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EC MICROBIOLOGY
Research Article

Endophytic Fungi, *Fusarium equiseti* Produce Amyrin with Cytotoxic Effect

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Received: June 10, 2025; **Published:** March 04, 2026

Abstract

An endophytic fungus was isolated from the medicinal plant, *Aerva lanata*, from Western Ghats region of India. The fungus was identified by morphology, scanning electron microscopy and molecular characterisation as *Fusarium equiseti*. The fungus was found to produce terpenoid when cultured in potato dextrose broth for 90 days. The compound was separated by column chromatography and identified by thin layer chromatography. The compound was further characterised by IR, NMR and LC-MS and was finally identified to be beta amyrin, a penta terpenoid. Cytotoxicity assay and cell cycle analysis were taken as end points for the study. The compound was found to induce cell death in colon cancer cell line SW620. In cell cycle analysis the Sub G_0 population was very high compared to the control population indicating cell death. The compound thus ensures the possible development of anticancer drug.

Keywords: *Endophytic Fungi; A. lanata; Terpenoid; Cytotoxicity; Apoptosis; Anticancer Metabolite*

Introduction

Evolutionary interactions between plants and microbes existed from the time immemorial. Dr. Charles Darwin was the first to study the evolutionary interactions between different organisms. Paul Erlich and Peter H Raven in 1964 coined the term co-evolution. Plant fungal interactions are believed to be present in all the plants on earth. The fungal partner finds a comfortable home inside the plants which is indeed an example of a beautiful mutualism. Co-evolution in fact affects the evolutionary pattern of both the partners. We studied the capacity of endophytic fungi to produce the same compound produced by the host plant [1].

Endophytic fungi are an important and assorted group of species that vary in mutualistic and green functions. More than 100 years of research has been carried out in these exceptional organisms. They have profound influence on plant ecology, strength, fruition and manifesting strong effects on community structure and diversity of associated organisms. Fossil records prove that this mutualistic association existed for > 400 Myr. Endophytes are well known for producing secondary metabolites with great therapeutic value. Over 1500 fungal metabolites are found to have antibiotic/antitumor activity in a recent literature survey [2].

Novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytes followed by purification and characterization of some of their natural products. Isolation of rohitukine, a chromane alkaloid possessing anti-cancer activity was reported from *Fusarium proliferatum* [3].

Aerva lanata is a perennial herb of the family Amaranthaceae. *Aerva lanata* is a valuable medicinal plant. The important medicinal properties include nephroprotection, cytotoxicity, immunomodulation, antidiabetic, antimicrobial and anti-inflammatory activities. Traditional claims inspired us to select the plant for the study. *Aerva lanata* functions well in treatment of hepatic cirrhosis, pancreatic gland; lowers the blood coagulability, thus preventing formation of thrombi in vessels [4-10]. *Aerva lanata* is known to produce amyrin. We isolated the true endophyte from the plant and cultured for the isolation of metabolites.

Materials and Methods

Chemicals

Oat meal agar was obtained from Himedia. Eagle's minimum essential medium (MEM) was obtained from HiMedia (India).

Collection of samples

Plant Samples were collected from six different districts selected at random, at each site during the months of April (pre-monsoon), May (monsoon) and October (post-monsoon) 2013 - 2014. Fifteen healthy plants were sampled from each district, altogether 90 plants in each sampling time. Sixty numbers of leaves each were selected randomly for inoculation from each districts for each season.

Isolation of endophytes

The samples were processed within 24 hrs of collection. The samples were washed thoroughly in running water, surface sterilized in 70% ethanol for 3 minutes and washed in sterile water for 20s. Sixty segments of leaf (0.5 cm diameter), stem (0.5 cm long), and root segments (0.5 cm long), were placed on oat meal agar (OMA) amended with streptopenicillin in petri dishes. The cultures were incubated at room temperature (26 +/- 2°C) in 12h light and 12 dark cycle for 28 days. The tissue segments were observed daily for fungal growth and the isolates were scored and transferred to OMA slants for preservation. Cultural characteristics of all the fungi isolated were recorded. From among the isolates, a white sterile fungus with pigmentation which showed host-specificity was identified by 28S rRNA gene amplification and sequencing.

