Chapter 2

Determination of *In Vitro* Cytotoxicity and Anti-Angiogenesis for a Bioactive Compound from *Aspergillus terreus* FC36AY1 Isolated from *Aegle marmelos* around Western Ghats, India

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Additional information is available at the end of the chapter

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Abstract

The biotechnological research mainly emphasis its investigation on searching new natural drugs at economical to human welfare. With this view in mind, this research has focused to develop a prospective bioactive compound isolating from an efficient endophytic fungus isolated from potential medicinal tree *Aegle marmelos*. The endophytic fungus was isolated from the medicinal tree and identified as *Aspergillus terreus* FC36AY1. This fungus produced maximum of crude metabolites and this was produced in Sabouraud's Dextrose Broth. The produced metabolites were extracted using acetone as a sole solvent and it was taken for the assessment of antimicrobial and antioxidant analysis. The crude metabolites exhibited maximum activity at least concentration and further the crude extract were taken for purification processes through chromatographic techniques. Through purification, five different fractions were eluted and those five different fractions were also assessed for antimicrobial and antioxidant analysis. From these analysis results, TA4 was found to be efficient fraction and it was characterized through FT-IR, GC-MS and UV-VIS analysis. The compound was taken for cytotoxicity determination in HT-29 cancer cells and anti-angiogenesis analysis was assessed through HET-CAM testing. The bio-activities study revealed that the compound TA4 has the ability to target the cancer cells in an efficient manner.

Keywords: *Aspergillus* sp., cytotoxicity, metabolites, HPLC

1. Introduction

There are numerous natural pigments in the world. They are collected from the sources such as plants, animals and microorganisms. The uses of natural pigments are increasing worldwide. The natural pigments have several affecting factors like temperature, pH, availability and cost. The natural pigments do not cause any serious health effects but it may cause some side effects only for the hypersensitivity persons. The natural pigments are also called as secondary metabolites in which the plants and microbes will produce in enormous by the metabolic production. Each living organism undergoes several pathways for the production of useful secondary metabolites. In this way the best and economical source is microbial metabolites and they have been extensively used in pharmaceutical and medicinal fields to treat various disorders and diseases. With this focus in mind, this research has been focused on the purification of secondary metabolites from an endophytic fungus which would be isolated from an efficient medicinal tree from biodiversified place.

Microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications. Among the molecules produced by microorganisms are carotenoids, melanins, flavins, quinines and more specifically monascins, violacein or indigo. Industries are now able to produce some microbial pigments for applications in food, cosmetics and textiles. Naturally, pigment producing microorganisms like fungi, yeast and bacteria are quite common [1]. The pigments producing microorganisms will produce the antibiotic and inhibit the disease causing pathogens. Antibiotics eliminate or prevent the growth and can therefore cure disease caused by bacterial infection. They cannot however treat viral infection such as common cold or nonbacterial inflammation. Among the microbial pigments the fungi placed a promising rank for its largest and efficient production of potential metabolites which could be applicable in different medicinal and pharmaceutical industries. To which, endophytic fungi explored an evidence in the production of medically used metabolites mainly from medicinal plants. An endophyte is an endosymbiont that lives within a plant for at least part of its life cycle without causing apparent disease [2]. Endophytes are ubiquitous and have been found in all species of plants however, most of the endophyte/plant relationships are not well understood [3]. Endophytes are also known to occur within lichens [4] and algae [5]. Many economically important grasses (e.g., *Festuca* sp. and *Lolium* sp.) carry fungal endophytes in genus *Epichloë*, some of which may enhance host growth [6], nutrient acquisition and may improve the plant's ability to tolerate abiotic stresses, such as drought, and enhance resistance to insects, plant pathogens and mammalian herbivores. Nowadays the studies have focused on endophytic fungi isolated from medicinal tree. From ancient time onwards the medicines have been prepared from trees and plants in order to prevent/or cure the diseases. In this regard the current research has been focused on developing the medicine or drugs from the endophytic fungi residing in potential medicinal plants/or trees. These drugs are nothing but the metabolites produced from the metabolic pathways by the organisms. As the endophytes (i) mimics the metabolism of the host plant, (ii) easy for industrial means and (iii) improvement in activity compared to host plant. The drug derived from endophytes explores the nature of the drugs derived from the medicinal plants and/or trees. The research will be discussed on the bioactive secondary metabolites produced by endophytic fungi through different production, and their usage in different medicinal fields as anti-angiogenic product.

2. Materials and methods

2.1. Isolation and identification of endophytic fungi

The potential endophytic fungus was isolated from a prospective medicinal plant *Aegle marmelos* from Western Ghats (Nilgiris cluster), Coimbatore. This fungus was found to exhibit highest antagonistic activity when compared to other 37 different endophytic fungi from the medicinal plant. Further the potential fungus was named as FC36AY1 and identified to be *Aspergillus terreus* FC36AY1 with the NCBI accession number KY807648. The FC36AY1 was taken for the production of metabolites in Sabouraud's Dextrose Broth (SDB) medium for 17 days of incubation at stationary phase in normal cycle. The pigmented crude metabolites extract was extracted using acetone as sole solvent and it was concentrated for further use. The crude metabolites extract was assessed for biological determination such as antimicrobial and antioxidant analysis [7]. The fungus FC36AY1 manifested maximum activity at least concentration so this was taken for further purification process. The mass production of pigmented secondary metabolites was carried out in SDB medium and the yield was calculated according to Mani et al. [7]. The crude extract was taken for purification process to elute the bioactive secondary metabolite through chromatographic techniques.

