WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.453
Volume 14, Issue 12, 557-579. Research Article ISSN 2277–7105

ANTIOXIDANT, ANTICANCER PROPERTIES OF *OCIMUM TENUIFLORUM* L., ASSOCIATED ENDOPHYTIC FUNGI

Jayabarath J.[1*] and Godwin Jeremiah I[2]

[1]Prof. & Head, Department of Biotechnology, [2]Department of Biotechnology Pavendar Bharathidasan College of Engineering & Technology Trichy-24.

Article Received on
03 May 2025,

Revised on 24 May 2025,
Accepted on 13 June 2025

DOI: 10.20959/wjpr202512-36089

***Corresponding Author**
Jayabarath J.
Prof. & Head, Department of Biotechnology, Bharathidasan College of Engineering & Technology Trichy-24.

ABSTRACT

Fungal endophytes have the ability to produce various type of biologically active novel compounds which can be used to treat various diseases. The diverse bioactive compounds produced from the fungal endophytes depends upon the geographical conditions. In this study, different samples of *Ocimum tenuiflorum* from three different sites of Chennai and Trichy, Tamilnadu. Nine different of endophytic fungi were isolated. The isolated endophytes produced secondary metabolites on production broth. The bioactive compounds were produced for nine isolates. The antioxidant activity of the fungal endophytic metabolites was assessed by DPPH, FRAP and NO assays. The cytotoxicity of the selected three isolates against HeLa cell lines were evaluated by performing MTT assay. The three selected isolates showed cytotoxicity against HeLa cells with IC_{50} value at the concentration of 165.98 µg/ml, 81.11 µg/ml and 256.43 µg/ml, respectively. The third isolate which have more prominent cytotoxic activity against HeLa cells was further selected for metabolite profiling by GC-MS. Nearly 20 bioactive compounds were characterized from the chosen isolate by GC-MS. Apoptosis of HeLa cell lines by the crude extract was assessed by DNA fragmentation assay which showed better cleavage pattern. Measurement of live and dead cells assay was carried out by flow cytometry analysis. The fungal identification was carried out using ITS region genotyping showing that the fungus was closely related with *Diaporthe eres.*

KEYWORDS: Fungal endophytes, DPPH assay, FRAP assay, NO assay, Cytotoxicity, MTT assay, Apoptosis, DNA fragmentation assay, GC-MS, Flow cytometry, ITS amplification.

INTRODUCTION

Nature of fungal endophytes

New variety of diseases, broad drug resistance of microorganisms and infections by unusual pathogens are rising as a threat to human, plant and animal health. Therefore, there is a greater need to discover new, safer and effective drugs to face these diseases. In the recent times, the drug discovery mechanism relied on microbes where 755 of the world's antibiotics have their origins. There is a need to explore new microbial habitats for the microorganisms that produce bioactive compounds. Endophytic fungi live within the healthy tissues of plant species. They are the important components of plant-microecosystems. Plants in Arctic, Antarctic, geothermal soils, deserts, oceans, mangroves and coastal regions can produce endophytic microorganisms. Approximately over 300000 higher plant species exist on the earth. Each plant of that crowd is a host to one or more endophytic microbes. It provides great opportunity for finding novel endophytic taxa and biotypes. There is rich and reliable biological diversity of endophytic fungi. They are very sourceful thing since last two decades for the production of biologically active products. Endophytic fungi are micro-sized fungi. They internally infect the plants without causing any symptoms in plants. They live in mutualistic mannerism for some of their life. They have the ability to produce wide range of enzymes and secondary metabolite compounds associated with those of their host plants.

Endophytic fungi derivatives for cancer treatment

Endophytes colonize internal tissues of all plant species and are highly diverse in nature. Bioactive natural compounds produced by endophytes have promising potential plant applicability in medicine, agriculture, and industry uses. The first discovery of Taxol (paclitaxel) as an anti-cancer drug was isolated from the endophytic fungi from the plant Taxus brevifolia Nutt. The approaches to attach phytochemicals to carrier molecules targeted for specific tumors, holds promise for effective treatment of tumors while avoiding their toxic side effects on normal healthy tissues. With the rapid identification of new proteins having significant regulatory effects on tumor cell cycle progression, molecules isolated from plants and other living organisms are proving to be important source of novel tumor suppressor, and these have potential for development into selective anticancer agents.

MATERIALS AND METHODS

Collection of samples

Samples were collected from the plant species *Oscimum tenuiflorum.* Healthy plants were chosen from different regions of tamilnadu and collected. The leaf parts of the plant were carefully collected from Trichy and Chennai region. They were taken in a clean sterile bag and processed within few hours after sampling. The isolation work was done from the fresh plant material to avoid contamination.

Surface Sterilization of Collected Samples

The plant samples collected in the sterile bags were rinsed in running water in order to remove dust and debris. After cleaning in water, the leaf parts were cut into very small pieces. Highly aseptic condition was required for the isolation of endophytic fungi. All the process was done in the laminar air flow chamber to prevent any contamination. Clean glasswares and mechanical things, such as scalpels, blades, forceps, etc were used.

The small pieces of samples were rinsed with 70% ethanol and 0.1% sodium hypochlorite. The concentration of the chemicals used and time of the treatment depends upon the type of tissues used. The samples were treated with sodium hypochlorite for 10 minutes. Then the samples were washed with ethanol for 1 minute. Then repeat it again by washing it in sodium hypochlorite and ethanol for 30 seconds to remove epiphytic microflora. Finally, the treated leaf samples were rinsed in sterile distilled water for three times. The wet leaf pieces were blotted on the sterile blotting paper aseptically.

Extraction of secondary metabolites

- The culture media and mycelia were separated by filtering the culture broths.
- Ethyl acetate solvent extraction procedure was performed to extract metabolites.
- Equal volume of the filtered media and the solvents was taken in the separating funnel
- The contents in the separating funnel were shaken vigorously for 10 minutes.
- The contents were extracted three times with the solvents.
- They were collected and sun dried to evaporate the excess solvents in the contents.
- After evaporation the metabolites were extracted using ethyl acetate.

ANTIOXIDANT SCREENING

DPPH Assay(1,1-diphenyl-2-picrilhydrazyl)

The effect of given samples on DPPH radical was estimated according to the procedure described by Von Gadow *et al.* (1997).

Two mL of 6 $\times 10^{-5}$ M methonolic solution of DPPH were added to 50 µl of a methonolic solution (10 mg /ml^{-1}) of the sample. Absorbance measurements commenced immediately. The decrease of absorbance at 515 nm was continuously recorded in a spectrophotometer for 16 min at room temperature. Methanolic solutions of pure compound [quercetin] were tested at 1 mg/ml concentration. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 m in duration as follows.

All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of Yen and Duh (1994).

$$\mathbf{IP = [(A_{C(0)} - A_{A(t)} / A_{C(0)})] \times 100}$$

Where $A_{C(0)}$ is the absorbance of the control at t = 0 min; and $A_{A(t)}$ is the absorbance of the antioxidants at t = 16 min.