Molecular characterization of the isolates

The fungal mycelium was subjected to genomic nucleic acid (DNA) extraction using a kit (Sigma) as per the directions of the manufacturer. Additionally, an ITS - 28S rDNA analysis was performed followed by a BLAST search. The ITS region of the fungus were amplified using Polymerase Chain Reaction (PCR) and the universal ITS primers. The DNA was sequenced at Unibiosys Lab, Kochi, Kerala. DNA sequencing was carried out with PCR amplicon. D1/D2 region was amplified by PCR from fungal genomic DNA using universal Primers [11-13]. The PCR reaction was performed as per the method of Rahjool., *et al.* [14]. The purified PCR product was subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer (Applied Biosystems, USA).

Scanning electron microscopy

Scanning electron microscopy was performed using the techniques described by Ezra., *et al. Fungi* was grown on OMA and was suspended in 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2 - 7.4) with Triton X. The fungal material was dried, coated with gold by sputtering technique and examined under JEOL 6100 SEM.

Isolation of the metabolites

The cell free extract was prepared by filtering the incubated culture grown in potato dextrose broth through muslin cloth and then filtered with Whatman No.1 filter paper. The PDB was mixed and shaken vigorously with equal amount of petroleum ether in a separating funnel. The dissolved fraction was separated and subjected to thin layer chromatography (TLC) using a mixture of ethyl acetate and petroleum ether in 5:4 ratio as the mobile phase. Single spot was detected under UV 480 nm. The compound was loaded on silica gel column. The column was prepared by making the slurry using isopropanol and allowed to set for a day. TLC was performed for each

fraction with same Rf values with the same solvent system for confirmation. IR was done at STIC facility, CUSAT (Cochin University of Science and Technology) and LC-MS analysis were done at SAIF, IIT, Madras, Chennai, India. The yield of the compound was 0.2 g/litre. For drug administration, 1 mg of the compound was dissolved in 2 µl of DMSO and 998 µl of sterile double distilled water.

Cell culture

SW620 cells were obtained from NCCL, Pune, cultured in complete Dulbecco's Modified Eagle Medium (cDMEM) composed of DMEM supplemented with streptomycin (100 mg/ml), penicillin (100U/ml) and 10%FBS. Cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Resazurin reduction assay

Cells were seeded in a 96 well plate at a density of 5000 per well and grown overnight. Cells were treated with increasing concentration of terpenoids ranging from (2 - 10 mg) in complete DMEM from the mother stock. Analysis of cytotoxicity after 48h treatment was determined by resazurin reduction assay with slight modifications [15]. At a concentration of 0.1 mg/ml, resazurin dye was added on to the media, incubated for 3h, for the reduction of blue dye resazurin to pink resorufin which is read at 570 - 590 nm. The data were analysed as percent control. IC_{50} was obtained by determining the concentration of compounds resulting in 50% inhibition of viability in colorectal cancer cells after 48h.

Cell cycle analysis

Propidium iodide staining and flow cytometry were used to assess the cell cycle distribution profile. The treated cells were washed with PBS-EDTA and then harvested using 0.25% Trypsin EDTA and then suspended in cDMEM. Cells were then washed with PBS and centrifuged at 500 x g at 4°C for 5 minutes, and resuspended in 300 µl propidium iodide (2 µg/ml) in the dark, incubated at 37°C for 1h. Data from 10,000 cells were collected for each sample. Data acquisition and analysis were performed on a flow cytometer (Beckman Coulter cell lab Quanta).

Results and Discussion

Fungal endophytes

Twelve cultures were obtained. The tissue cultures constantly yielded one white sterile fungus which was identified as *Fusarium equiseti* through molecular techniques (Figure 1-4). The fungus was recovered from during all seasons and from all localities. Other isolates viz., *Alternaria sp*, *Colletotrichum* sp., *Aspergillus niger, Phomopsis sp. Rhizopus* sp. and several other strains of white, grey and brown sterile mycelium were infrequent and not isolated from all the localities. Hence, *Fusarium equiseti* is apparently the only host-specific endophyte of the plant.

