

Bioprospecting potential and textile dyeing of Mycobial red pigment from novel microorganism *Nigrospora oryzae*

Israel Mani

Madurai Kamaraj University

Myithili Thangavel

Madurai Kamaraj University

M. Joe Xavier Sneha

Madurai Kamaraj University

Mohan Pandi

an_pandi@rediffmail.com

Madurai Kamaraj University

Akash Surendrababu

Madurai Kamaraj University

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Abstract

Natural pigments are getting prominence because of the hazardous effects from the use of synthetic colouring chemicals. Microbial pigments, in addition to their vast biological activities, provide an easily accessible alternative supply of natural colours. A total of twenty-four fungal cultures were isolated from the leaf tissues of *Senna auriculata*, out of which a distinctive strain (FNG1) exhibited an extracellular pigmentation of reddish orange. The isolate was identified as *Nigrospora oryzae* based on morphological traits and a molecular phylogenetic study of a combined Internal Transcribed Spacer (ITS) and β -tubulin analysis. The crude red pigment was the most soluble in ethyl acetate. The natural pigment was characterised and partly purified using thin-layer chromatography. As a result, the partly purified fungal pigment *Ergo*, was used in further bioprospecting investigations. The antimicrobial activity of the partly purified sample was evaluated against eight human pathogens, with the maximum zone of inhibition identified in *Leucobacter* AA7 (200–500 $\mu\text{g/mL}$). DPPH scavenging activity of the compound increased from 38.2–67.9% with an IC_{50} value of $34.195 \pm 2.33 \mu\text{g/mL}$. The cytotoxicity of partially purified fungal pigment inhibited cancer cells while having little effect on non-cancerous HEK293 T cells. The gas chromatography–mass spectrometry (GC–MS) provided evidence that the compound produced had antibacterial and anticancer properties. Cotton fabrics have the highest staining capacity for crude mycobial pigment, which is fast and exhibited negative cytotoxicity. This is the first report from novel microorganism *N. oryzae*.

Introduction

Natural pigments are colours that are sourced from nature and are primarily generated by plants, animals, and microorganisms (Samanta et al., 2009). The pervasive use of colour in many facets of human life, such as clothes, food, and furniture, has made life more "colourful." The use of pigments as colouring agents may be traced back to prehistoric periods, as evidenced by a large quantity of archaeological data (Narsing Rao et al., 2017). The ability to see colours serves a multitude of functions in living creatures, including camouflaging defence systems, aposematic warning signals that promote sexual attraction, and our food choice tendencies. Science, art, and philosophy are just few of the fields that study colour because of its significance, attraction, and profound effect on human instincts and inclinations (Garber Jr et al., 2016, Elliot AJ & Maier M A, 2014). Natural colours, generally termed pigments, are the end products of metabolic processes in living organisms. Mukherjee et al., in 2017 stated that carotenoids, anthocyanins, and a lot more are types of such metabolites. Identical colours to those found in nature, such as beta carotene and canthaxanthin, are synthetically produced. On the other hand, inorganic colours are also produced and include titanium dioxide, gold, silver, and other similar elements. Natural pigments have been accumulating for a long time, and as people became even more aware of the adverse effects of synthetic colourants, they were increasingly concerned about their continuing growth. Natural pigments such as carotenoids, chlorophylls, quinones, flavonoids (anthocyanins), and phycobiliproteins are used to produce a broad spectrum of colours. Microorganisms are the most

promising of these because of the relative simplicity of culture and extraction, as well as the genetic variety they contain (Higuera-Ciapara, 2006)

In contrast to most other microbes, fungi have the unique ability to produce powerful colours that may be utilised as a dye or to colour food (Babitha et al., 2007). Since they produce chemicals with excellent light and chemical durability, a diverse colour range, a high yield, and a reliable supply, fungi have attracted the curiosity of researchers as a potential natural source of pigments (Fouillaud et al., 2016; Chen et al., 2017). Fungi are responsible for the synthesis of a multitude of colors, many of which have biological properties and some of which are cytotoxic. Fungi are responsible for the production of a diverse array of pigments, many of which exhibit many biological features, some of which are cytotoxic (Durán et al., 2002). Even in the most hostile settings, the existence of fungi makes it feasible for those fungi to synthesise new secondary metabolites since fungi are such a varied and prolific group of eukaryotic creatures on the planet. Fungal life is pervasive and may be found in both terrestrial and aquatic environments (Ramesh et al., 2019). *Aspergillus*, *Penicillium*, *Fusarium*, and *Trichoderma* are just a few of the fungal species which may create a spectrum of colors as intermediary metabolites (Atalla et al., 2011). Carotenoids, polyketides, polyketide-derivates/azaphilones and melanins are all examples of fungal pigments (Meruvu et al., 2021). It is currently obvious that a single species of fungus is capable of producing a variety of distinct pigments, each of which has a unique set of biological characteristics. Fungi's ability to produce these colours serves a vital function in the organism (Mukherjee et al., 2017). To withstand extreme conditions, fungus produce melanin, which also protects them from UV rays (Kunwar et al., 2012). *Monascus* is responsible for the creation of six unique pigments, each of which is produced from polyketides and serves to distinguish the colours yellow, orange, and red yellow pigments include monascin and ankaflavin. The two typical orange *Monascus* pigments, monascorubrin and rubropunctatin, are essential precursors in the synthesis of food colourants (Feng et al., 2012). Fungi are next to plants in the number of different pigments that they contain, with over one hundred having been identified thus far. One of the most essential qualities that can be used to identify different kinds of fungus is the variety of colours that they could take on. Its own pigments protect it from harmful ultraviolet (UV) radiation and may also provide resilience to infection from microorganisms. The pigments found in fungus are quite distinct from those found in higher plants, which do not include chlorophyll or the anthocyanins that are responsible for the myriad of colours seen in flowers (Mukherjee et al 2017).