FRAP Assay

- The stock solutions included 300mM acetate buffer (3.1 g $C_2H_3NaO_2$ $3H_2O$ and 16mL $C_2H_4O_2$), pH 3.6, 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40mM HCl, and 20mM $FeCl_3$ $6H_2O$ solution.
- The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5mL TPTZ solution, and 2.5mL $FeCl_3$ $6H_2O$ solution and then warmed at 37ºC before using.
- Then the extracts (50mL) were allowed to react with 2850 mL of the FRAP solution for 30 min in the dark condition.
- Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm.
- The standard curve was linear between 25 and 800 mM Trolox.
- Results are expressed in mM TE/g fresh mass.
- Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

Nitric oxide scavenging activity

- Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH was measured by Griess reaction (Marcocci *et al*., 1994).
- The reaction mixture (3ml) containing sodium nitropruside (10mm) in phosphate buffer saline and the test extract (10, 25, 50 and 100µg/ml) was incubated at 25ºC for 150min, after incubation 1.5ml of the reaction mixture was removed and 1.5ml of the Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% Napthylethyline diamine hydrochloride) was added.
- The absorbance of the chromophore formed was read at 546 nm. Percent inhibition of nitric oxide scavenging was calculated using the formula.

Percentage Inhibition = (A of Control – A of Sample) /A of Control× 100.

SECONDARY SCREENING FOR ANTIOXIDANT POTENTIAL FOR SELECTED ISOLATES

After the primary screening of the selected isolates for the DPPH, FRAP and NO assays revealed that different spectrum of antioxidant potential of the individual isolates. Among the 9 tested isolates, isolate 2, 3 and 9 showed prominent antioxidant potential were selected for further secondary screening to confirm its ability. The selected three isolates were individually grown on 50 mL of PDB and incubated for three days in an orbital shaker at room temperature. After growth period, the culture free supernatant was obtained upon centrifuged at 10000 rpm for 10 min (Remi, Mumbai, India). The culture filtrate was twice extracted with ethyl acetate using separating funnel. Thus resulted organic layer was evaporated using rotary evaporation at vacuum (Buchi, Switzerland). The crude metabolites were obtained was used for further antioxidant potential using the methodologies given elsewhere in this chapter and anticancer activity also checked.

IN VITRO ANTICANCER ACTIVITY AGAINST HELA CELL LINES

Preparation of HELA cell suspension

A subculture of HeLa cell line in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To th disaggregated cells in the flask 25 mL of DMEM with 10% FCS was added. The cells suspended in the medium by gentle passage with the pipette and the cells homogenized.

Seeding of cell

One mL of the homogenized cell suspension was added to each well of a 24 well culture plate along with different concentration of tested sample (0 to 200 µg/mL) and incubated at 37°C in a humidified CO_2 incubator with 5% CO_2. After 48 hrs incubation the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells cytotoxicity assay was carried out.

Cytotoxicity assay

The assay was carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5- diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48 h incubation the wells were added with MTT and left for 3 h in room temperature. All wells were removed the content using pipette and 100µl SDS in DMSO were added to dissolve the formazan crystals, absorbance's were read in Readwell touch micro plate reader at 540 nm.

Mechanism of action of crude extract of isolate 3 on Cell line

The HeLA cell line were cultured in DMEM medium supplemented with 10% FBS and antibiotics (penicillin 0.1 µg/µL and streptomycin 0.1 µg/µL) in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C. The selected isolate 3, crude extract was added at the concentration of IC50 (~100µg/mL) to check the mechanisms of action towards the cancer cell line.

DNA extraction protocol

Because of floating apoptosis cells, the media of cultures was collected and centrifuged at 5000 rpm for 5 minutes, the supernatant was discarded and added 500 µL lysis buffer, after then, 500 µL of lysis buffer was added to vacant plates, incubated in room temperature for 10 minutes and harvested lysate cells from plates/flasks into the tubes harboring pellet of centrifuged cells then incubated in 65°C for 5 minutes. After cooling in room temperature for 5 minutes, 700 µL chloroform-iso amyl alcohol was added, and then centrifuged at 12000 rpm for 5 minutes. The aquatic (upper) phase was transferred into new micro-centrifuge tubes. An equal volume of cold isopropanol was added into tubes, and mixed gently by inversion. The tubes were then centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded, and the pellet was air-dried for 30 minutes. Then, the dried DNA was dissolved in

50 µL distilled water. The DNA samples were electrophoresed on a 1.5% agarose gel and examined the gel and photographed by an ultraviolet gel documentation system (Orange, Bangalore, India).

Flow cytometry for Cell viability

Trypsinized cells with 1 mL trypsin-EDTA at 37oC for 5 minutes until cells were detached completely. Then, 10% FBS-DMEM medium was added to terminate the trypsinization, which were then gently pipetted to avoid the formation of cell clumps. The cells were transferred into an Eppendorf tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, then the cells were washed with PBS (1X). Afterward, 5 µL annexin V-FITC was added (Cat. No. 88-8005). After 15 minutes incubation at room temperature in the dark, the cells were centrifuged at 1000 rpm for 5 minutes. Then, the cell pellet was resuspended in 200 mL annexin V binding buffer, and the cells were counter-stained with 5 µL propidium iodide (PI) before analysis. The cells were analyzed using FACS-Calibur flow cytometry (Becton Dickinson, San Jose, USA) with emission filters of 515-545 nm for FITC (green) and 600 nm for PI (red). A total event of 10000 cells per sample were acquired, and the data were analyzed with Cell Quest software (Becton Dickinson, San Jose, USA).

GC-MS SCREENING FOR THE SELECTED ISOLATE 3

Gas Chromatograph: A Shimdzu GC-2010 Plus gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35°C, hold for 2minutes, then ramp at 20°C per minute to 450°C and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode). The sample was dried under nitrogen and derivatized with 300 µl of trimethylsilane at room temperature for 20 min. The derivatized sample was dissolved in 1 ml of toluene. A volume of 1 µl was injected at the time of analysis.

Mass Spectrum: A Direct connection with capillary column metal quadrupole mass filter prerod mass spectrometer operating in electron ionization (EI) mode with software GCMS solution ver. 2.6 was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 1000 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were

acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 1000 at 1 second per scan.

Mass spectrometry library search

Identification of the components of the compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instrument's software. GC/MS metabolomics Database was used for the similarity search with retention index.

FUNGAL IDENTIFICATION BY GENOTYPING USING 18S rRNA FOR SELECTED ISOLATE EF 3

Isolation of genomic DNA

The genomic DNA was isolated from a fungal isolate by following the method of Moller et al. (1992). The fungal culture was grown in 100 ml PDB for 5 days. The culture was harvested and the mycelial mat was separated by filtration using Whatman No. 1 filter paper. Then mycelium was ground separately in pestle and mortar using liquid nitrogen. About 50 mg of the powdered mycelium was transferred into a microtube contained 500 µl of TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS). To which, 50 µg proteinase K was added and incubated for 1 h at 60°C with occasional gentle mixing. To the above mixture, 140 µl of 5 M NaCl was added to adjust the salt concentration to 1.4 M. Then 65 µl of 10% CTAB (Cetyl Trimethyl ammonium bromide) was added and incubated for 10 min at 65°C. To the above mixture, 700 µl of Chloroform and isoamyl alcohol (24:1) was added, mixed gently, incubated for 30 min at 0°C and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was transferred to a 1.5 ml tube; to which 225 µl of 5 M NH4Ac was added, mixed gently, incubated on ice for 30 min and centrifuged at 10,000 rpm for 15 min at 4°C. Then the supernatant was transferred to a fresh tube, 510 µl of isopropanol was added to precipitate the DNA and centrifuged immediately for 10 min at 10,000 rpm. The supernatant was removed; the pellet was washed twice with cold 70% ethanol, air-dried and suspended in 50 µl TE buffer.

