAGGATATCAAATCG
TGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCAC

E. Consensus Sequence of S2 (1495 bp)

CCGTTCTGGGTTATAAATAGTAGATCGCTCTATCATGCAGTCGAGCGAACAGACAGAGAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGCAACCTACCCTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAATAATCTATGTCACCTCATG
GTGACATACTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGA
CGATGCGTAGCCAACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAGGAAGGTTTTCGGATCGTAAAACTCGTTTGTAAGGGAAGAACAAGTAC
AGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
GTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACACGATTTGATATCCTGGTGGTGCACGCGCTGAAAGC
TGAGTGCTAAAAGTGAGGGGGTGTCCGCCCCTTAGTGTTGCAGATAACACACTTAGCACTCTGCGCGGGGAGTGCGGTGGCACGAGTGAA
AATCAAAAGAATAGACGGGGGCCCGCACACGCGGTGGAGCGCGTGGTGTATTTTGCAGCAACGCGCAGAGCACCACCACGTCTTGACATC
TCGTTGGCCACCGTAAAAATATAGTTTTCCCTTTGGGGACAACGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTCGC
GAGAGGGAGCTAATCCCATAAAGTCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTTGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAGTCGGTGAGGTAAC
CTTTTGAGCCAGCCGCCGAAGGTGGAGGTGTGTTTAATAAAAGACAGTTAATCCC

Blast Data

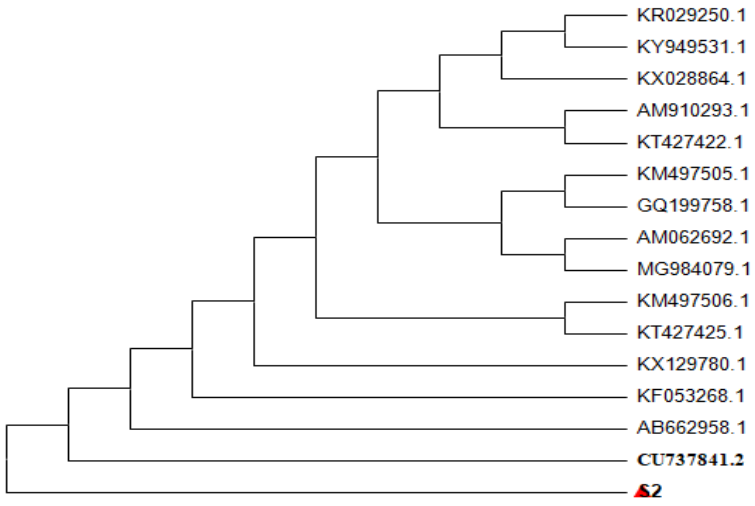
G. SequenceAlignmentsView

Accession	Description	Max score	Total score	Query coverage	E value	Maxident
CU731441.3	Phaeosphaeria nodorumAF2B-3B	2230	2290	96%		92%
CU737841.2	**Acremonium strictum**ACT2	2259	2289	95%		98%
KM497505.1	Acremonium sp.KACI5	2227	2287	96%		93%
KX028864.1	Alternariasp.VKMB-751	2275	2285	95%		95%
KM497506.1	Pestalotiopsis sp.TS4I2C	2295	2285	96%		95%
AM062692.1	Fusarium oxysporum18SrRNAgene,isolate p227	2273	2283	95%		95%
KR029250.1	Penicillium sp.ACT2J6-13	2249	2279	95%		96%
KY949531.1	Aspergillus tamarii	2239	2279	95%		96%
KF053268.1	Acromonium sp. xoACT233AS	2249	2279	95%		97%
AB662958.1	Athelia sps	2219	2279	95%		94%
GQ199758.1	Alternariasp. 210_56	2229	2279	95%		91%
AM910293.1	Apiosporina strain R-31030partial	2264	2274	95%		92%
MG984079.1	Cerrena unicolorMSP58	2234	2274	95%		95%
KT427425.1	Chrysomyxastrain PNPB4/WL	2254	2274	95%		91%
KT427422.1	CiboriniastrainMIRB3/WL	2254	2274	95%		89%