When it comes to stimulating economic growth and providing new career opportunities, the textile sector is unrivalled. It is of tremendous importance to the textile industry because fungal pigments have high colourfastness and staining qualities; they merit manufacture under controlled circumstances, have no seasonal changes, and are biodegradable (Mapari et al., 2010). The dyeing industries have the responsibility of minimising the negative impact they have on the surrounding ecosystem. The textile industry has high hopes for fungal dyes because of their many potential benefits; yet, they have fallen short owing to their inconsistent fixation. Moreover, the commercial dyeing of fungal pigments is not standardised since there are no recognised methods. Hence, various innovative fungal pigments and a reliable method for standardising the industrial dyeing of fungal dyes are required for use in standardised

industrial applications. This should be done as soon as possible. It has been suggested that stable and consistent fungal pigments might replace synthetic dyes in the textile industry. This is due to the pigments' potential usage as replacements in the textile industry. Certain pigments have the potential to absorb ultraviolet light, and when used as textile dyes, they may protect human skin from the harmful effects of UV radiation (Venil et al., 2020). The objective of the research focuses on the exploration of the pigment that is produced by the fungal strain that has been utilised for textile dyeing, along with the characterization of the pigment after it has been purified and assessments for its cytotoxicity.

Materials & Methods

Isolation of pigment producing endophytic fungi

The mature, disease-free leaves of *Senna auriculata* were gathered from the Nagamalai hills in the Madurai region of TamilNadu, where they were analysed for endophytic fungi. The area of investigation lies in 9°54'N latitude and 78° 00' E longitude and at an elevation of 150m. The hill is 1500 ft at its tallest point and stretches for over four kilometres. The leaves were cleaned for 5 minutes under running tap water before being sliced into small leaf fragments (5 mm²). Surface sterilisation was carried out using 70% ethanol for 30 seconds, 90 seconds in 4% sodium hypochlorite, followed by three washes in sterile distilled water. Fifty leaf segments were plated on agar containing potato dextrose (PDA) supplemented with 50 gmL⁻¹ streptomycin. The plates were incubated at 25⁰ C (± 2° C) with a 12:12 light and dark cycle to monitor fungal growth (Sundaresan et al., 2019). Pure cultures were raised from the emerging hyphal tips and used for further studies. Suitable control plates were maintained throughout the study to validate the surface sterilization process.

Identification of pigment-producing endophytic fungi using morphological and molecular identification.

A light microscope was used to analyse the morphology of an isolated pigment-producing endophytic fungus strain grown on PDA medium. Extraction of genomic DNA was performed for molecular identification according to the procedure outlined by (Cenis, 1992). The fungal strain was identified by amplifying the internal spacer region spanning the ITS1-5.8S-ITS2 segment with universal ITS primers (ITS1-forward primer (5' TCCGTAGGTGAACCTGCGG 3') and ITS4-reverse primer (5' TCCTCCGCTTATTGATATGC 3')). Polymerase chain reaction (PCR) was carried out in a 25 L reaction including 1 µL of DNA template, 1.25 µL of Forward and Reverse Primers, and 12.5 µL of 2x DNA master mix (Amplicon). PCR amplification was carried out in a Himedia's Prima-96™ Thermocycler instrument with the following reaction conditions: Denaturation at 94° Celsius for 4 minutes, 30 cycles for thirty seconds at 94° Celsius for denaturation, annealing at 58.2° Celsius for 1 minute, extension at 72° Celsius for 2 minutes, and extension at 72° Celsius for 7 minutes (White et al., 1990). The amplicons were confirmed on 1% agarose gels, purified and sanger sequencing was carried out by Bio kart India Pvt. Ltd., Bangalore, India. The amplified sequence was analysed using NCBI and BLAST, and a phylogenetic tree was constructed using MEGA X.

ITS2 Secondary Structure prediction of endophytic fungi and phylogenetic analysis

The ITS2 region of the fungal isolate was extracted from the ITS2 Database (<http://its2.bioapps.biozentrum.uni-wuerzburg.de/>) (Ankenbrand et al., 2015). The ITS2 sequence and the secondary structure for all the sequences were exported to 4SALE V1.7.1 for generating synchronous alignment of sequence and secondary structures. Compensatory Base Change (CBC) were analysed and the genera specific consensus secondary structure were modelled. The phylogenetic tree was reconstructed using Phangorn package implemented in the R framework as previously reported. The phylogenetic tree was rebuilt by using the Phangorn tool that was functioning within the R environment (Schliep, 2011). The tree was built using the maximum Likelihood (ML) technique using a General Time Reversible (GTR + I + G) substitution model. The 1000 repetitions were used to calculate the branch support values in the phylogenetic trees (Sundaresan et al., 2019).