2.2. Partial purification of the bioactive secondary metabolite

2.2.1. TLC

The crude extract was subjected to TLC (thin layer chromatography) [8]. About 5 μL of the crude extract was applied 1 cm above from the lower edge of the thin layer chromatography slides and dried. It was immersed to a depth of 1 cm in the solvents. The different solvent system tested for movement of the pigment was chloroform:methanol and petroleum ether:ethyl acetate. The best solvent system for the separation of the components in the crude extract was taken. The solvent front was marked and R_f value was calculated.

2.2.2. High performance liquid chromatography (HPLC)

The active fraction was further purified through preparative high performance liquid chromatography (HPLC) (Shimadzu-1100 series), manual injector with quaternary pump, photodiode array detector equipped with C_{18} column (4.6 × 250 mm) with 5 μL of pore size with the flow rate of 1 mL/min and mobile phase of acetonitrile:H_2O (80:20) at 427 nm.

2.3. Antioxidant activity

The purified fractions were taken for antioxidant analysis for determining the prospective secondary metabolite.

2.3.1. DPPH radical scavenging assay

The antioxidants present in fungal crude metabolites were aliquot into different concentrations (20–100 μg) to determine extract's ability to scavenge of 2,2-diphenyl-1-picrylhydrazyl

(DPPH) radicals using the method of Mani et al. [7] in triplicates. DPPH solution (1 mM DPPH radical solution in 95% ethanol) was added to the crude metabolites extracts and made upto 1 mL, vortexed well, and then incubated for 30 minutes in dark hood at room temperature. After incubation, the samples were poured into microfuge tubes and centrifuged for 5 min at 13,500 rpm at RT. The absorbance of each sample at λ = 517 nm was measured and 1 mL of 95% EtOH/MeOH was used as a control, and DPPH were used as reference compounds. The antioxidant activity is given as percent (%) DPPH scavenging assay was calculated using the formula: [(control absorbance − extract absorbance)/(control absorbance) × 100]. The fungi exhibiting the maximum antioxidant activity at minimum concentration and antagonistic profile were taken for further identification studies.

2.3.2. Reducing power assay

Total reducing power was determined as described by Oyaizu [9] in triplicates [7]. 1 mL of sample solution at different concentrations (20–100 μg) was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture and centrifuged at 3000 × *g* for 10 min. The supernatant (5 mL) was mixed with 1 mL of ferric chloride (0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power.

2.3.3. Metal chelating activity

The metal chelating activity was analyzed by the method of Dinis et al. [10] with slight modification in triplicates [7]. The reaction was performed in HEPES buffer (20 mM) at pH 7.2. Various concentrations (20–100 μg) of samples were mixed with a solution of 12.5 μM ferrous sulfate solution. Addition of 75 μM ferrozine was to initiate the reaction and the mixture was shaken vigorously and incubated for 20 min at room temperature. After incubation the absorbance was measured at 562 nm. Ascorbic acid was used as the reference compound and the percentage chelating capacity was calculated as; % chelating activity = $[(A_0 - A_1)/A_0] \times 100$ where, A_0 = absorbance of the blank; A_1 = absorbance of the sample.

2.3.4. Superoxide anion radical scavenging assay

Measurement of the superoxide anion radical scavenging capacity of the eluted fractions were essential according to the method described by Liu et al. [11] using a minor modification. The principle of this method is that superoxide radicals are generated in phenazine methosulfate (PMS)-nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated with 3.0 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1.0 mL of NBT (50 μM) solution, 1.0 mL NADH (78 μM) solution and samples of the compound MM4 (20–100 μg/mL) in methanol. The reaction was initiated by adding 1.0 mL of phenazine methosulfate (PMS) solution (10 μM) to the mixture. The absorbance at 560 nm was measured against a blank. Ascorbic acid was used as a standard. The scavenging activity was calculated by: [(Abs control − Abs sample)/Abs control] × 100.

2.3.5. Hydroxyl radical scavenging assay

The scavenging activity for hydroxyl radicals recommended by Yu et al. [12] was followed with minor changes using the fractions. Reaction mixture contained 0.6 mL of 1.0 mM Deoxy ribose, 0.4 mL of 0.2 mM phenyl hydrazine, 0.6 mL of 10 mM phosphate buffer (pH 7.4). It was incubated for 1 h at room temperature. Then 1 mL of 2–8% TCA, 1 mL of 1% TBA and 0.4 mL of compound (at different concentrations) were added and kept in water bath for 20 min. The absorbance of the mixture at 532 nm was measured with a spectrophotometer. From the readings, the hydroxyl radical scavenging activity was calculated as: [(Abs control – Abs sample)/Abs control] × 100.