Distance Matrix

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
S2	**1**		0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009	0.009
CU737841.2	**2**	0.048		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
KX129780.1	**3**	0.049	0.001		0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
KM497505.1	**4**	0.049	0.001	0.000		0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
KX028864.1	**5**	0.049	0.001	0.000	0.000		0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
KM497506.1	**6**	0.049	0.001	0.000	0.000	0.000		0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
AM062692.1	**7**	0.049	0.001	0.000	0.000	0.000	0.000		0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
KR029250.1	**8**	0.050	0.001	0.001	0.001	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
KY949531.1	**9**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001		0.000	0.001	0.000	0.000	0.000	0.000	0.000
KF053268.1	**10**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000		0.001	0.000	0.000	0.000	0.000	0.000
AB662958.1	**11**	0.049	0.002	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001		0.001	0.001	0.001	0.001	0.001
GQ199758.1	**12**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001		0.000	0.000	0.000	0.000
AM910293.1	**13**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000		0.000	0.000	0.000
MG984079.1	**14**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000		0.000	0.000
KT427425.1	**15**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000		0.000
KT427422.1	**16**	0.049	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	

Phylogenetic Tree:



The evolutionary history was inferred using the Neighbor-Joining method [1]. The bootstrap consensus tree inferred from 1000 replicates [2] is taken to represent the evolutionary history of the taxa analyzed [2]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1410 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [4].

Fig. Clustal W alignment with consensus sequences of S2 Vs *Acremonium strictum* strain ACT2 (AccessionNumber CU737841.2)

Screening of endophytic fungal isolates for antioxidant potential

All the twenty isolates were grown in three different media viz.PDB and MEB The culture filtrates of all the fungal isolates showed total antioxidant activity. It was observed that out of 20 isolates maximum antioxidant activity was observed in PD20 (**Table 1).**

Table 1: Screening of Endophytic fungi for their Antioxidant Potential

Isolate number	100µl	200µl	300µl
PD1	0.224	0.278	0.352
PD2	0.233	0.265	0.309
PD3	0.261	0.302	0.345
PD4	**0.368**	**0.437**	**0.957**
PD5	0.268	0.289	0.387
PD6	0.272	0.294	0.314
PD7	**0.382**	**0.515**	**1.001**
PD8	0.219	0.227	0.356
PD9	0.237	0.258	0.434
PD10	0.320	0.333	0.365
PD11	0.182	0.264	0.584
PD12	0.287	0.299	0.639
PD13	0.219	0.245	0.777
PD14	0.161	0.181	0.675
PD15	0.274	0.282	0.789
PD16	**0.371**	**0.555**	**1.813**
PD17	0.268	0.345	0.927
PD18	0.282	0.280	0.666
PD19	0.309	0.395	1.113
PD20	**0.412**	**0.666**	**1.882**

Out of twenty isolates screened for their antioxidant potential, only four isolates PD4, PD7, PD16 and PD 20 exhibited best activity and were selected for further studies. The culture filtrates of these four isolates were subjected to sequential extraction in organic solvents - Hexane, chloroform, ethyl acetate and chloroform: methanol. The Total Antioxidant Activity (TAA) and DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging activities of these organic solvents were determined. The chloroform: methanol extract of all the four isolates PD4, PD7, PD16 and PD20 showed total antioxidant activity. Highest activity was recorded for PD20. The ethyl acetate and hexane extract of PD7 did not exhibit any antioxidant activity. PD 16 did not exhibit antioxidant activity **(Table 2, 3**). Maximum activity was recorded for PD 20 and the total antioxidant activity increased with concentration hence, the activity is concentration dependent.