Extraction of Fungal pigment

Endophytic fungal isolates were cultured in 200ml of a broth made from potato dextrose (PDB) for pigment synthesis. About, 5mm diameter Agar plugs containing mycelia (7 days old) was used as the inoculums and the flasks were inoculated and incubated for 21 days at 25⁰ C ($\pm$ 2 °C) with 12:12 hours light and dark cycle. After incubating for 21 days, the fungal culture containing the mycelia was filtered using filter paper. From the filtrate of the fungal culture, the extracellular components were extracted using ethyl acetate (1:2 V/V) (Gokul Raj et al., 2015). After collecting the organic phase, the solvent was removed from the mixture by passing it through a rotary vacuum evaporator at low. For further bioprospecting research, the remaining dry material was used.

Characterization of Crude Mycobial pigment

Solubility test

Tests for solubility was carried out through a modified form of the procedure outlined by (Ahmad et al., 2012). Crude fungal pigment was dissolved in the concentration of 1mg/ml with following solvents Acetone, Benzene, Chloroform, Ethanol, Ethyl acetate and Methanol. The mixer was vortex for 5 mins at the room temperature. The $\lambda_{\max}$ for each solution was measured from (300–600 nm) by UV – 8453 Agilent spectrophotometer where the $\lambda_{\max}$ of the red orange pigment was recorded at (450–550 nm).

Purification of Mycobial Pigment

TLC Separation and Sample Preparation

The fungal crude extract was combined with ethyl acetate before being loaded onto thin-layer silica gel plates (Silica gel F254, Merck, Germany) for solvent-based separation (chloroform: methanol – 9:1 ratio). The bioactive components were subsequently extracted by drying the running lane. Both 254 nm and 366

nm UV light systems were used to observe the chromatogram in the TLC chamber. Based on the spot resolution, the optimal mobile phase was selected. The silica residue was centrifuged to remove the remaining particles, and the resulting supernatant was collected. Biological activity was assessed in the silica-free supernatant. R_f was determined by dividing solute distance by solvent distance.

Assessment of antibacterial assay

The anti - bacterial activity was evaluated using the agar well diffusion technique, according to (Perez, 1990). The fungal crude extract and partially purified fungal pigment was determined against eight distinct human pathogen bacterial strains: *Escherichia coli* MTCC 1687, *Bacillus cereus* ATCC14579, *Klebsiella aerogenes* MTCC 4031, *Salmonella typhi* A MTCC 733, and *Staphylococcus aureus* MTCC 7443, *Enterobacter cloacae* GS1, *Leucobacter* AA7. The bacterial cultures (8- hour old) were swabbed (sterile cotton swabs) on the Muller Hinton Agar (MHA) plates. The plates were perforated six times with a sterile corkborer of six-millimeter diameter. Each well had 50µl of partly purified fungal extract (200– 500 µg/mL) applied to it and left to diffuse at ambient temperature. The antibiotic streptomycin used as a standard for bacterial species while water was used as control. The plates had been incubated for twenty-four hours at 37⁰ C. The triplicate plate was kept.

Assessment of Antioxidant activity

The antioxidant capacity of the partially purified extracts of pigment-producing fungus was evaluated using the DPPH and reducing power assays. Each study was carried out three times, with the findings averaged.

DPPH Assay

The 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) approach was used to evaluate the scavenging of free radicals' activity of the partly purified fungal extract, as described by (Rehehati et al., 2019). A 100 µL fungal extract 10– 100 µg/mL was diluted with methanol and mixed with a 100 µL DPPH solution (0.5 mmol/L in 95% methanol) on a 96-well microtiter plate for 30 minutes at room temperature. Fungal extract at different concentration (10µL-100µL) was diluted with methanol and was added with 100µL DPPH solution in 96-well microtiter plate. The plate was incubated in the dark for 30 mins at room. After 30 minutes, the absorbance at 515 nm was assessed. The following formula was used to calculate the percentage of free radicals that were neutralized:

$$\% \text{ Scavenging of DPPH free radical} = [(A_0 - A_1)/A_0] \times 100$$

where A₀ = absorbance of the control, and A₁ = absorbance of the test extracts. The positive control, ascorbic acid in methanol, was employed to assess the radical scavenging capacity of the fungal extracts.

Reducing power assay

To evaluate the extract's reductive potential, it was examined in accordance with (Oyaizu, 1986). Phosphate buffer of pH 6.6 (0.2 mol/L) and Potassium Ferricyanide (2.5 mL, 1% w/v) were added to 1 mL of distilled water containing a fungal extract of varying concentrations (10–100 g/mL), the mixture was centrifuged for 10 minutes at 3000 rpm. The absorbance was assessed in spectrophotometer at 700 nm after mixing the surface layer (2.5 mL) with equal parts of filtered water and Ferric chloride (0.5 mL, 0.1% w/v). High absorption value, implying a higher reductive potential.

Anticancer activity

Cell line and culturing

The HeLa cervical cancer cell, Human Embryonic Kidney (HEK 293 T) and A549 Lung cancer cell lines were acquired from the NCL cell collection in Pune. HeLa, HEK 293 T, and A549 cells were used for the experiments. These cells were cultured in (DMEM, Himedia), to which were administered 10% heat-inactivated FBS (Himedia) and 1% antibiotic cocktail (penicillin (10,000 units/mL), fungizone (10,000 g/mL), and streptomycin (25 g/mL)). Cell monolayers were cultured in tissue culture flasks in a humidified 37°C, 5% CO₂ gas incubator.