2.3.6. Chemical characterization of bioactive secondary metabolite

The bioactive purified fraction which evinced maximum antioxidant activity at least concentration was dissolved in acetone and it was taken for UV-analysis using UV-Vis scanning spectroscopy for the detection of single peak. Scanning was performed between 200 and 800 nm wavelength. Later the fraction was subjected to structural elucidation of the compound. 1 mg of purified fraction was dried and analyzed for Infrared (IR) spectra using FTIR spectroscopy. The important IR bands of symmetric and asymmetric stretching and stretching frequencies were studied to determine the presence of functional groups.

The gas chromatography-mass spectrometry was done by advanced equipment (Thermo GC—Trace ultra VER: 5.0, Thermo MS DSQ II). The column used in this experiment was DB 5-MS capillary standard non-polar column with the dimension of 30 mts, ID-0.25 mm and the film was 0.25 μm. The carrier gas used was helium, with the flow rate at 1.0 mL/min and the temperature was as oven temperature 70°C which was raised to 260°C at 6°C/min. The sample of 1 μL which was purified from HPLC analysis was taken and injected for the experiment.

2.4. *In vitro* studies

2.4.1. MTT assay

The cytotoxic effect of the bioactive compound was studied using cancer cell lines. The HT-29 cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 50 μg/mL CO_2 at 37°C. Cells (1×10^5/well) were plated in 24-well plates and incubated in 37°C with 5% CO_2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24 h. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100 μL/well (5 mg/mL) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) were added and incubated for 4 h. After incubation, 1 mL of DMSO was added in all the wells. The absorbance at 570 nm was measured with UV-Vis Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required to inhibit 50% of cells (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula 1:

$$\%\text{cell viability} = \left(A_{570} \text{ of treated cells}/A_{570} \text{ of control cells} \times 100\right) \quad (1)$$

Graphs were plotted using the % of cell viability as Y-axis and concentration of the sample in X-axis. Cell control and sample control was included in each assay to compare the full cell viability in cytotoxicity assessments.

2.5. Determination of HET-CAM test [Hen's egg test on the chorio-allantoic membrane (HET-CAM) of chick eggs]

In order to understand the inflammatory tissue reactions of metabolite coated materials on the live tissues, the materials were placed on the surface of Chorio-Allantoic membrane (CAM) of embryonated chick eggs. The inflammatory response on CAM was evaluated by direct evaluation method. Freshly laid fertile eggs were collected from the chicken farm and incubated at 36–37°C for 8 days before implanting (implantation day: 9th day) the sample materials such as compound TA4, acetone, positive and negative solutions. During the incubation time, the eggs were turned twice daily. On the day of implantation (9th day after laid), the eggs were candled to determine the position of the air sac and the embryo. A square, with sides approximately 18–20 mm, was marked on the shell where the chorio-allantoic membrane was best developed. Using a dental drill fitted with a straight hand-piece the sides of the marked square were drilled. In one corner of this large triangle a second smaller square was drilled, with sides of approximately 5 mm. A small slit was drilled in the shell over the air sac.

2.5.1. Application of test sample, solvent, positive and negative control on CAM

Aseptic technique was used for the implantation of the test sample on biomaterial as filter paper discs. For dropping the material onto chorio-allantoic membrane the egg was mounted on a stand, with the drilled area of shell uppermost; a straight Hagedorn's needle was gently inserted under one corner of the smaller square of shell and this square was raised and removed. The shell and shell membrane circumscribed by the larger square were then removed, and the sterile pre-measured size of sample was inserted and carefully lowered on to the exposed membrane. In order to implement the implanted sample TA4, 0.3 mL of the substance (positive and negative control) was applied to the surface of the CAM on separate eggs. 0.1 N NaOH was added on the CAM of separate egg as a positive control and 0.9% NaCl was used as an appropriate negative control. After a 20-s exposure period, the CAM is rinsed with 5 mL of water.

2.5.2. Direct evaluation of CAM time for development of observed endpoints after exposure to the test substance

A procedure used to evaluate the time for development of endpoints after exposure to the test substance was to continually observe the CAM during the 5-min observation period and record (typically in seconds) the time at which each of the endpoints developed. Therefore, two separate time values (after 2 and 18 h of incubation) were obtained and recorded for each egg (one time value for each endpoint).

3. Results

3.1. Purification of secondary metabolite

3.1.1. Thin layer chromatography

The concentrated crude metabolite extract was subjected to thin layer chroamtography and the best solvent system which separated maximum compounds as band was chloroform:methanol:toluene:acetic acid and at 95% about five visible bands got separated (**Figure 1a**)—R_f value was calculated for obtained peaks and tabulated (**Table 1**).

3.1.2. High performance liquid chromatography (HPLC)

The crude extract was forced for preparative HPLC analysis about 6 different peaks were obtained at 427 nm (**Figure 1b**) and this range corresponds to the result of UV spectrum analysis. The eluted fractions from HPLC analysis were assessed for antimicrobial profile. Among the five fractions (TA1–TA5), fraction 4 (TA4) was found to contain highest antimicrobial profile on comparing to other fractions (**Table 2**). The fraction 4 explored highest activity against *C. albicans*, *K. pneumoniae*, *E. coli*, *S. epidermidis* and *S. typhi.* From these results it is evident that this particular fraction was able to control the growth of gastro intestinal and skin pathogens.