Table 2: Total antioxidant activities of filtrates of selected isolates in various solvents

Isolate no./conc.	Hexane			Chloroform			Ethyl acetate			Chloroform:Methanol		
	100	200	300	100	200	300	100	200	300	100	200	300
PD4	0.007	0.024	0.037	0.186	0.288	0.359	0.041	0.096	0.096	0.09	0.018	0.044
PD7	0.012	0.037	0.017	0.008	0.010	0.025	0.008	0.009	0.010	0.028	0.039	0.051
PD16	0.004	0.008	0.021	0.009	0.017	0.025	0.059	0.087	0.099	0.237	0.377	0.954
PD20	0.006	0.010	0.019	0.006	0.009	0.018	0.018	0.032	0.048	0.009	0.017	0.021

Table 3: DPPH Scavenging Assay –percentage inhibition (I%) of culture filtrates of selected isolates in various solvents

Isolate no./conc.	Hexane			Chloroform			Ethyl acetate			Chloroform:Methanol		
	100	200	300	100	200	300	100	200	300	100	200	300
PD4	36.28	44.57	57.72	40.11	47.22	59.67	43.27	49.16	62.7	42.11	52.19	64.19
PD7	36.37	48.29	57.80	44.12	48.99	60.47	44.69	50.78	62.90	44.32	52.85	65.31
PD16	46	50.13	59.65	47.37	50.15	61.12	46.30	51.47	63.52	47.20	53.11	68.81
PD20	47.20	51	61.44	48.19	51.17	63.34	47.23	52.13	64.22	48.68	54.79	69.00

Hence, PD20 was selected for further studies i.e., DPPH radical scavenging, reducing power, and anticancer activity.

Antioxidant capacity of chloroform: Methanol extract

In the present study DPPH free radical scavenging activity and ferric reducing antioxidant power (FRAP) assay were used to determine the antioxidant capacity of the chloroform: methanol extract **(Table 4)**

Table 4: Total antioxidant activity chloroform: methanol (6:3) extract of PD20 dissolved in DMSO

Isolate no./conc.(µg/ml)	**100**	**200**	**300**
PD20	1.8641	3.112	5.226

Table 5 : DPPH scavanging activity (I%) of chloroform: methanol (6:3)extract of PD20 dissolved in DMSO

Isolate no./conc.(µg/ml)	12.5	25	37.5	50	75	100	125	150
PD20	15.42	24.32	36.7	51.5	66	69.9	77.8	80.9

IC_{50} is 76.2 µg/mL

The antioxidant activity of chloroform:methanol extract in terms of percentage of inhibition was 66% The IC_{50} was found to be 76.2 µg/ml while IC_{50} value for ascorbic acid was 26.6 µg/ml. It was observed that the activity increased with increasing concentration **(Table 5)**

In the present study the reducing power of the ferrous ion was found to be maximum at 3000 µg/ml (695 eq to ascorbic acid µm/l/g) (**Table 6**).

Table. 6 Reducing Power of chloroform: methanol (6:3) extract of PD20 dissolved in DMSO

Concentration of sample(µg/ml)	Conc.of ferric chloride with reference to ascorbic acid as std.(µm/lt/gm)
1000	224
2000	437
3000	695

When Total Phenolics was done using mentioned protocol, it was observed that maximum activity was at 3000µg/ml which was found to be 88.5mg/gdw when tannic acid was used as standard (**Table 7**)

Table 7 : Total Phenolics Activity of chloroform: methanol (6:3) extract of PD20 dissolved in DMSO

Concentration of sample(µg/ml)	**Total phenolic content equivalent to Tannic acid (mg/gdw)**
1000	37
2000	62
3000	88.8

It was observed that in terms of equivalent to quercetin as standard, maximum amount of flavonoids was present in 3000 µg/ml (2.8 mg/gdw) (**Table. 8)**

Table 8: Total Flavonoids Activity of chloroform: methanol (6:3) extract of PD20 dissolved in DMSO

Concentration of sample(µg/ml)	**Total Flavonoid content equivalent to Quercetin (mg/gdw)**
1000	0.9
2000	2.1
3000	2.8

Anticancer activity

Since chloroform and methanol fraction of isolated fungi had maximum antioxidant potential so it was further processed for anticancer activity.