Figure 1: *Aerva lanata plant growing in wild.*

Figure 2: *Endophytic fungi isolated from A. lanata.*

Figure 3: *True endophytic fungi, fusarium equiseti from Aerva lanata.*

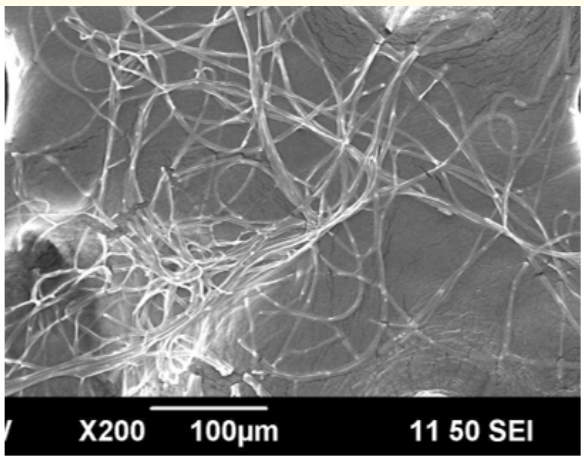

Figure 4a: *Scanning electron microscopic image of F. equiseti.*

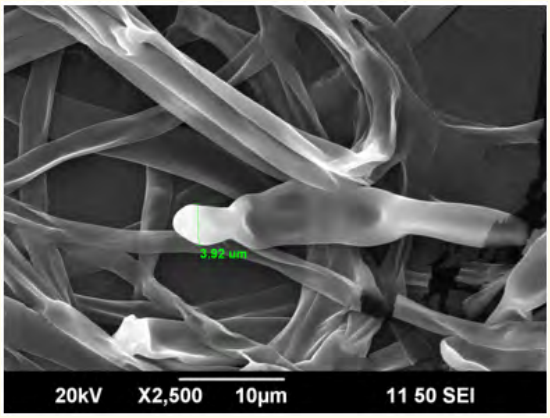

Figure 4b: *Scanning electron microscopic image of F. equiseti with chlamydospores.*

Cultural characteristics

The colonies of *F. equiseti* were white, cottony, irregular margin with pigmentation. The culture was fast growing. Chlamydospores were observed in SEM analysis wit coiled network (Figure 1-4, table 1 and 2). The consensus sequence showed 100% similarity on BLAST analysis. The sequence is deposited in NCBI library with the accession number KM823610.

Plant Name	Months collected	Sample site	Materials collection
Aerva lanata	April, June and October	Peyad, Pathanapuram, Thodupuzha, Puzhakkal, Thaliparampa and Kanhangad	Whole plant

Table 1a: *Particulars of sample collection.*

Fungi/Location	Kanhangad			Thaliparamba			Thodupuzha			Thrissur			Pathanapuram			Peyad		
	M	J	N	M	J	N	M	J	N	M	J	N	M	J	N	M	J	N
Fusarium equiseti	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fusarium sp.	-	-	+	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+
Sterile grey mycelium	+	-	+	-	+	-	+	-	-	+	+	+	+	+	+	+	-	-
*Contains a group of 3 morphotypes none of which were isolated constantly from any locality during any season. +: Present; -: Absent. M: May, J: June, N: November.																		