3.2. Determination of antioxidant properties

3.2.1. DPPH radical scavenging activity

The bioactive compound of the present study showed a concentration dependent antiradical activity by inhibiting DPPH radical (**Figure 2a**). The decrease in absorbance of the DPPH radical

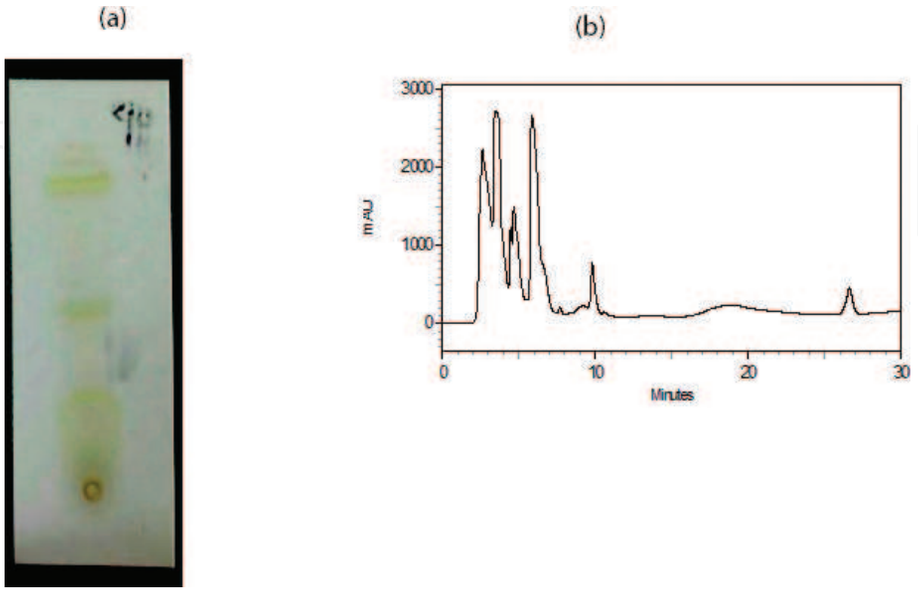

Figure 1. (a) TLC of crude extract and (b) chromatogram showing different peaks in *Aspergillus* sp. extract.

S. no.	Bands	R_f value (%)
1	I	0.046
2	II	1.125
3	III	1.184
4	IV	1.25
5	V	1.8
6	VI	1.956
7	VII	3.461
8	VIII	9.000

Table 1. R_f value of TLC for crude extract.

S. no.	Pathogens	Zone of inhibition (in cm)				
		TA1	TA2	TA3	TA4	TA5
1	*Staphylococcus aureus*	0.5	0	1.2	1.2	1.2
2	*Klebsiella pnuemoniae*	0	0	0.9	1.4	0.9
3	*Staphylococcus epidermis*	0.9	0	0.8	1.6	0
4	*Pseudomonas aeruginosa*	1.1	0	1.2	1.1	0
5	*Enterococcus faecalis*	1.2	0	2.1	2.1	0
6	*Bacillus subtilis*	1	0	0	2.3	1.3
7	*E. coli*	0	0	0	1.0	1.2
8	*Proteus mirabilis*	0	0	1.1	0.9	0.9
9	*Shigella sp*	0	0	0.9	0	0.7
10	*Salmonella*	0	0	0	0	0.8
11	*Candida albicans*	0	0	0	2.1	0.9
12	*Aspergillus terreus*	2.1	0	0	2.3	0

<0.5 cm: no inhibition activity.

Table 2. Antimicrobial activity for eluted fractions.

caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a color change from purple to yellow. Also, a lower value of IC_{50} (concentration at which the 50% scavenging activity is obtained) indicates a higher antioxidant activity at lower concentration. The IC_{50} concentration of purified fraction was found to 56 μg/mL.

3.2.2. Reductive power ability

The reducing ability of a bioactive compound generally depends on the presence of reductones, which exert the antioxidant activity by breaking the free radical chain by donating a