The present study revealed the anticancer and cytotoxic potential of PD-20 on breast cancer cells MCF-7 and the report was compared with cisplastin at various concentrations. It was noticed that PD-20 with a concentration ranging from 10 to 200 µg/ml resulted in dose dependent decrease in cellular viability of cancer cells with IC_{50} value of 193± 12.4 µg/ml while cisplatin treatment revealed IC_{50} value of 10.00± 0.02 µg/ml. The variation between the positive drug and samples is because the positive drug is a pure and so it

will require lower concentration to inhibit the growth of cancer cells. Alternatively higher concentration of samples resulted in 50% inhibition of cancer cells. Screening of cytotoxicity of PD-20 on 3T3 cells revealed that it was marginally toxic to cells even at higher concentration. Overall in MCF-7, the viable cells were around 70.63% at 100µg/ml which decreased to 40.19% which reveals the fact that it is toxic at increasing concentration of test sample. When compared to 3T3 cell lines, it was observed that the cells were viable at 65.68% at 500µg/ml which proved its non toxicity **(Tables 9-11)**

Table 9: Showing percent cell viability of standard (Cisplstin)(MCF7 breast cancer cell line)

Concentrations(µg/ml)	% Cell Viability
5	58.02±0.03
10	50.46±0.02
25	43.78±0.01
50	39.91±0.01
100	21.46±0.009
250	17.17±0.006
500	7.53±0.004

Table 10 : Showing cell viability of tested sample against MCF7 breast cancer cell line.

Tested concentrations (µg/ml)	% Cell Viability
10	91.69±0.09
25	84.28±0.08
50	79.57±0.08
100	70.63±0.07
200	40.19±0.04
Control	100

Table 11 : Percent cell viability against 3T3 fibroblast cell line

Tested concentrations (µg/ml)	% Cell Viability
25	95.54±0.1
50	92.46±0.1
100	75.36±0.1
250	71.88±0.09
500	65.68±0.06
Control	100.00

The physiological role of endophytic fungi isolated from plant and their mechanism of communication with the host and other endophytes and organisms associated with the plant flora is yet to be elucidated (Strobel, 2018). Further , the microbial diversity which are having their habitat in different plant species, along with the variety of the natural products that endophytic fungi synthesize, creates the opportunity for the innovation of new natural products with various biotechnological roles (Fadiji and Babalola, 2020). Further , various reports have been proved the importance of endophytic microorganisms in host endurance, since endophytes directly control bioactive compounds like , resist extreme temperatures and scarcity of water , along with occurrence of phytopathogens (Xia et al., 2022). Therefore, the conventional application of the plant and the region in which it harbor are crucial criteria for reproduction of endophytes (Santos et al., 2015).

Gurgel et al ., 2023 proved free radical scavenging and antimicrobial potential of endophytic fungi correlated with *Arrabidaea chica* (Bignoniaceae). They reported total of 107 endophytic fungi were incubated in liquid cultures and the bioactive compounds were purified with ethyl acetate. Screening for free radical scavenging potential using the DPPH antioxidant assay showed that the *Colletotrichum* sp. CG1-7 endophyte isolates showed potential efficacy with an EC50 of 11 µg/mL, which is equivalent to quercetin (8µg /mL). The FRAP assay inveterate the antioxidant efficacy of the fungal extracts. The occurrence of phenolic compounds and flavonoids in the active isolates was proved using TLC. Their observations reported that 2 of the fungi isolated from *A. chica* possessed tremendous antimicrobial and antioxidant efficacy.

The observations of the DPPH assay of the 2 endophytic fungi, MA and MB endophytic extract proved that at a dose level of 100 µg/ml, showed antioxidant activity with % Inhibition of 55.1 and 48.3% respectively.

In the present study DPPH free radical scavenging activity and ferric reducing antioxidant power (FRAP) assay were used to determine the antioxidant capacity of the chloroform: methanol extract. The antioxidant activity of chloroform: methanol extract in terms of percentage of inhibition was 66% The IC_{50} was found to be 76.2 µg/ml while IC_{50} value for ascorbic acid was 26.6 µg/ml. It was observed that the activity increased with increasing concentration. In the present study the reducing power of the ferrous ion was found to be maximum at 3000 µg/ml (695 eq to ascorbic acid µm/l/g).When total phenolics was done using mentioned protocol, it was observed that maximum activity was at 3000µg/ml which was found to be 88.5mg/gdw when tannic acid was used as standard. It was observed that in terms of equivalent to quercetin as standard, maximum amount of flavonoids was present in 3000 µg/ml (2.8 mg/gdw).