ITS PCR primers

In this study, ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ was used as a forward primer and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ was used as reverse primer (White et al., 1990).

Polymerase chain reaction (PCR)

Polymerase chain reaction was performed in a thermelcycler to produce multi copies of a specified DNA using the following reaction mixture.

Amplification was carried out with an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min using a thermocycler (iCycler; Bio-Rad Laboratories, CA). PCR products were analyzed on 1% agarose gel for amplicons in 1X TBE buffer at 100 V. The amplified product was sequenced using ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

ITS sequence analysis

The sequences of these ITS regions were compared against the sequences available from GenBank using the BLASTN program (Altschul et al., 1990) and were aligned using CLUSTAL W software (Thompson et al., 1994). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. Phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap analysis was done based on 1000 replications. The MEGAX package (Kumar et al., 2018) was used for all analyses.

RESULTS AND DISCUSSIONS

Isolation of endophytic fungi from *O. tenuiflorum*

From the surface sterilized sample pieces nine different morphotypic fungi were obtained from three different localities derived *O. tenuiflorum* leave samples (Figure 1). The *O. tenuiflorum* sample from Site 1 gave 2 isolates, site 2 yielded 3 isolates and site 3 given 4 isolates (Figure 2; Table 1).

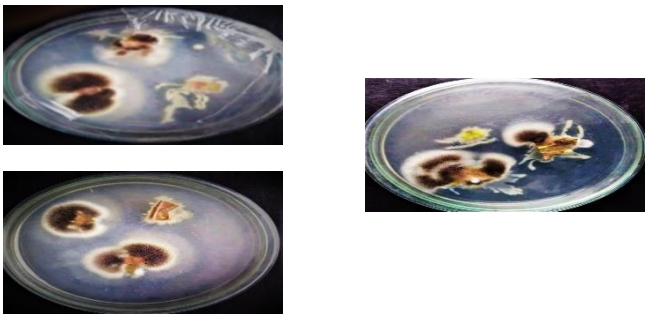


Figure 1: Isolation of fungal endophytes from different *O. tenuiflorum* leaves sample.

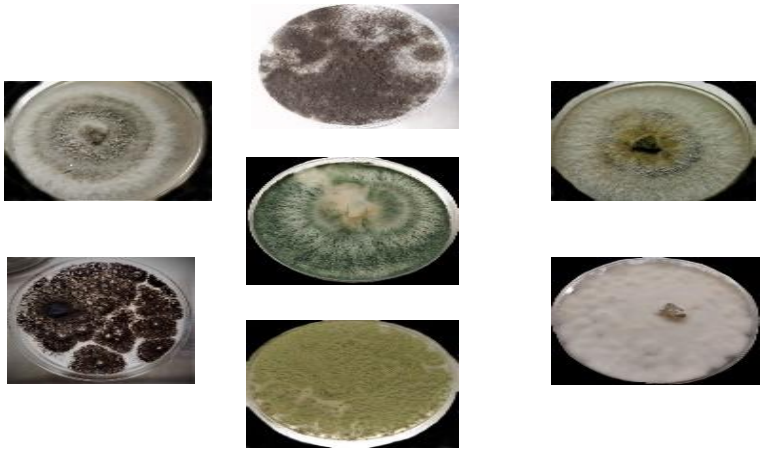


Figure 2: The pure fungal endophytic isolates from different locale derived *O. tenuiflorum* leaves sample.

Table 1: Different endophytic isolates from different locale derived *O. tenuiflorum* leaves sample.

Site	Isolate Code	Morphology
1	EF 7	White in colour, sporulative
	EF 8	White in colour, sporulative
2	EF 1	Dark green in colour, non sporulative
	EF 2	Light green in colour, sporulative
	EF 3	Yellowish white in colour, Non-sporulative
3	EF 4	Pure white colour, Non-sporulative
	EF 5	Brown in colour, sporulative
	EF 6	Greenish white in colour, Sporulative
	EF 9	Black in colour, sporulative

FERMENTATION OF ENDOPHYTIC FUNGI ON PRODUCTION BROTH

To check the pharmacological activities of the selected endophytic fungi from *O. tenuiflorum* leaves derived samples were grown in production broth (Figure 4). Three days old culture broth was extracted and obtained crude metabolites were tested for various antioxidant assays.

Figure 3: Fermentation of metabolite broth.

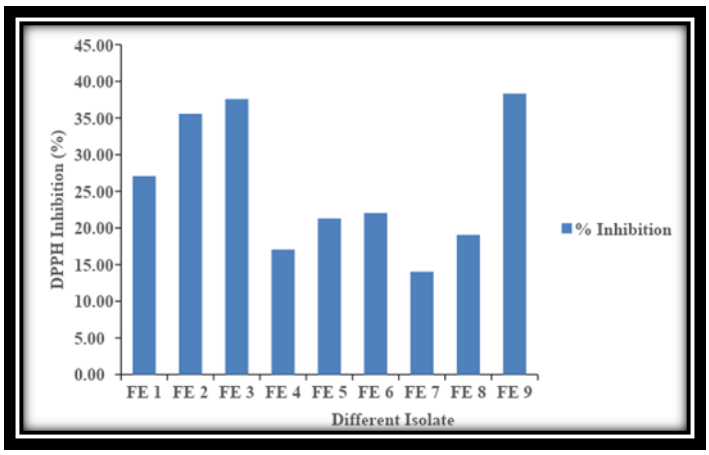
ANTIOXIDANT SCREENING

DPPH Assay

The antioxidant potential of different endophytic fungal isolates from *O. tenuiflorum* leaves derived were tested against DPPP free radical scavenging ability. It was confirmed that, all the endophytic fungal derived crude extracts possess the free radical scavenging ability with some extent. None of them showed no scavenging ability (Figure 5). The highest DPPH free radical scavenging ability was found in the crude extracts of isolate 2, 3, 7 and 9 (Table 2)

Table 2: DPPH free radical inhibition percentage ability of selected fungal endophytes crude extract.

Isolate code	Absorbance @ 515 nm	Control OD @515 nm	Inhibition (%)
FE 1	0.398	0.698	42.98
FE 2	0.279	0.698	60.03
FE 3	0.259	0.698	62.89
FE 4	0.421	0.698	39.68
FE 5	0.476	0.698	31.81
FE 6	0.355	0.698	49.14
FE 7	0.344	0.698	50.72
FE 8	0.497	0.698	28.80
FE 9	0.311	0.698	55.44



FRAP Assay

The antioxidant potential of different endophytic fungal isolates from *O. tenuiflorum* leaves derived were tested against FRAP radical scavenging ability. It was confirmed that, all the endophytic fungal derived crude extracts possess the free radical scavenging ability with some extent. None of them showed no scavenging ability (Figure 5). The highest FRAP radical scavenging ability was found in the crude extracts of isolate 1, 2, 3, and 9 (Table 3).