Cytotoxicity evaluation by MTT assay

The MTT analysis was made to examine the sample's potential cytotoxicity. A confluent HeLa, HEK 293 T, and A549 cell monolayer was distributed at a density of 1×10^4 cells/well in 96-well plates. The cells were cultured for 24 and 48 hours at 37°C in a 5% CO₂ incubator with crude and partly purified extract at various doses (25–250 µg/mL). A medium control, which was a blank medium, and a cell control, which were cells that had not been treated with an extract, were also included in the same plates. The MTT colorimetric test was utilized to assess the vitality of the cells, as the protocol outlined by (Mosmann, 1983), with few modifications. Each well contained 10 µL solution of MTT (10 mg/mL in phosphate buffered saline (PBS)) for 4 hours incubation at 37°C in an incubator with 5% CO₂. To dissolve the MTT formazan, approximately 200 µL of dimethyl sulfoxide were added for every well. The absorbance at 595 nm was read using a microplate reader (BioTek- Agilent, USA). Using the formula below, the optical density value was converted to a percentage of viability:

$$\text{Percentage of cell viability} = (\text{A595 of treated cells} / \text{A595 of control cells}) \times 100.$$

The average results of the triplicates were used to figure out the IC₅₀ concentration and the percentage of living cells.

Dual Acridine Orange and Ethidium Bromide (AO/EB) Fluorescent staining

Acridine orange and ethidium bromide double stain was used as described by Gohel et al., 1999. AO/EB labelling distinguishes between living and dead cells by monitoring cellular and nuclear changes. A total of 2×10^4 HEK293 T, HeLa, and A549 cells were introduced into each well of a 6-well culture plate and

incubated for 24 hours. Following a 24-hour incubation at 37⁰ C with 5% CO₂, the cells were treated with the fungal extract at the IC₅₀ concentration. The cells were rinsed with PBS before being stained with AO (100 µg/ml) and EB (100 µg/ml). The untreated cells serve as a baseline for comparison. The living and apoptotic cell morphology was studied using a fluorescent microscope (Ti Eclipse) at a magnification of 400.

GC-MS analysis

Metabolite profiles were determined using an Agilent GC 7890A / MS5975C equipped with an Agilent DB5MS column (30m x 0.25mm x 0.5m) and 99.99% pure helium gas (carrier gas) flowing at 1 ml/min using the gas chromatography mass spectrometry (GC-MS) method. The temperature of the column was adjusted from its initial setting of 50 °C / min to 300 °C at a rate of 12°C/ min and maintained there for 2 minutes. An injection volume of 1 µL was employed (split ratio of 5:1), and a constant temperature of 250°C was maintained at the injection port's base. The mass spectral scan range was set between 50 and 550 (m/z) and the ionization voltage was 70 eV. Compounds were identified by searching for mass spectra in the National Institute of Standards and Technology's USA library database, and then classified into appropriate groups using a web-based natural product classifier. (<https://npclassifier.ucsd.edu/#>) (Kim et al., 2021).

Textile Dyeing

The lab scale dyeing studies were carried out on cotton fabric employing the fungal pigment extract with mordanting. A mordant is a chemical or material that is used to increase the affinity of cloth and extracted dyes. In this study, numerous mordants were used, including Aluminium potassium sulphate (alum), ferrous sulphate, lemon juice (lemon), and myrobalan. Experiments with mordanting used a pre-mordanting procedure that included simultaneously treating the mordant and the liquid extract. The extract in liquid form without the mordant served as a control. Mordant was utilized at a concentration of 5%. Experiments with mordants and dyes were carried out in a water bath heated to (60° C) for 1 hour. The material to liquor ratio (MLR) is 1:50 (fabric weight: extract in w/v). Once the procedure was done, the cotton was removed and air dried. The cloth was washed under running water and air-dried. Once the procedure was done, the cotton was removed and air dried. The cloth was washed under running water and air-dried.

Wash Fastness and rubbing tests

Test for wash fastness and rubbing was carried out in AZO Textile Dyeing Laboratory, Tirupur, Tamilnadu, India. At 30°C, the color wash fastness after washing and rubbing was tested using the ISO 105 C06 (MODIFIED-A2S): 2010 and ISO 105X12: 2016 methods. Colorfastness after being washed. The resistance to washing and rubbing was rated on a scale of 1 to 5. On this scale, 1 denotes very poor, 2 denotes poor, 3 denotes moderate, 4 denotes good, and 5 is the best. Dyed cotton textiles were then clipped (1.5 cm) following the fastness and rubbing tests in order to make the shade card (Kannathasan & Kokila, 2021).