Table 1b: *Fungal endophytes obtained from the leaves of A. lanata in different seasons and locations.*

Plant	Frequency of occurrence of endophytes (value-%)	Colonisation rate (value-%)	Isolation Rate (value-%)
A. lanata	98.8	80	86

Table 2: *Frequency, colonisation and Isolation rate of endophytes from A. lanata.*

Consensus sequence data

AGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCG

AGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTT

GTAGAGGATGCTTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGA

GGGTGAGAGCCCCGTCTGGTTGGATGCCAAATCTCTGTAAAGCTCCTTCGACGAGTC

GAGTAGTTTGGGAATGCTGCTCTAAATGGGAGGTATATGTCTTCTAAAGCTAAATAC

CGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGA

AAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGTTTATGACCAGACT

TGGGCTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCAGTCCAGGCCAGAT

CAGTTTTCGCCGGGGGATAAAGGCTTCGGGAATGTGGCTCTCTCCGGGGAGTGTTAT

AGCCCGTTGCGTAATACCCTGGCGGGGACTGAGGTTCGCGCATCTGCAAGGATGCTG

GCGTAATGGTCATCAACGACCCGTCT.

IR and mass spectrum

The minor peaks at 2948.31 cm^{-1}, 2837.18 cm^{-1} and 2164.90 cm^{-1} indicates the presence of –C-H stretching vibrations of alkanes. The major peak at 3415.35 cm^{-1} indicates –N-H stretching. The peak at 1650.48 cm^{-1} indicates –C=O stretching. The peak at 668.39 cm^{-1} indicates (C-S-C) stretching. Similarly, the peaks at 1454.81 cm^{-1}, 1022.32 cm^{-1} indicates =C-H broad and strong bend. The band 1650 indicates from LC-MS analysis the molecular weight of the compound is determined to be 203.99 kDa (Figure 5 and 6). The mass spectrum of the compound was found to be β-amyrin according to the published spectrum of the molecule from plant *Dorstenia arifolia* of the family Moraceae [16]. The molecular ion peak at m/z 203 and m/z 189 are characteristic fragments of β-amyrin, a triterpenoid (Figure 7).