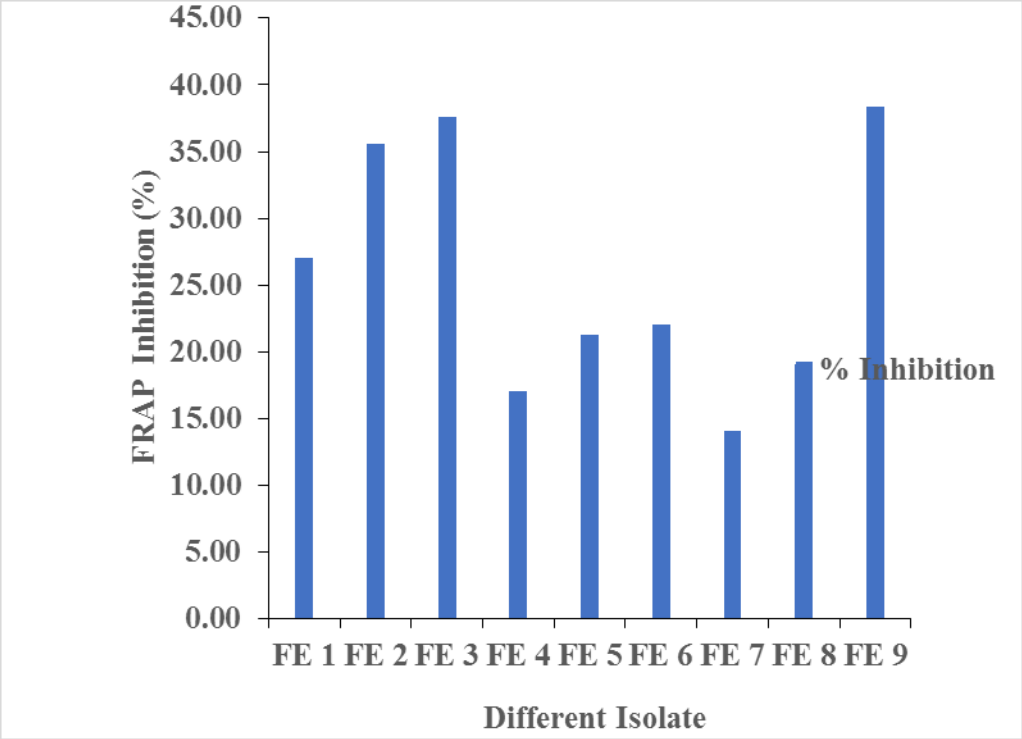


Figure 4. FRAP radical scavenging ability of selected fungal endophytes crude extract.

Table 3: FRAP radical inhibition percentage ability of selected fungal endophytes crude extract.

Isolate code	Absorbance @ 593 nm	Control OD @ 593 nm	Inhibition (%)
FE 1	0.291	0.399	27.07
FE 2	0.257	0.399	35.59
FE 3	0.249	0.399	37.59
FE 4	0.331	0.399	17.04
FE 5	0.314	0.399	21.30
FE 6	0.311	0.399	22.06
FE 7	0.343	0.399	14.04
FE 8	0.323	0.399	19.05
FE 9	0.246	0.399	38.35

NO Assay

The antioxidant potential of different endophytic fungal isolates from *O. tenuiflorum* leaves derived were tested against NO radical scavenging ability. It was confirmed that, all the endophytic fungal derived crude extracts possess the free radical scavenging ability with some extent. None of them showed no scavenging ability (Figure 7). The highest NO radical scavenging ability was found in the crude extracts of isolate 2, 3, 6 and 9 (Table 4).

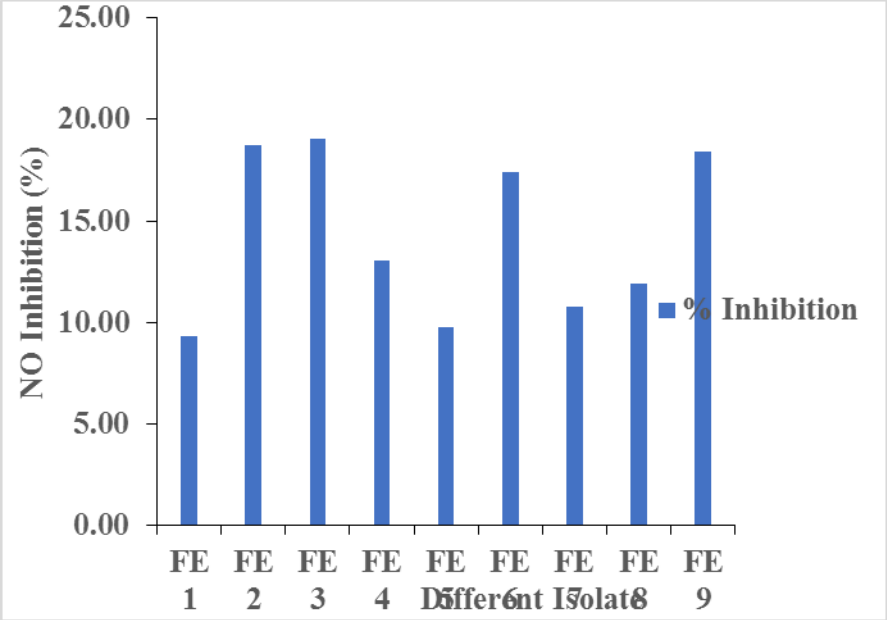


Table 4: NO radical inhibition percentage ability of selected fungal endophytes crude extract.

Isolate code	Absorbance @ 546 nm	Control OD @ 546 nm	Inhibition (%)
FE 1	0.557	0.614	9.28
FE 2	0.499	0.614	18.73
FE 3	0.497	0.614	19.06
FE 4	0.534	0.614	13.03
FE 5	0.554	0.614	9.77
FE 6	0.507	0.614	17.43
FE 7	0.548	0.614	10.75
FE 8	0.541	0.614	11.89
FE 9	0.501	0.614	18.40

SECONDARY SCREEENING FOR ANTIOXIDANT POTENTIAL OF SELECTED ISOLATES

After the primary screening of the selected three isolates such as EF 2, EF 3 and EF 9 were tested for antioxidant and cytotoxicity ability. The crude metabolites thus obtained from the selected three isolates were tested for the radical scavenging ability against DPPH, FRAP and NO radicals. The results of the various radical scavenging ability of the selected three isolates are given in Table 5. The antioxidant potential of the isolates was ranged to EF 3 to EF 2 and EF 9 (Figure 8).

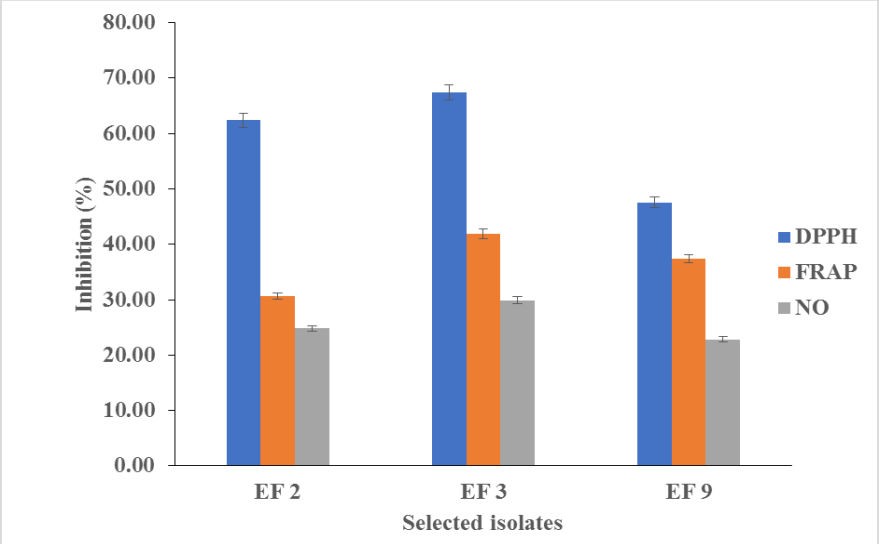


Figure 5: The antioxidant potential of the isolates.