Results

Isolation and Identification of Mycobial Pigment

The plant specimen for the study was collected from Nagamalai hills in Madurai district, where the area of investigation lies in 9°54'N latitude and 78° 00' E longitude and at an elevation of 150m. A total of twenty-four isolates were isolated from *Senna auriculata* leaf tissues, including one unique strain (FNG1) that generated an extracellular red orange colour. Macroscopic and microscopic examination of the FNG1 isolate revealed that its colonies were flat, whole bordered, floccose in the centre, and comprised of grey aerial mycelia that aged to black and produced a soluble red orange pigment. According to these characteristics described above, strain FNG1 was identified as one certain species of the genus *Nigrospora*. The FNG1 isolate's identification was confirmed using molecular techniques. A neighbour-joining tree was constructed from the ITS and β - tubulin sequencing and their closet matching references sequencing was matched with NCBI database and the phylogram of combined sequences showed in **Figure. 1** using MEGA X. Using the ITS2 database, ITS2 secondary structure prediction was employed. *Nigrospora* sequences had a length of 159 base pairs (bp) in their ITS2 region, and four helices were present in their secondary structures. The homology modelling was found to be similar with the *Nigrospora oryzae*. No CBCs could be discerned among the species pairs of *Nigrospora* (**Table S1**), nevertheless they formed well supported clades in the phylogenetic tree (**Figure. S1**), which confirms the species as *Nigrospora oryzae*.

Characterization of crude fungal pigment

Solubility test and fungal pigment purification

The fungal crude pigment was tested in a variety of solvents to check their solubility. The coloured crude extract was soluble in a wide variety of solvents and exhibited a vivid red colour in Acetone, Benzene, Chloroform, Ethanol, Ethyl acetate, and Methanol, with a maximum absorbance range of 400-550 nm. The highest solubility was found in Ethyl acetate **Figure. 2A**. The crude extract was purified, characterised, and preliminary purified using TLC **Figure. 2B** A red orange colour was detected in UV radiation between the wavelengths of 254 and 366 nm. The red orange spot was verified by a single spot. As a result, the partly purified fungal pigment was employed for additional bioprospecting investigation.

Antimicrobial and antioxidant potential of partially purified fungal pigment

Positive results were found when a partially purified fungal pigment was evaluated against eight human pathogens **Figure. 3** The greatest zone of inhibition observed was in *Leucobacter* AA7, which was tolerant to all concentrations (200-500 $\mu\text{g/mL}$), whereas *B. cereus* ATCC14579 produced the zone of inhibition from 300-500 $\mu\text{g/mL}$. *Salmonella typhi* A MTCC 733, *Staphylococcus aureus* MTCC 7443 formed the zone of inhibition at the range of 400-500 $\mu\text{g/mL}$. There was no obvious zone of inhibition found against *Escherichia coli* MTCC 1687, *Pseudomonas aeruginosa* MTCC 1688, *Enterobacter cloacae* GS1, or *Klebsiella aerogenes* MTCC 4031, as shown in the **Table 1**.

The partly purified fungal pigment's DPPH radical scavenging ability was denoted in the **Figure.4A** DPPH radical absorbance reductions induced by the antioxidant were a result of radical scavenging via hydrogen donation. There was a noticeable shift in colour from purple to yellow as a result of the reaction. The extract showed scavenging activity from 38.2% to 67.9%. The IC_{50} values were calculated to be $34.195 \pm 2.33 \mu\text{g/mL}$, which was equivalent to the standard, as shown in the **Table 2**.

In the assay for reducing power, the ability to reduce was assessed by the rate at which Fe^{3+} was converted to Fe^{2+} . The reaction mixture's greater absorbance was a measure of the samples' enhanced reduced power capability. During the reducing power assay, the yellow test solution changes to various hues of green and blue in response to the reducing power of the antioxidant samples. **Figure. 4A** shows the reducing power of the fungal extract. The extract had strong reduced power, which increased with concentration. The IC_{50} values were determined to be $46.515 \pm 3.57 \mu\text{g/mL}$, which was comparable to that of standard, showed in the **Table 2**.

Cell Cytotoxicity/Viability Assay

The cytotoxicity of the partly purified fungal pigment was evaluated using the HeLa, A549, and HEK 293T cell lines. The IC_{50} value was plotted in the graph **Figure. 5** The IC_{50} value of HeLa cell line for the partially purified compound (24H- 786 $\mu\text{g/mL}$, 48H- 589 $\mu\text{g/mL}$), A549 cell (24H-714 $\mu\text{g/mL}$, 48H-497 $\mu\text{g/mL}$), for HEK293 T cell line (24H-798 $\mu\text{g/mL}$, 48H-609 $\mu\text{g/mL}$). Cancer cells were significantly inhibited by the extracts. At the same time, meagre cytotoxicity was observed on the non-cancerous HEK293 T cells, which suggest that it is safe to use in the food and pharmaceutical formulations. As a result, the isolate has the potential to be used as an anticancer therapeutic agent.

The viability of cells was determined using AO/EB dual staining at the IC_{50} concentration for 24 hours in HeLa, A549 and HEK 293 T cell line. Using the fluorescence imaging the cellular variation was observed. The control cells continually exhibited a green florescence light, indicating their viability. Late-stage apoptosis was identified by orange-stained cells, whereas necrotic cells were marked by red staining. When comparing with the control cells, The AO/EB-stained apoptotic cells also showed nuclear breakage, chromatin condensation, cytoskeletal disintegration and plasma membrane blebbing **Figure. 6**