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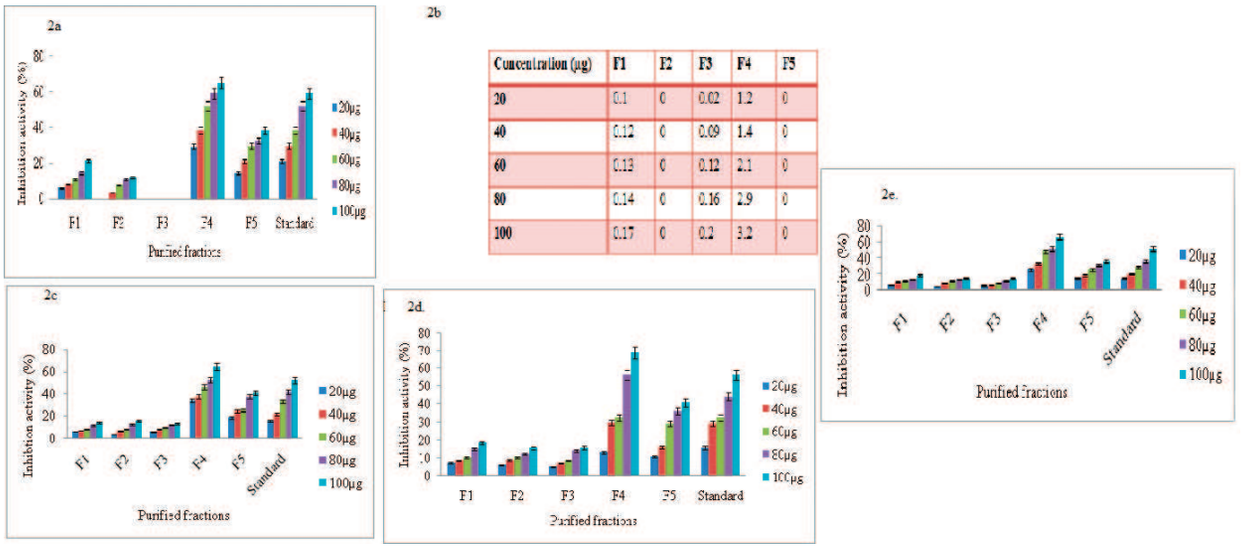

Concentration (µg)	F1	F2	F3	F4	F5
20	0.1	0	0.02	1.2	0
40	0.12	0	0.09	1.4	0
60	0.13	0	0.12	2.1	0
80	0.14	0	0.16	2.9	0
100	0.17	0	0.2	3.2	0

Figure 2. (a) DPPH radical scavenging activity, (b) reductive power ability of purified fraction, (c) superoxide radical scavenging activity, (d) hydroxyl radical scavenging activity, and (e) metal chelating activity.

hydrogen atom. In this study, the reductive ability of the compound TA4 had a maximum reductive power and this was observed by increasing OD units (**Figure 2b**). This confirmed the increasing reducing power through increasing OD units. When compared to the standard ascorbic acid, TA4 exerted a similar activity. This activity was concurrent with the investigation of ***Liu et al. (2007) with the same concentration of 20–100 µg/mL.

3.2.3. Superoxide radical scavenging activity

The superoxide radicals are generated by PMS and it was assessed using NBT. **Figure 2c** explains that the bioactive compound has a maximum scavenging mechanism at minimum concentration. The 50% of inhibition concentration value was found to be 24 µg/mL. The IC_{50} concentration of purified fraction was found to 68 µg/mL.

3.2.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging assay showed the ability of the bioactive compound and standard ascorbic acid in inhibiting hydroxyl radical mediated deoxyribose degradation in a Fe^{3+} EDTA ascorbic acid and H_2O_2 reaction mixture (**Figure 2d**). The IC_{50} value of the compound was found distinctly increased with increased concentration when compared to the standard. Hydroxyl radicals are the major active oxygen species causing enormous biological damage by lipid peroxidation in cells. The IC_{50} concentration of purified fraction was found to 75 µg/mL.

3.2.5. Metal chelating activity

The rate of color reduction was measured which was used in the estimation of chelating activity of the coexisting chelator. In the current analysis, the absorbance of Fe^{2+} ferrozine complex was decreased in a dose dependent manner which can be meant in other way as the activity increased with the increasing concentration from 20 to 100 µg/mL and the IC_{50} concentration of purified fraction was found to 70 µg/mL (**Figure 2e**).

3.3. Chemical characterization of the secondary metabolite

UV-Visible spectrum of the purified pink colored compound was recorded using acetone solution in the range of 200–700 nm showed a single peak for the purified fraction (**Figure 3a**). The spectral measurement showed that the compound had registered its absorption band in the region 427 nm. The peak in the wavelength of 427 nm showed that this is the active fraction. IR spectrum has proven to be the most effective way to give the information about the functional groups present in the compound.

Figure 3b depicted the FT-IR report for the purified fraction. In that, the stretching frequencies of the IR spectrum recorded the highest peak at 1711.92 which correspond to C—O (carbonyl/ketone) stretching. The region 1360.41 denotes the region —C—H (sp^3 configuration and alkane group) and the region 1221.06 records CN stretching (aromatic primary amine group or phenolic group). The region 1426.80 and 1093.19 denotes —C—H (sp^3 configuration with vinyl C—H) and C—O (alcohol group) respectively. The frequencies from 3600 to 3200 cm^{-1} denoted the alcohol group present in the compound and in the same way the frequencies recorded from 3000 to 2850 cm^{-1} denoted the alkane stretching.

3.3.1. GC-MS analysis

The gas chromatography-mass spectrometry (GC-MS) was analyzed for purified fraction of TA4. The chromatogram was obtained with 4 major peaks in TA4 (**Figure 3c** and **d**). Finally from the results of UV-spectra, FT-IR, GC-MS, analysis we conclude the compound of TA4 was found to be octadecenoic acid 4-hydroxy methyl ester C19H38O3.