Table 5: Various scavenging activities of three isolates.

Assays	Isolate code	Different OD	Control OD	% Inhibition
DPPH	EF 2	0.254	0.675	62.37
	EF 3	0.218	0.675	67.70
	EF 9	0.354	0.675	47.56
FRAP	EF 2	0.278	0.401	30.67
	EF 3	0.233	0.401	41.90
	EF 9	0.251	0.401	37.41
NO	EF 2	0.455	0.605	24.79
	EF 3	0.424	0.605	29.92
	EF 9	0.467	0.605	22.81

IN VITRO ANTICANCER ACTIVITY AGAINST HELA CELL LINES

The in-vitro cytotoxicity activity results of the crude extracts of selected isolates such as EF 2, EF 3 and EF 9 were tested against HeLa cells. The cytotoxicity of the crude extracts was showed remarkable inhibition of cancer cell lines over untreated control sample. The cytotoxicity of the crude extract showed dose dependant manner on the inhibition of cell lines, the increasing of sample concentrations showed little increment of cytotoxicity and it was clearly observed in results (Table 6). It was evident that the samples tested at high as 200 µg/ml showed cytotoxicity activity as high as 49.01%, 21.97% and 61.53 by EF 2, EF 3 and EF 9, respectively. It was proven that the high cytotoxicity effect of the test sample showed cell disintegration after 48 h of treatment against the selected tested cell lines even at lower concentrations (Figures 9-11). In this cell lines studies, cytotoxicity effect was observed in tested sample concentrations in 48 hours treatment, it also revealed that all tested concentration of test samples shown high cytotoxicity over the tested cell line (Figures 12-

14). The IC50 of the tested crude extracts from selected isolates of EF 2, EF 3 and EF 9 against HeLa cells was calculated as 165.98 µg/ml, 81.11 µg/ml and 256.43 µg/ml, respectively.

Table 6: Cytotoxicity of samples against HeLa cell lines.

Sample code	Title	Concentration								
		0	1.562	3.125	6.25	12.5	25	50	100	200
EF 2	% Viability	100.00	82.55	77.55	74.33	70.29	67.35	58.86	54.48	49.01
	% Cytotoxicity	0.00	17.45	22.45	25.67	29.71	32.65	41.14	45.52	50.99
	IC_{50} (ug/ml)	156.88								
EF 3	% Viability	93.43	84.80	81.79	78.58	74.20	70.09	68.90	66.67	61.53
	% Cytotoxicity	5.34	18.41	28.61	32.92	41.07	46.06	50.99	56.26	78.03
	IC_{50}(ug/ml)	81.11								
EF 9	% Viability	93.43	84.80	81.79	78.58	74.20	70.09	68.99	66.67	61.53
	% Cytotoxicity	6.57	15.20	18.21	21.42	25.80	29.91	31.01	33.33	38.47
	IC_{50}(ug/ml)	265.43								

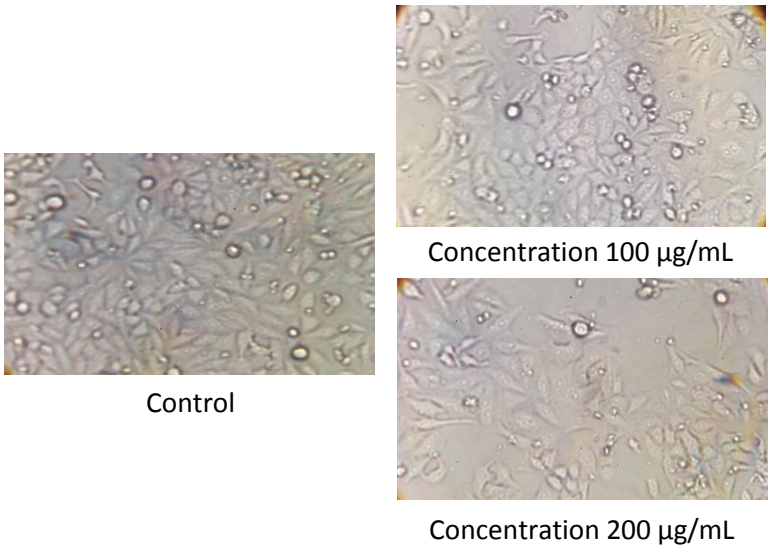


Figure 6: Cytotoxicity effect of crude extract of EF 2 against HeLa cell lines.

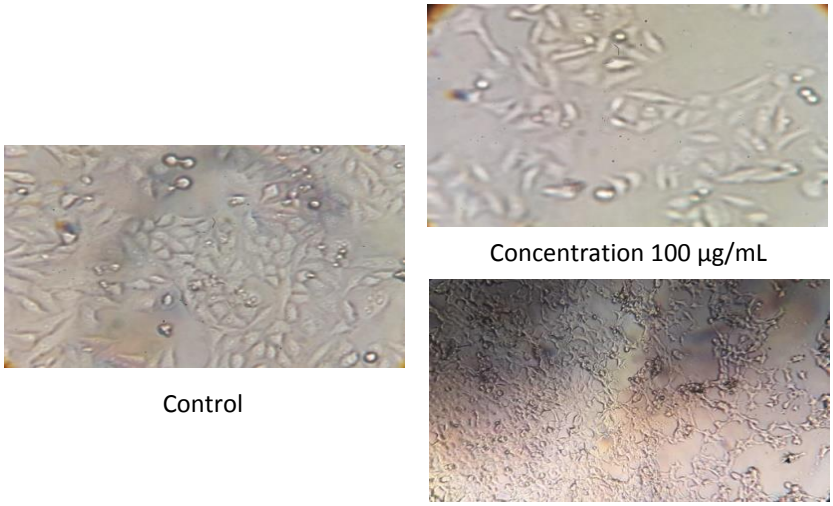


Figure 7: Cytotoxicity effect of crude extract of EF 3 against HeLa cell lines.

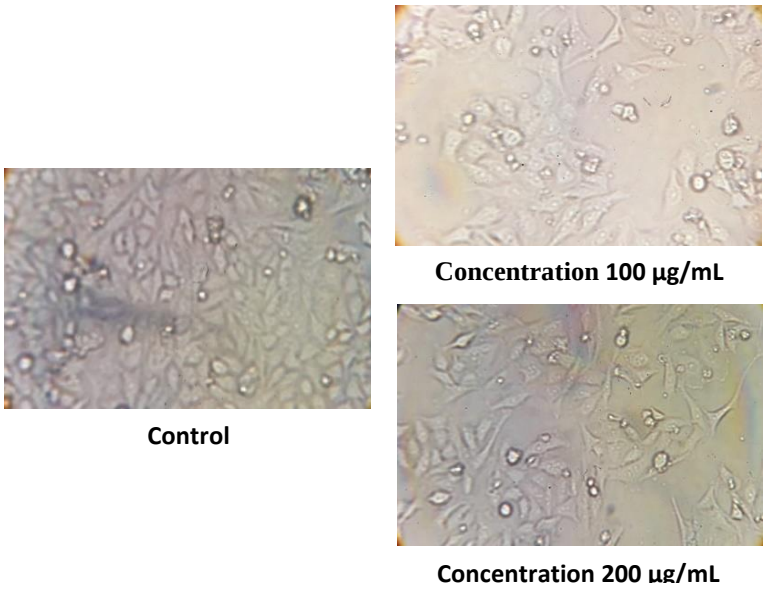


Figure 8: Cytotoxicity effect of crude extract of EF 9 against HeLa cell lines.