GC-MS analysis

The GC-MS peaks' spectral properties were compared to those of compounds in the NIST library, and then the NP classifier method was used to categorise the compounds according to their similarities to natural products. Metabolites were classified into five major classes based on their pathways such as: alkaloids (43.8%), fatty acids (18.8%), shikimates and phenylpropanoids (18.8%), polyketides (6.3%), and terpenoids (12.5%) showed in the **Figure.4B**. The metabolites recognized in the GCMS are Hexanoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester, Benzenemethanol, .alpha.-(phosphinomethyl)-, 2-Methyl-2-vinyloxirane, 2-Butenenitrile, 2-chloro-3-(4-methoxyphenyl)-, o-Methoxy-.alpha.,.alpha.-dimethylbenzyl alcohol, Phenol, 2,4-bis(1,1-dimethylethyl), Benzoic acid, 4-ethoxy-, ethyl ester, 2,4-Dimethoxyphenyl isocyanate, Acetamide, 2-(3,4-dimethoxyphenyl)-N-(9-oxo-9H-fluoren-2-yl)-, 7,9-Di-tert-butyl-1-oxaspiro (4,5)

deca-6,9-diene-2,8-dione, 1H-Indole, 1-(trimethylsilyl)-5-[(trimethylsilyl)oxy]-, Phthalic acid, 2,7-dimethyloct-7-en-5-yn-4-yl isobutyl ester, Octadecanoic acid, 4,8,12-trimethyl-, methyl ester, 1H-Indole, 5-methyl-2-phenyl-, 5-Methyl-2-phenylindolizine, Trimethyl[4-(1,1,3,3-tetramethylbutyl)phenoxy]silane, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, Tetrasiloxane, decamethyl-, 1,1,1,3,5,5,5-Heptamethyltrisiloxane, 2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-. Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester has the highest area percentage **Table 3**.

Textile dyeing

Color fastness to laundering and rubbing

Table 4 shows the results of a study comparing the wash fastness to laundering and rubbing of cotton fabric coloured using various mordanting methods and temperatures. In Wash fastness to laundering the Sample 1 & 2 shows the grey scale rating of 1, where else Sample 3 & 5 shows 1-2 scale rating and sample 4 shows 2 scale rating, which emphasize that among the sample 4 only has little high scale rating when compared to other samples. In Fastness to rubbing (Wet and Dry) sample 1 shows the grey scale rating of (-4-, 4-5), Sample 2 (-3-, 4-5), sample 3 (2-3, 3-4), sample 4 (-4-, 4-5) and sample 5 (3-4, -4-), here sample 4 has shown the highest rating and the least rating was observed in the sample 3. Standard testing techniques revealed that cotton fabric coloured with Fungal extract after being pre-treated with lemon had excellent fastness against rubbing (Wet and Dry), as shown by a grey scale grading of (4, 4-5). But all the dyed fabrics showed poor fastness to laundering. The dyed cotton fabrics are shown in the **Figure. 7**. Due to the difficulty associated with the washing and rubbing resistance of naturally coloured samples, the findings for wash fastness and stains on textiles were adequate.

Discussion

A growing desire for products derived from natural sources has resulted in an emerging market for natural colourants. Endophytic fungus generates a wide range of chemically distinct colours, they are investigated as a potential source because of their easy accessibility (Westphal et al., 2018). *Senna auriculata* (L.) Roxb., a Fabaceae family member, is a traditional therapeutic plant used in India's Ayurveda and Siddha medical systems to cure a number of ailments. Almost every part of the plant has been studied for its potential therapeutic value, including the flowers, leaves, seeds, barks, and roots. It was once used to treat metabolic disorders, skin disorders, diabetes, asthma, rheumatism, dysentery, and skin ailments. (Nille et al., 2021). An endophytic fungus *Nigrospora sp.*, was discovered to generate a deep red orange colour extracellularly isolated from *S. auriculata* in the present study. Early literature had revealed that pure cultures of fungi such as *Blakeslea*, *Eurotium*, *Fusarium*, *Isaria*, *Monascus*, *Nigrospora*, *Paecilomyces*, *Penicillium*, and *Talaromyces*, where the colored pigment that has been released in solid and liquid mediums. (Velmurugan et al., 2010, Arumugam et al., 2014, Torres et al., 2016, Lebeau et al., 2017, Suwannarach et al., 2019, Mishra et al., 2021).

In this study, microscopic examinations, together with phenotypic and genotypic features, allow the fungus to be classified as *Nigrospora sp.* when cultured on PDA media at 28°C, the mycelium of this

species expands fast, forming cottony white colonies. Colonies mature in 3–4 days without sporulation, and during this time red orange metabolites are secreted extracellularly. When left to incubate longer, the red extracellular pigments are bleached and the colour changes to a brownish tan. The use of ITS markers as DNA barcodes in the accurate identification of a wide variety of fungus, with a barcode gap that is most clearly defined between interspecific and intraspecific changes, has been described in the past (Schoch et al., 2012). The fungal isolate's ITS and beta-tubulin sequences were compared to those in the NCBI database to construct a phylogenetic tree. In a multitude of investigations, ITS2 ribotyping has been shown to be superior to other methods for determining phylogenies (Sundaresan et al., 2019). In addition to the clades created in the phylogenetic tree, ITS2 secondary structure prediction and CBCs provided extra support for the clades formed in the phylogenetic tree.