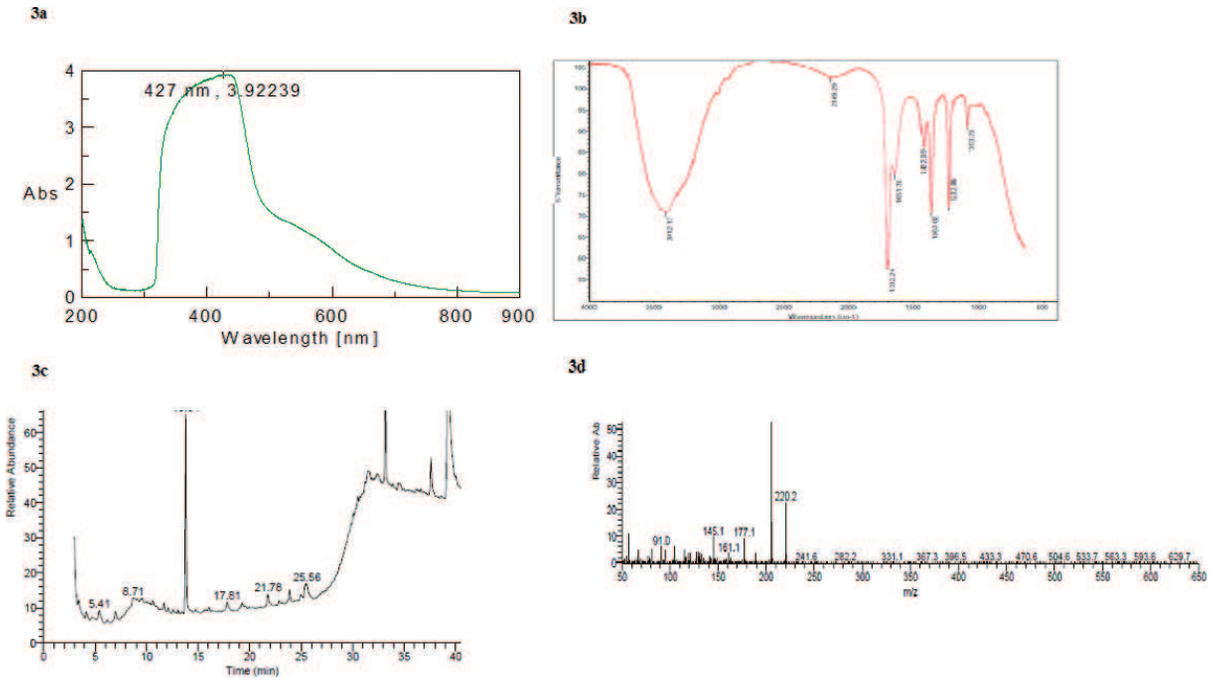

Figure 3. (a) UV-VS of TA4, (b) FT-IR spectra for TA4, (c) GC-MS of TA4, and (d) GC-MS RA peak of TA4.

3.4. *In vitro* studies

3.4.1. MTT assay

The present investigation has been carried out for a potential bioactive compound TA4. The studies have been performed on HT-29 colon cancer cell lines with the control of triton X 100 (**Table 3** and **Figure 4**). This result was similar to the investigation of Yuvaraj et al. [13] reported the IC_{50} at nearest concentration. The present study has showed that *A. terrus* FC36AY1 acetonic fractions could extensively inhibited cell proliferation architecture in dose dependent manner. This report demonstrating the *in vitro* anticancer activity of the TA4 from methanolic extract of *A. terrus* FC36AY1 providing a scientific basis for its effects on human health which is similar to Yuvaraj et al. [13], TA4 was found to be inhibited maximum number of cells. So TA4 was taken for further studies.

Sample concentration (µg/mL)	Average OD at 540 nm	Percentage viability
Control	0.3855	
6.25	0.3044	78.96239
12.5	0.2941	76.29053
25	0.2427	62.9572
50	0.2077	53.87808
100	0.1545	40.07782

Table 3. MTT assay.