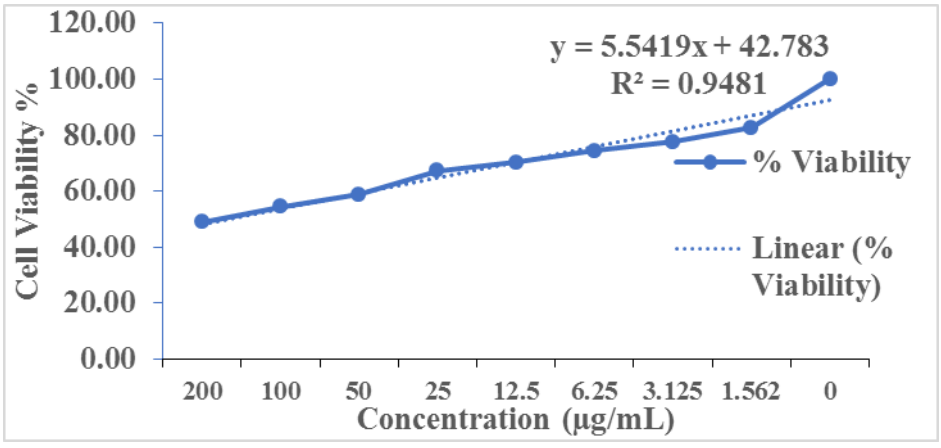


Figure 9: Cell viability percentage of crude extract of EF 2 against HeLa cells.

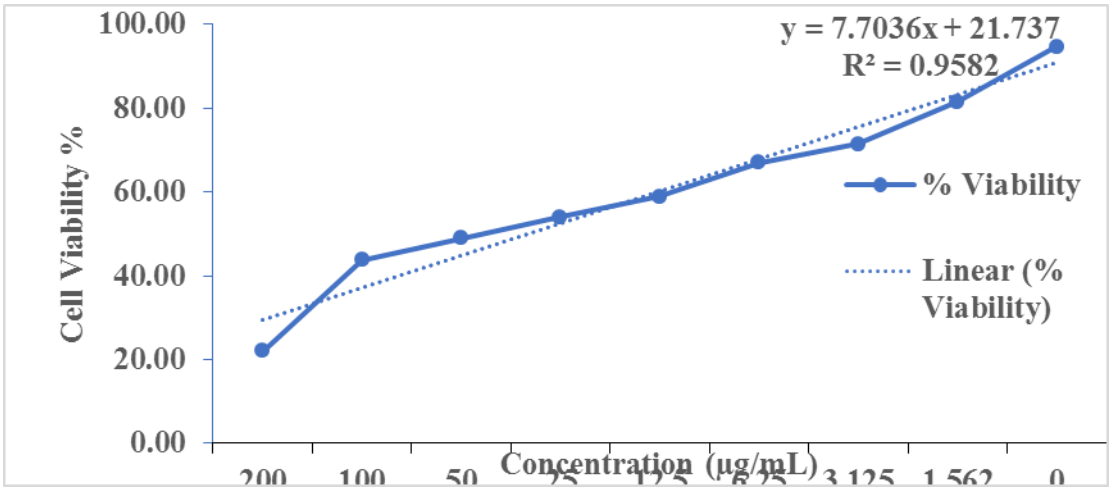


Figure 10: Cell viability percentage of crude extract of EF 3 against HeLa cell lines.

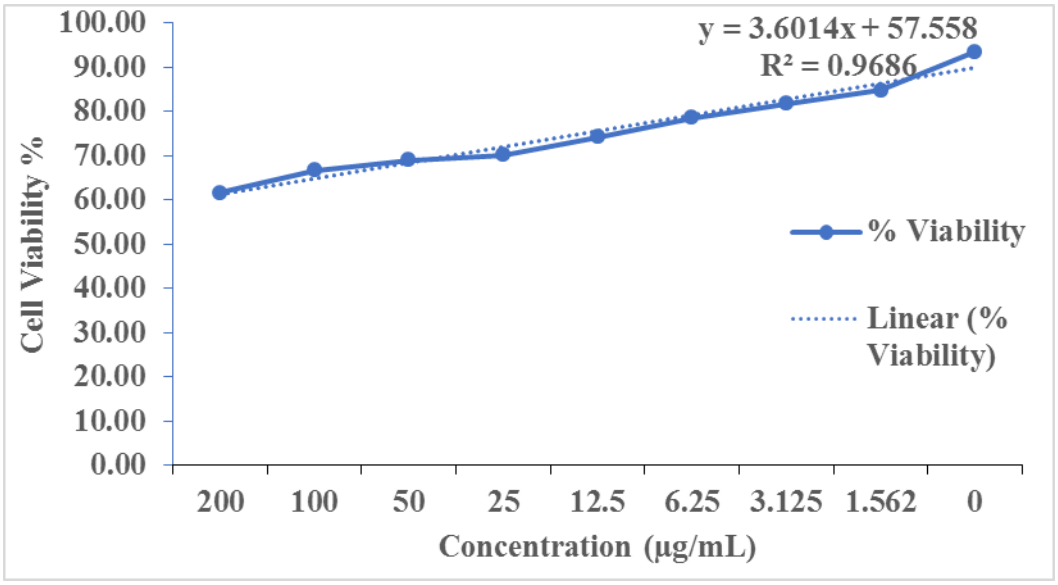


Figure 11: Cell viability percentage of crude extract of EF 9 against HeLa cell lines.

Selection of best isolate for further process

Based on the antioxidant activities and cytotoxicity against HeLa cell lines clearly revealed that all the three isolates were good candidate for the future drug development. However, the isolate EF 3 showed more prominent cytotoxic activity against HeLa cells and IC50 concentration also very remarkable since, taken for the further mechanism of action and GC-MS metabolite profiling and molecular identification (Figure 12).

Figure 12: Colony morphology of selected isolate EF 3 on Potato dextrose agar plate.

DNA Fragmentation assay

Apoptosis can be visualized as a ladder pattern of 180 – 200bp due to cleavage by the activation of a nuclear endonuclease by standard agarose gel electrophoresis. The crude extract from selected isolate EF 3 showed a very good spectrum of the DNA cleavage pattern which was seen as ladder formation (Figure 13). Therefore, showed the formation of the DNA ladder in gel electrophoresis by induction of apoptosis in HeLa cell line.