The crude red orange pigment was solubilized in a variety of solvents, with Ethyl acetate showing the highest rate of solubility. This conclusion is consistent with previous research that revealed that varying solvents altered the solubilization potential of crude microbial pigments. Pigments produced by *Epicoccum nigrum*, *Lecanicillium aphanocladii*, and *Penicillium flavigenum* were found to be soluble in ethyl acetate by (Souza et al., 2016). According to a study by **Arumugam et al., 2014**, *Nigrospora sp.* is capable of producing an orange-red pigment with a high solubility in ethyl acetate and similar observations were also recorded in the *Aspergillus sp.* by (**Narendrababy and Shishupala, 2017**).

Microbial pigments exhibit distinct bioprospecting properties, including antioxidant, antibacterial, anticancer, anti-inflammatory, antiangiogenic, and immunosuppressive activities. In light of these pharmacological advantages, the scope of this research was expanded to include an examination of pigment's cytotoxic and antioxidant capacities (El-Sayed et al., 2022). Pawle and Singh, 2014) reported that Ethyl acetate extract of the endophytic fungus *Nigrospora sp.* suppressed bacterial growth. In the present study, the bactericidal activity of partly purified red orange pigments was assessed using the well diffusion method. Sensitivities to red orange pigments have been seen in both gram-negative and gram-positive bacteria, although the sensitivity varies depending on the organism. *Leucobacter* AA7 was susceptible for all the concentration and showed maximum zone of inhibition. The red orange pigment was most effective to the gram-positive pathogens. The red orange pigments rendered several gram-negative and gram-positive bacteria inactive.

In addition to antibacterial action, the partly purified fungal extract displayed radical scavenging activity and power reduction capacity. When examined by the DPPH free radical scavenging experiment, the partly purified fungal extract had an IC_{50} value of $34.195 \pm 2.33 \mu\text{g/mL}$, with the greatest scavenging activity at 67.9%. Pawle and Singh, 2014) showed in their investigation that the radical-scavenging DPPH activity of *Nigropsora sp.* extract exhibited an IC_{50} value of $9.28 \mu\text{g/mL}$. In the reducing power test, the ability of a compound to reduce is contingent not only on the electron donor but also on the molecule's capacity to quench free radicals (Singh & Rajini, 2004). The partially purified fungal extract also showed great reducing power potential with a IC_{50} value of $46.515 \pm 3.57 \mu\text{g/mL}$. According to Khalil et al., 2020, their results showed that 29 of the 13 endophytic fungi's crude extracts had a reducing power activity, which ranging from 0.14–12.13 mg/mL of fungal extract. The antioxidant compounds aid in the

prevention of cancer, along with ageing and the mechanisms that lead to neurodegeneration Xing et al., 2005.

According to (Salvador-Reyes and Luesch, 2015) research, one method for determining a potential for a new treatment is to first extract naturally occurring bioactive molecules and then test those compounds to determine if they have any pharmacological value. Several different endophytes have been linked to the development of new compounds that have shown promise in anticancer studies by Elkhoully et al., 2021. The MTT test was used to evaluate the cytotoxicity of a partially purified fungal extract against two human carcinomas (A549, HeLa cell line, and HEK 293 T as a control). Fungal pigment extract showed the strongest cytotoxic effect in a dose-dependent activity. Upon treatment the fungal pigment extract was more effective in A549 & HeLa cells and was less cytotoxicity to the control cells. The study was similar with Lu et al., 2012, where A549 and HeLa cells showed antitumor activity of 82.4% by endophytic fungal cultures obtain from *Actinidia macrosperma*. For HeLa cells, the IC_{50} values for *T. purpureogenus* and *N. sphaerica* extracts were below 200 $\mu\text{g/ml}$, whereas the IC_{50} values for *Aspergillus sp.* and *T. stipitatus* extract for HeLa cells were $30.1 \pm 1 \mu\text{g/ml}$ and $117.2 \mu\text{g/ml}$, respectively (Taritla et al., 2021). When evaluating the safety of a cytotoxic treatment is appropriate to use, the assessment of the drug's impact on non-cancer cell lines is one of the most essential factors. Efficient cytotoxic therapies induce distinct pathways in malignant and non-cancerous tissues, making tumor cells more exposed to the drug's effects (Navya et al., 2019). When the anticancer activities of melanin pigment were examined on HEK 293 T cells after 24 hours, the percentage of cell viability was 17.70.1 when 100 g/mL of melanin was utilised. The highest IC_{50} value ($\mu\text{g/mL}$) was recorded as $64.11 \pm 0.00 \mu\text{g/mL}$ (Polapally et al., 2022). The partially purified fungal pigment showed robust anticancer activity against A549 and HeLa cells while being relatively noncytotoxic to HEK 293 cells, making it a promising candidate for application as an antitumor drug at the same time for other industrial and pharmaceutical applications.