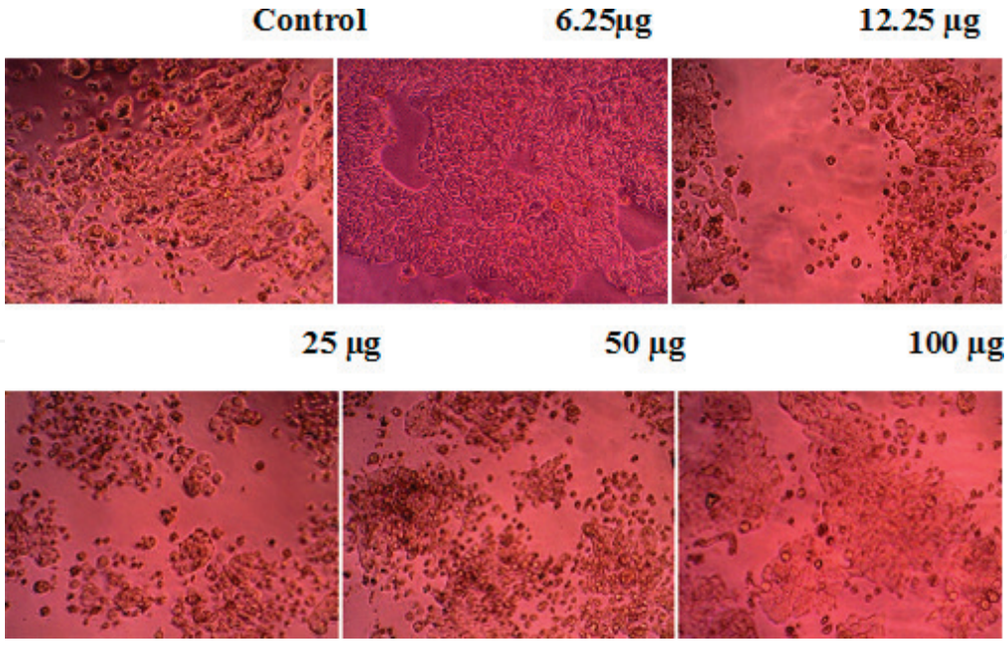

Figure 4. MTT analysis of TA4.

Sample	For 2 h		For 18 h	
	No. of vessels in untreated CAM	No. of vessels in treated CAM	No. of vessels in untreated CAM	No. of vessels in treated CAM
Negative control—acetone	18	10	18	05
Sample—1–200 μL	12	10	12	08
Sample—1–400 μL	10	05	13	03
Positive control—NaOH	11	11	11	11

Table 4. Anti-angiogenesis effect of TA4 in HET-CAM test.

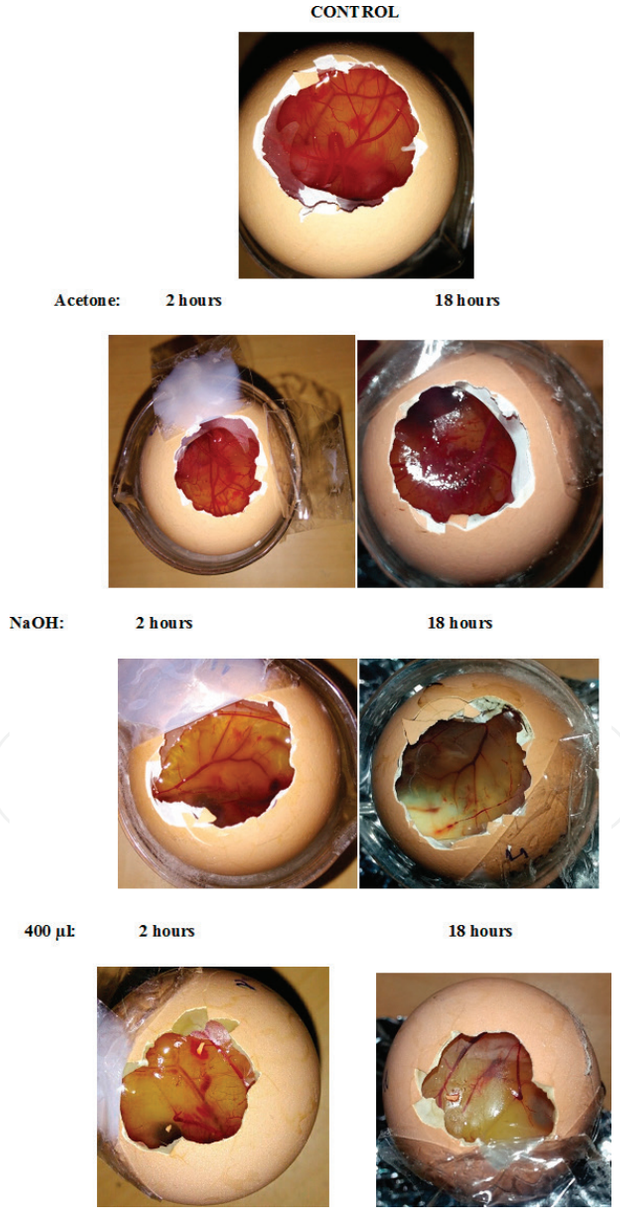

Figure 5. HET-CAM test on chick embryo control.

3.5. HET-CAM test on chick embryo

The CAM assay is a perceptive, easily feasible, and cheap in vivo check for enquiries of the anti-angiogenic promise of individual compounds. The compound TA4 inhibited the angiogenesis at an interval of 2 and 18 h (**Table 4** and **Figure 5**). This evinced that the compound has the good anti-angiogenic potentiality which could be explored as anticancer agents.

4. Discussion

In our current scenario there are many new and interesting bioactive metabolites applied as antibiotics, antiviral, anticancer and antioxidant compounds, which are of pharmaceutical, industrial and agricultural importance. Those have been investigated, reported and characterized from several fungal endophytes especially from medicinal plants/trees. Endophytic fungi have the ability to pulse up a plethora of secondary metabolites, typically dependent on the stage of development and environmental factors ranging from nutrient concentrations to light and temperature. The biosynthesis of pigmented secondary metabolite(s) is directly related to cultural conditions that include biomass in the production phase and duration of the incubation periods [14].