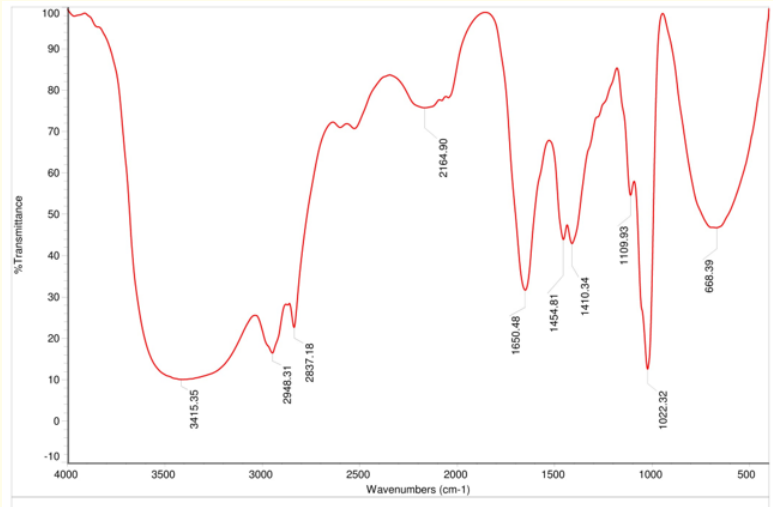

Figure 5: *IR spectrum of terpenoid isolated from F. equiseti.*

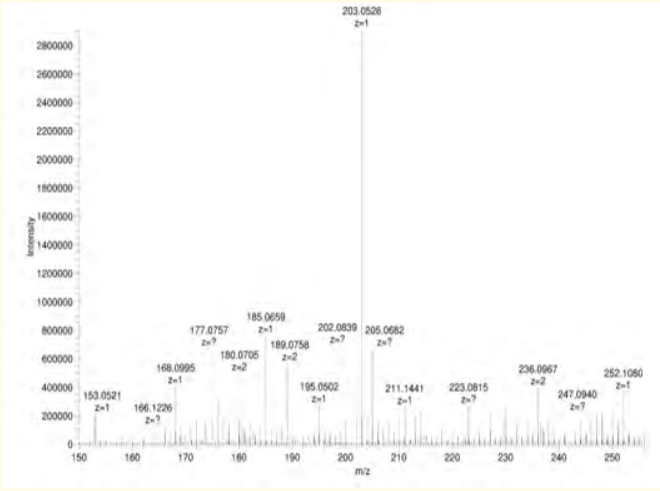

Figure 6: *LS-MS of terpenoid isolated from F. equiseti.*

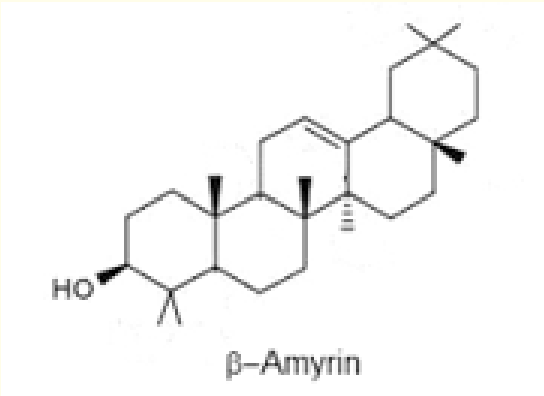

Figure 7: *Structure of beta amyrin.*

Resazurin reduction assay

The terpenoids were cytotoxic to the SW620 cells in a dose dependent manner after 48h of incubation. The IC_{50} value was found to be 164.250 µg (Table 3).

Concentration of terpenoid in µg	% death
100	69.3603
200	60.78263
300	45.50383
400	35.54813
500	21.41964
IC_{50}	164.520 µg

Table 3: *Resazurin assay. IC_{50} value of terpenoid in colon cancer cell line SW 620.*

Cell cycle analysis

Treatment with the terpenoids at 100 µg/ml, increased the population of SW 620 cells at sub G_0 phase with a concomitant decrease in the G_0-G_1, S and G_2M phase when compared to the control population (Figure 8-10). The population of SW 620 cells were 45.8%, 8.4%, 3.58 and 1.35 for Sub G_0, G_0-G_1, S and G_2 M phase respectively in the terpenoid treated cells compared to 1%, 63.09%, 13.7% and 1.35% in the untreated cells. Increased sub-G_0/G_1 population indicates cell death, a hallmark of apoptosis. It is 44 times higher when compared to the untreated group confirming the cell death obtained in the resazurin reduction assay.

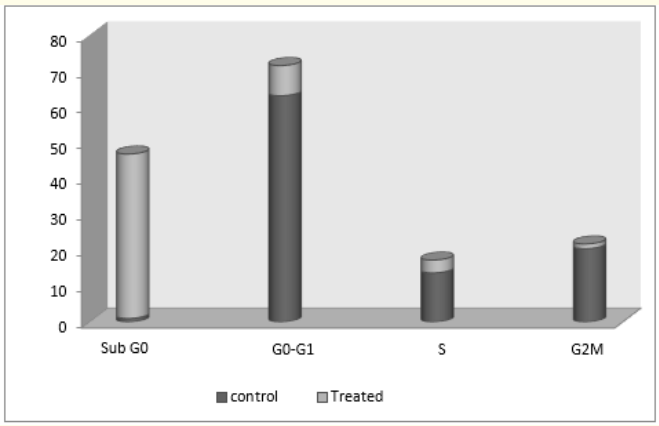

Figure 8: *Quantitative results of flow cytometry analysis in different stages of cell cycle in the presence and absence of terpenoids in SW620 cells.*