Talukdar et al., 2021 reported isolation and identification of an endophytic fungus *Colletotrichum coccodes* through molecular techniques thus producing tyrosol from *Houttuynia cordata* Thunb using ITS2 RNA secondary structure and various bioinformatics tool. They reported that on this basis it has been identified as *Colletotrichum* sp. and characterized by its genomic ITS rDNA and ITS2 sequences. Phylogenetic analyses proved clustering of its isolate with *Colletotrichum coccodes*. Species of *Colletotrichum* have been considered as plant pathogens. Therefore, to confirm the endophytic lifestyle of the isolate, ITS2 RNA secondary structure is one of the most prominent tool. The result proved that this isolate exhibited variations in the folding pattern including motif structures when compared to those of pathogenic *C. coccodes*. Molecular tools possessing phylogenetic analyses that including various other conventional techniques have got lot of success for peculiar identification and characterization of fungi and have further created a revolutionary breakthrough in identification and classification of endophytic fungi (Sarwar et al., 2019). Moreover, the ITS region which possess of a highly conserved 5.8S rRNA and variable regions ITS1 and, ITS2, is one of the broadly recommended as phylogenetic markers for fungal species identification (Xu and Adamowicz, 2016). Further, the spacer regions like ITS1 and ITS2 are prominently useful in analysing and identifying fungal pathogens from clinical specimens and environmental tests. Also, current research possessed the sequence variability in ITS2 is peculiar pertinent for phylogenetic reconstruction and species variation in eukaryotes including in fungi (Iwen et al., 2002).

Wu et al.,2015 reported the efficacy of endophytic fungi for its anti-cancer potential isolated from of *Morinda citrifolia* Linn. They reported the cytotoxic potential of organic extracts isolated from the fermented broths of individual endophytes was then tested against 3 major cancers that effects humans. 12 different endophytic fungal species were isolated from the leaves and 3 from the fruit. Three of the leaf endophytes showed potency against the growth of human carcinoma cell lines LU-1 (lung), PC-3 (prostate), and MCF-7 (breast) with IC50 values of ≤10 µg/mL.

In the present investigation the anticancer and cytotoxic potential of PD-20 on breast cancer cells MCF-7 and the report was compared with cisplastin at various concentrations. It was noticed that PD-20 with a concentration ranging from 10 to 200 µg/ml resulted in dose dependent decrease in cellular viability of cancer cells with IC50 value of 193± 12.4 µg/ml while cisplatin treatment revealed IC50 value of 10.00± 0.02 µg/ml. The variation between the positive drug and samples is because the positive drug is a pure and so it will require lower concentration to inhibit the growth of cancer cells. Alternatively higher concentration of samples resulted in 50% inhibition of cancer cells. Screening of cytotoxicity of PD-20 on 3T3 cells revealed that it was marginally toxic to cells even at higher concentration. Overall in MCF-7, the viable cells were around 70.63% at 100µg/ml which decreased to 40.19% which reveals the fact that it is toxic at increasing concentration of test sample. When compared to 3T3 cell lines, it was observed that the cells were viable at 65.68% at 500µg/ml which proved its non toxicity.

References

1. Plett, J. M., and Martin, F. M. (2018). Know your enemy, embrace your friend: using omics to understand how plants respond differently to pathogenic and mutualistic microorganisms. Plant J. 93, 729–746
2. Saijo, Y., Loo, E. P., and Yasuda, S. (2018). Pattern recognition receptors and signaling in plant–microbe interactions. Plant J. 93, 592–613
3. Strobel G.and B. Daisy, "Bioprospecting for microbial endophytes and their natural products," Microbiology and Molecular Biology Reviews, vol. 67, no. 4, pp. 491–502, 2003
4. Chen J, Wang H and Guo S X 2012 Isolation and Identification of Endophytic and Mycorrhizal Fungi from Seeds and Roots of *Dendrobium* (Orchidaceae) Mycorrhiza 22 297–307