An investigation of Strobel and Daisy [15] suggested the endophytic fungi since such plants may harbor unique and rare endophytes capable of producing important bioactive metabolites with multiple applications. First and foremost pharmaceutical applied drug Taxol, an anticancer drug was derived from *Taxus*, a gymnosperm is an important anticancer plant. Several endophytic fungi isolated from *Taxus* spp., worldwide have been reported to produce important bioactive metabolites [16, 17]. This current investigation is focused to produce the pigmented crude secondary metabolites and purify a bioactive compound through the antioxidant assessment which could be taken for anticancer applications by *in vitro* and *in vivo* studies. The isolated and identified endophytic fungus FC36AY1 was a prospective and potential strain on analyzing antagonistic and preliminary antioxidant analysis. The fungus explored highest activity in both the assessment. The antagonism revealed the extent of this analysis ability increased as the endophytic fungal colonies matured when compared to immature colonies. The significant inhibition in the growth of fungi without direct contact of mycelia suggests that the prevailing antagonisms may be due to the production of inhibitory substances by the fungi or due to the competition for nutrients or both [18]. However, the mechanisms of inhibition in colony growth of the tested fungi were not addressed in our studies. This was similar to the investigation of Tayung et al. [17] investigated an endophytic fungus *Fusarium* sp. with highest antimicrobial and antioxidant analysis isolate from India. The potential strain was taken for the identification which showed up the organism was *Aspergillus terreus* FC36AY1 with the NCBI BankIt ID accession number was KY807648.

The study focused to produce the mass cultivation of pigmented crude secondary metabolites using SDB as production medium on which the strain FC36AY1 produced about 2.31 U/g in 1 L of production medium. The produced crude pigments were extracted using acetone as sole solvent from the fungal mat (biomass). This was similar to the study of Mani et al. [7] which explored that the extraction of pigmented secondary metabolites was done only in mid polar

solvents. The crude pigmented metabolites were experimented for the purification process through HPLC analysis on which the crude was separated into 6 different fractions. Those fractions were taken for the assessment of antimicrobial and antioxidant activities. The 4th fraction TA4 was found to scavenge more free radicals at minimum concentration in a dose dependent manner and this was similar to the investigation of Samaga et al. [19]. In living organisms, oxidative stress created by reactive oxygen species (ROS) resulting from metabolism, in the form of superoxide anion (O^{-2}), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) and nitric oxide (NO) leads to conditions like cancer, stroke, myocardial infarctions, diabetes, septic and hemorrhagic shock, and neurodegenerative diseases by inducing biomolecular oxidations. Therefore, effective free radical scavenging molecules are needed by food and pharmaceutical industries. The fraction TA4 exhibited effective free radical scavenging activity comparable with that of BHA. The free radical scavenging activity of this particular fraction could be attributed to the presence of phenolic compounds [7]. This fraction was taken for the elucidation purpose through FT-IR and GC-MS analysis, on which TA4 showed similarity with 96–100% for the mass spectra of the compound and FT-IR exhibited the functional groups present in the compound and this was similar to the study of Liu et al. [11]. From this characterization, we found that the compound was octadecenoic acid 4-hydroxy methyl ester C19H38O3. This compound was taken for cytotoxicity analysis on HT-29 colon cancer cell lines, the compound TA4 explored its activity by showing a promising strategy of killing the cancer cells in an efficient manner. This was similar to the study of Devi and Prabakaran [20] in which they had experimented on four different cancer cell lines by using a potential bioactive compound from endophytic fungus.

The compound was taken for determination of anti-angiogenesis on chick embryo and this HET-CAM test exhibited maximum anti-angiogenesis by inhibiting maximum number of blood vessels. This manifesting that the compound TA4 has the ability to target the cancer cells and destruct by inhibiting the angiogenesis mechanism which is an important thing in oncology. The drug should have the ability to kill the cancer cells and also destruct the angiogenesis mechanism which could lead to metastasis i.e., taking the cancer cells to other parts of the body through the blood vessels and colonize in some particular area of the human body. In this case, we have found a potential bioactive compound from an efficient endophytic fungus possessing the ability to target the angiogenesis and avoid the metastasis. This is the first report that a bioactive compound TA4 from *A. terrus* FC36AY1 possessing the ability of anti-angiogenesis which was identified through HET-CAM analysis. From this investigation, we are concluding the compound TA4 evincing the ability to target the cancer cells and also it possess multi-functionality in medicinal fields.

5. Conclusion

The current scenario of the biotechnological research is searching for a new natural drug which could efficiently target on different diseases. This research mainly focuses on cancer study by isolating and characterizing a bioactive compound which exhibit its cytotoxicity and anti-angiogenesis against cancer cell lines. The compound TA4 manifested its characteristics by exploring its activities in different analysis. Finally, this investigation concludes that the compound TA4 has broad variety of bio-activities which emphasis in pharma and medicinal fields.

Conflict of interest

The authors declare that we do not have any conflict of interest.

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