Lane A: DNA Ladder 1 kb; B: Control Cell; C: Crude extract of isolate EF 3

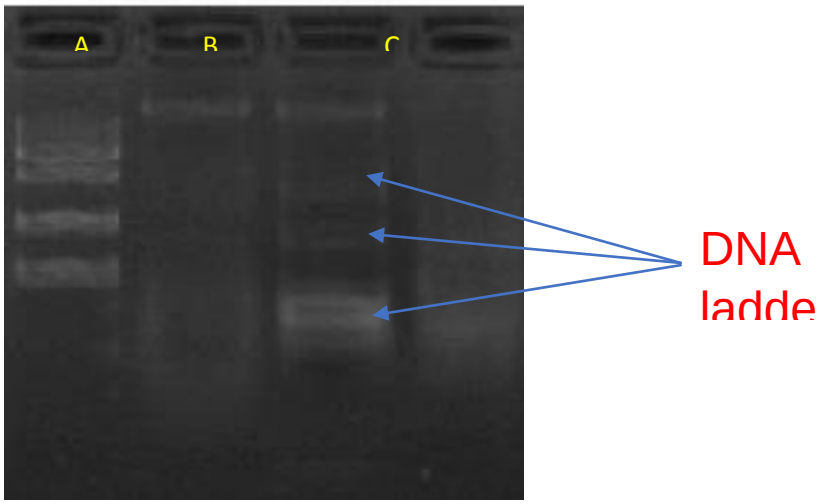


Figure 13: DNA fragmentation assay against HeLa cell lines by selected isolate EF 3 crude extract.

Flow cytometry for Cell viability

The measurement of live and dead cell assay was performed in a flow cytometry analysis using IC50 concentration of the selected isolate EF 3 crude metabolites. The process applied

here allows classification of each event according to both, live stain and dead stain and hence allows the exclusion of double positive (region III) and double negative (region IV) events. However, to be consistent with usually applied procedures, where live or dead cells are identified by staining with a single dye. It was confirmed that, almost 67.31% cell viability as seen in the control sample set whilst, 40.37% cells were viable at the treated concentrations (Figure 14).

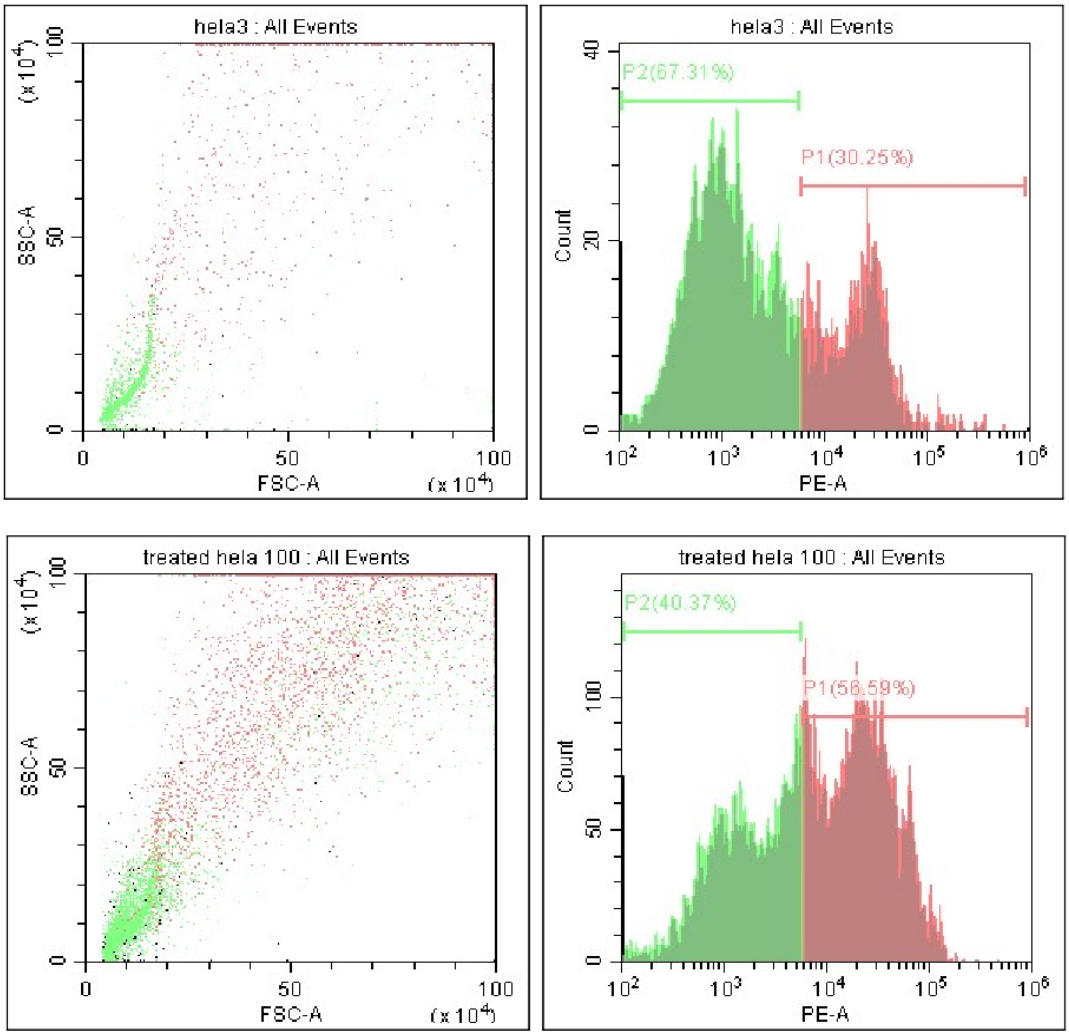


Figure 14: Flow cytometry for cell viability upon addition of crude extract at IC50 Concentration of Isolate EF 3.

GC-MS analysis of the selected isolate EF

The potent bioactive compounds of crude extract of selected isolate EF 3 were analysed using GC-MS (Figure 15). The prevailing compound in the extract was Butanoic acid, 2-methyl-, 3-methyl butyl ester (4.57%), Pyridine, 2,4-dimethyl- (16.55), Oxalic acid, 6-ethyloct-3-yl propyl ester (4.29%), Heptafluorobutyric acid, undecyl ester (5.66%), Benzene, 1,1'-[1,2-

ethenediylbis(sulfonyl)]bis-, (Z)- (17.55%), Thiophene-2-carboxylic acid [2-(5-methoxy-2-methyl-1H-indol-3-yl)-ethyl]-amide (8.58%), Methyl 12-hydroxy-9-octadecenoate (5.48%), Stigmastan-3,5,22-trien (5.97%), Bicyclo[4.1.0]heptane-7-carbohydrazide, N2-(3- allyl-2-hydroxybenzylideno)- (8.95%).

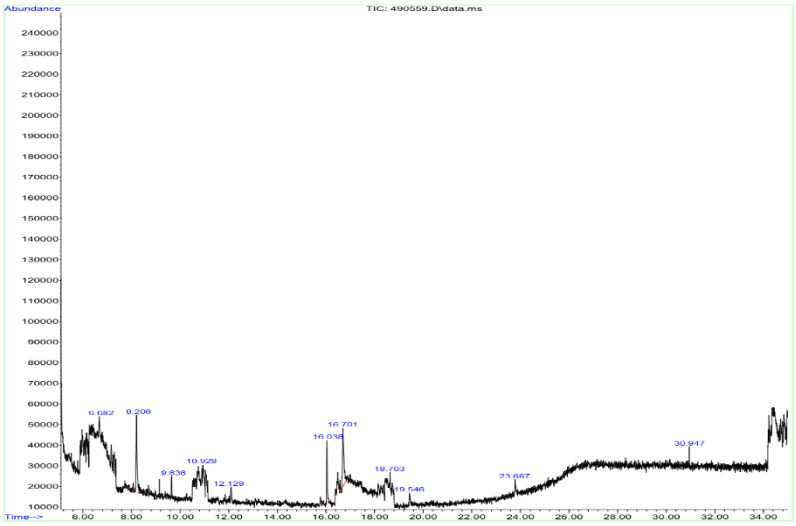


Figure 15: GC-MS total ion chromatogram of selected isolate EF 3 crude metabolite.