5. Khan SA, Al Kiyumi AR, Al Sheidi MS, Al Khusaibi TS, Al Shehhi NM, Alam T. "*In vitro* inhibitory effects on α-glucosidase and α-amylase level and antioxidant potential of seeds of *Phoenix dactylifera* L.," *Asian Pac J Trop Biomed* 2016; 6 (4):322–9
6. Hridoy M, Gorapi MZH, Noor S, Chowdhury NS, Rahman MM, Muscari I, Masia F, Adorisio S, Delfino DV and Mazid MA. Putative anticancer compounds from plant-derived endophytic fungi: a review. Molecules 2022; 27: 296
7. Crouch J.A. , B. B. Clarke, and B. I. Hillman, "Phylogenetic relationships and fungicide sensitivities of *Colletotrichum graminicola* isolates from turfgrass in North America," International Turfgrass Society Research Journal, vol. 10, pp. 186–195, 2005
8. Guha GV, Rajkumar V, Kumar RA, and Mathew L (2010). Aqueous extract of *Phyllanthus amarus* inhibits chromium (VI)-induced toxicity in MDA-MB-435S cells. Food Chem.Toxicol48: 396-01
9. Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal.Biochem 269 (2):337-341
10. Oyaizu M (1986). Studies on products of the browning reaction, antioxidative activities of browning reaction products prepared from glucosamine, Jpn. J. Nutr44:307-15
11. Boyom F. F., Fokou P. V. T., Tchokouaha L. R. Y., et al. Repurposing the open access malaria box to discover potent inhibitors of *Toxoplasma gondii* and *Entamoeba histolytica*. Antimicrobial Agents and Chemotherapy. 2014;58(10):5848–5854
12. Strobel G.and B. Daisy, "Bioprospecting for microbial endophytes and their natural products," Microbiology and Molecular Biology Reviews, vol. 67, no. 4, pp. 491–502, 2003
13. Fadiji A E and Babalola O O 2020 Exploring the Potentialities of Beneficial Endophytes for Improved Plant Growth *Saudi J. Biol. Sci.* **27** 3622–33
14. Xia, Y.; Liu, J.; Chen, C.; Mo, X.; Tan, Q.; He, Y.; Wang, Z.; Yin, J.; Zhou, G. The Multifunctions and future prospects of endophytes and their metabolites in plant disease management. Microorganisms 2022, 10, 1072
15. Santos, I.P.; Silva, L.C.N.; Silva, M.V.; Aragão, J.M.; Cavalcanti, M.S.; Lima, V.L. Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae). Front. Microbiol. 2015, 6, 350
16. Gurgel, R. S., de Melo Pereira, D. Í., Garcia, A. V. F., Fernandes de Souza, A. T., Mendes da Silva, T., de Andrade, C. P., & Albuquerque, P. M. (2023). Antimicrobial and antioxidant activities of endophytic fungi associated with *Arrabidaea chica* (Bignoniaceae). *Journal of Fungi*, *9*(8), 864.
17. Talukdar, R., Padhi, S., Rai, A. K., Masi, M., Evidente, A., Jha, D. K., & Tayung, K. (2021). Isolation and characterization of an endophytic fungus *Colletotrichum coccodes* producing tyrosol from *Houttuynia cordata* Thunb. using ITS2 RNA secondary structure and molecular docking study. *Frontiers in Bioengineering and Biotechnology*, *9*, 650247.
18. Sarwar, S., Firdous, Q., and Khalid, A. N. (2019). "Importance of molecular and phylogenetic analyses for identification of basidiomycetes," in Recent Advances in Phylogenetics, ed. Y. Zubaida (London: IntechOpen), doi: 10.5772/ intechopen.80671
19. Xu, J., and Adamowicz, S. (2016). Fungal DNA barcoding. Genome 59, 913–932.
20. Iwen, P. C., Hinrichs, S. H., and Rupp, M. E. (2002). Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med. Mycol. 40, 87–109
21. Wu, Y., Girmay, S., da Silva, V. M., Perry, B., Hu, X., & Tan, G. T. (2015). The role of endophytic fungi in the anticancer activity of *Morinda citrifolia* Linn.(Noni). *Evidence-Based Complementary and Alternative Medicine*, *2015*.

Conflict of interest

Authors declare that there is no conflict of interest