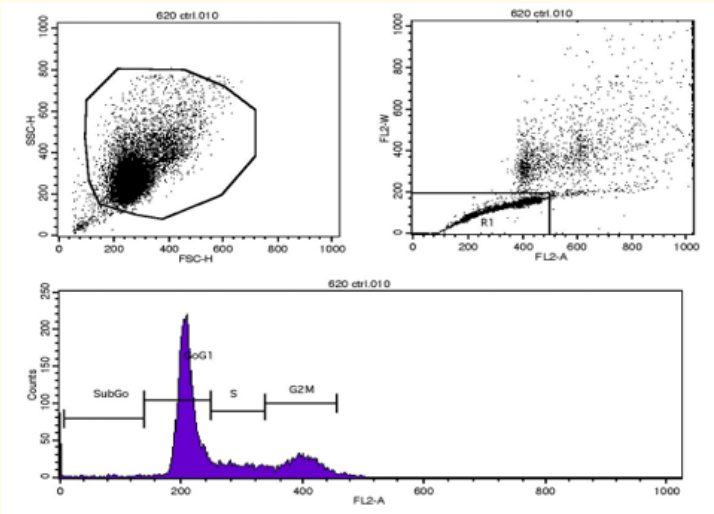

Figure 9: *Flow cytometry data of control group, SW 620 cells without treatment.*

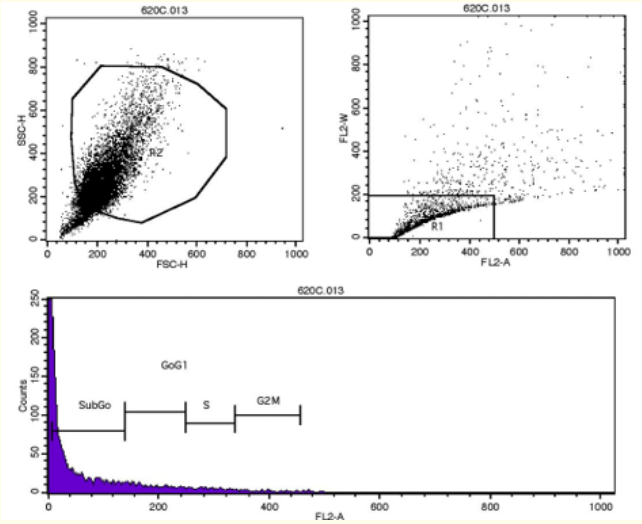

Figure 10: *Flow cytometry data of cells treated with terpenoid at 100 µg.*

Herein we report for the first time, the production of the penta carbon triterpenoid, β-amyrin from the endophytic fungi, *Fusarium equiseti.* Our present study indicates that the terpenoid isolated from the endophytic fungi *Fusarium equiseti* is capable of inducing cell death indicating the possible development of anticancer drug. Studies have suggested that phenolic, methoxy and ketone groups in its chemical structure are very important for their biological activities [17]. Earlier reports suggest that beta amyrin isolated from the plant *Betula utilis* was found to possessed cytotoxic activity in different cell lines [18]. A large number of terpenoids exhibit cytotoxicity against a variety of tumor cells and cancer preventive as well as anticancer efficacy in preclinical animal models [19]. The pentacyclic triterpenoids exhibit a broad spectrum of antitumor activities which make them an important class of natural products that can be used as models for anticancer drug development.

Endophytic fungi are a precious resource of rare and valuable compounds which possess a broad range of therapeutic properties. After the discovery of taxol from the endophytic fungus, *Taxomyces andreanae,* research on endophytes gained importance. Tropical endophytes are a target for study in recent years but few studies have addressed the therapeutic potentials of these fungi. Endophytic fungi capable of producing compounds with anticancer property can be used as a substitute for plants with these properties. Taxol is an expensive anticancer drug extracted from the plant Pacific yew, *Taxus brevifolia*. This would prevent over exploitation of the plants which may lead to extinction of such plants. *F. equiseti* is capable of synthesising a vast range of secondary metabolites [20,21].

Our rationale for studying endophytic microbes as prospective sources of novel medicines is to tap this unexplored area of biochemical diversity. Endophytes can protect the plant by virtue of the antimicrobial compounds that they produce. There are possibilities that the drugs derived out of a plant endophyte will have reduced toxicity compared to drugs developed from chemicals. Plants are a treasure house of microbes having bioactive molecules with reduced toxicity towards higher organisms. The success of chemotherapeutic agents is attributed to its potentialities in inducing the apoptosis in malignant cell populations [22]. Therefore, it is important to promote the development of therapies that involve compounds from endophytic fungi. Modern technologies are to be used to understand this important resource and make it available for the benefit for the mankind.

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