Fungal identification using ITS region genotyping

Genomic DNA was isolated from the selected isolate EF 3 was shown in Figure 19. Approximately 25 kb genomic DNA was obtained. The obtained genomic DNA was subjected to the amplification of ITS region amplification and it was amplified well and observed in a 1% agarose gel electrophoresis (Figure 20). The expected amplicon size was ~ 600 bp and also been recoded the same size in the obtained amplicon. The amplified product was purified using Exo-sap method and run in an ABI Prism gene sequencer. About 512 base pairs was obtained in the sequence through forward primer set (Figure 21). The sequences were analysed /BLAST program at NCBI website showed sequences similarities with existing fungal 18S rRNA gene sequences up to 100% sequences similarities. Closed similar species 18S rRNA gene sequences were obtained and used for CLUSTAL W alignment. The CLUSTAL W aligned sequences were analysed Nieghbour joining method and found that the sequences that obtained from endophytic fungus was closely related with *Diaporthe eres* with a single clade (Figure 16)

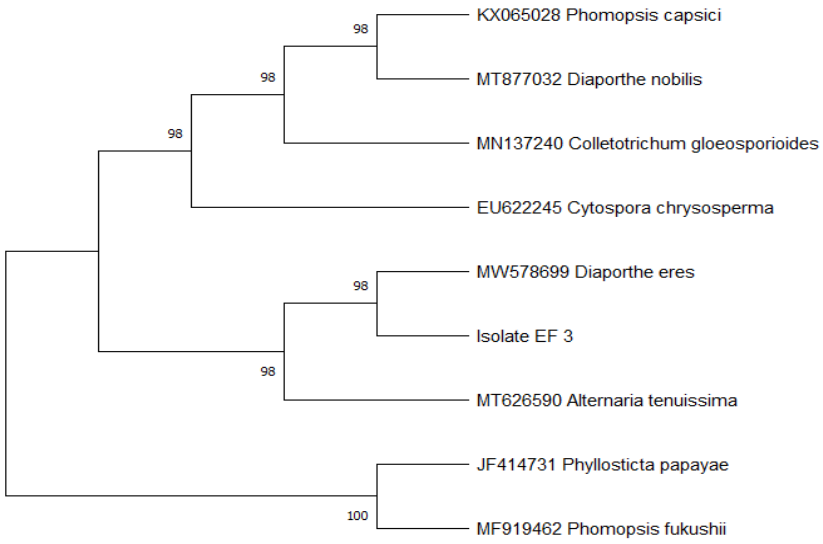


Figure 16: Phylogeny tree analysis of selected isolate EF 3 ITS 1 and ITS 4 regions.

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 0.06723175 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There was a total of 512 positions in the final dataset. Phylogenetic analyses were conducted in MEGAX (Kumar et al., 2018).

Based on the BLAST analysis in the NCBI and phylogeny tree clearly revealed that the selected endophytic fungus belongs to the taxa ***Diaporthe eres***.

CONCLUSION

In this study, we collected samples from different sites and it produced nine typr of endophytic fungi. Then the fungi were fermented to produce secondary metabolites. The metabolites were extracted and carried out antioxidant assays. The EF 3 showed highest antioxidant activity when compared to EF 2 and EF 9 based on the various assays. In cytotoxicity assay, EF 3 showed very prominent cytotoxicity against HeLa cell lines with the IC_{50} value of 81.11ug/ml. Apoptosis were identified by DNA fragmentation assay. The viability of the cells was calculated using flow cytometry. And also, the EF 3 isolate have

nearly 20 compounds which was characterized by GC-MS profiling. The genomic DNA was collected and the ITS regions were amplified. Based on the BLAST analysis in the NCBI and phylogeny tree analysis, that the selected sample EF 3 belonggs to the taxa ***Diaporthe eres.***

REFERENCE

1. Moller; E.M., Bahnweg; G., Sandermann; H and Geige H.H. 1992," A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues". Nucl Acids Res, 20: 6115-6116.
2. White; T.J., Bruns; T., Lee; S. and Taylor, J.W, 1990." Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics". In: PCR Protocols: A Guide to Methods and Applications, Innis, M. A., D. H. Gelfand; J. J. Sninsky; T. J. White (eds.), Academic Press, Inc., New York, 315-322.
3. Sneath PHA; Sokal RR (1973), "Numerical Taxonomy". Freeman, San Francisco.
4. Felsenstein J (1985)," Confidence limits on phylogenies: An approach using the bootstrap". Evolution, 39: 783-791.
5. Kimura M (1980), "A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences". Journal of Molecular Evolution, 16: 111-120.
6. Kumar S; Stecher G; Li M; Knyaz C; Tamura K. (2018). MEGA X: "Molecular Evolutionary Genetics Analysis across computing platforms". Molecular Biology and Evolution, 35: 1547-1549.
7. Camila R; Carvalho; Vivian N. Gonçalves; Cristiane B. Pereira; Susana Johann; Isis V. Galliza; Tânia M. A. Alves; Ana Rabello; Marcos E.G. Sobral.
8. Wu-Yang Huang; Yi-Zhong Cai; Jie Xing; Harold Corke; Mei Sun," A potential antioxidant resource: Endophytic fungi from medicinal plants" 61, Article number: 14 (2007).
9. M Govindappa; M Lavany; P Aishwarya; Kavya Pai; Prathiksha Lunked; B Hemashekhar; BM Arpitha; YL Ramachandra; Vinay B. Raghavendra," Synthesis and Characterization of Endophytic Fungi, *Cladosporium perangustum* Mediated Silver Nanoparticles and their Antioxidant, Anticancer and Nano-toxicological Study", 2020; 10: 928–941.
10. Wu-Yang Huang; Yi-Zhong Cai; Kevin D. Hyde; Harold Corke; Mei Sun," Endophytic fungi from *Nerium oleander* L (Apocynaceae): main constituents and antioxidant activity", 2007; 23: 1253–1263.

11. T. S. Sadananda; M. Govindappa; Y. L. Ramachandra, In vitro Antioxidant Activity of Lectin from Different Endophytic Fungi of Viscum album L., 626-643.
12. Budiono Budiono; Elfita Elfita; Muharni Muharni; Heni Yohandini; Hary Widjajanti,"Antioxidant Activity of Syzygium samarangense L. and Their Endophytic Fungi", 2012.
13. Nelson H. Caicedo; NAndres F. Davalos; Paula A. Puente; Ana Y. Rodríguez; Paola A. Caicedo,"Antioxidant activity of exo-metabolites produced by Fusarium oxysporum*:* An endophytic fungus isolated from leaves of Otoba gracilipes", 2018.
14. Buvaneshwari, V; Indira priyadarshini R," Characterization and transformation of the gene encoding laccase from isolated white rot fungus", 2011; 1-41.
15. Arora; Daljit singh; Chandra; priyanka, "Antioxidant potential of some soil fungi", 2012; 50: 46-61.
16. Govindappa M; Ramachandra Y L; Umashankar T, "Screening of anticancerous conpounds from endophytes of crotalaria sps", 2015; 32(54): 32-56.
17. Kamili; Azra N; Nawchoo; Irshad A; Humeera Nisa, "Isolation identification and culturing of endophytic fungi from few species of genus Artemisia for the production of bioactive fungal metabolites", 2018; 44(45): 42-54.