The presence of a wide range of phytochemical groups was confirmed by Gas chromatography of the partly purified fungal compounds, many of which have been shown to have activity against malignancy, microbes, and possess antioxidant and other activity. Majorly the metabolites are classified into five classes such as alkaloids, fatty acids, shikimates and phenylpropanoids, polyketides and terpenoids. Hexanoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester possess antibacterial activity, but their mechanisms are yet to known (Aji et al., 2022). Cognitive functions, neural plasticity, and Cholinergic transmission are just a few of the many neurological processes that benzenemethanol, α -(phosphinomethyl)- aids to (Olasehinde et al., 2019). It has been discovered that the naturally occurring substance phenol 2, 4-bis (1, 1-dimethylethyl) serves numerous purposes in the fields of agriculture, food processing, and medicine. In the field of medicine, it is used for its antioxidant, anticancer, antifungal, and antibacterial characteristics, as well as its ability to protect against the cognitive dysfunction caused by trimethyltin (TMT) (Ren et al., 2019). Benzoic acid-4-ethoxy-ethyl ester is a potential natural additive for preventing food deterioration microorganisms (Arokiyaraj et al., 2018). Antimicrobial and anticancer activity was reported in 7,9-di-tert-butyl-1-oxaspiro [4.5] deca-6,9-diene-2,8-dione and Phthalic acid, 2,7-dimethyloct-7-en-5-yn-4-yl isobutyl ester- (El-Fayoumy et al., 2021, Eid et al., 2018). Octadecanoic acid, 4,8,12-trimethyl-, methyl ester-

naturally occur as Stearic acid ester and major possess antioxidant, anti-inflammatory and anticancer activity (**Ganesh et al., 2017**). Hemolytic, pesticide, flavour, antioxidant properties are reported in Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (Tyagi & Agarwal, 2017). Antifungal, antimicrobial activity, anti-inflammatory, antioxidant and anticancer properties are reported in methyl-2-phenyl-1H-indole- (Salim, 2018, Mannaa and Kim, 2018). The compound Trimethyl [4-(1,1,3,3, - Tetramethylbutyl) Phenoxy] Silane acts as vitamin D derivatives to cure rickets and antioxidant potential (**Abed & Salman, 2022**). According to Sami 2022 the 2,4,6-Cycloheptatrien-1-one,3,5-bis-trimethylsilyl- possess Antibacterial, nematocidal and used as binder, 1,1,1,3,5,5,5-Heptamethyltrisiloxane- showed Anti-inflammatory, anti-microbial and used as an additive.

According to studies, *Nigrospora sp.* Red orange pigment might be employed as a textile dye. The red pigment imposed the most strain on cotton fibres, as measured by how lengthy the fabric remained the colour after washing. Additionally, studies have revealed that the fungal pigment might be utilised to create natural textile colours (Suwannarach et al., 2019). When the fungal extract is combined with water, it is easy to bond and display its colour. When mordant is present, the net positive charge of cotton yarn rises, facilitating the rapid drawing of pigment down the fibre orientation. It was observed that fungal extract dye combined with pre-mordanting reveals that lemon juice produces the finest colour fastness. It can also be demonstrated that pre-mordanting with myrobalan produces better results than other synthetic mordants, such as alum and ferrous sulphate. It is possible to notice that. On the other hand, both ferrous sulphate and alum, which are synthetic mordants, exhibit poor colour fastness on the cotton fabric, but alum performed much better than ferrous sulphate. Myrobalan and alum, on the other hand, performed much better than ferrous sulphate. Similarly, (**Zubairu et al., 2015**) reported that the use of lemon juice in a pre-mordanting process improved the fabric's colourfastness. Concurrently, red pigments which were produced by *Nigrospora aurantiaca*, *Penicillium purpurogenum*, *Alternaria alternata*, *Scytalidium cuboideum*, *Aspergillus niger*, *Talaromyces verruculosus*, and *Trichoderma virens* demonstrated the maximum degree of staining and exceptional fastness to washing in cotton textiles (Suwannarach et al., 2019, Sastrawidana et al., 2016, Hinsch et al., 2015, **Anchana devi, 2014**, Devi and Karuppan, 2015, Chadni et al., 2017). In addition, these fungal pigments have no negative effects on the cloth and cause no irritation to the human skin. As a result, there is potential for the variety of applications by using fungal pigments to expand into the clothing and textile sectors.

Conclusion

There is a significant need for more environmentally friendly alternatives to synthetic colourants because of the irreparable harm that they do to ecosystems and human health. Fungal pigment, one of the microbial pigments, has recently been investigated for its use in textile dyeing. It is obvious that, A fungal endophyte, *Nigrospora sp.*, cultured from *S. auriculata* was identified to synthesize an extracellular reddish orange pigment. The results obtained from the studies highlights that the pigment possess antioxidant, antimicrobial, and anticancer properties confirms the promising applications in the pharmaceutical and food industries. The bioactive metabolites found in the partly purified pigment and their medicinal potential were discovered using GC-MS analysis. Fungal pigments are effective

substitutes for synthetic colours in cotton garments. The research shows that fungal pigments may be utilised as non-toxic cotton textile dyes. These dyes offer exceptional colour fastness to washing and rubbing and may be used on cotton. As a result, the findings of our research indicate that this pigment has prospective applications in the textile and pharmaceutical sectors. Pigment-producing microbes play an important part in the advancement of biotechnology and may potentially be employed in the textile, cosmetic, food, feed, and pharmaceutical industries.

Declarations

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AUTHORSHIP CONTRIBUTION STATEMENT

Israel Mani: Investigation, Methodology, Writing – original draft & editing, Visualization. **Myithili Thangavel:** Investigation, Methodology, Conceptualization, Writing – review & editing, Visualization. **Akash Surendra Babu:** Investigation, Methodology, Conceptualization. **M. Joe Xavier Sneha:** Writing – review & editing. **Mohan Pandi:** Supervision, Writing – review & editing.

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DATA AVAILABILITY STATEMENT

The data supporting the findings are provided in the supplementary file.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest regarding the present study.

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Tables

Table. 1 Antibacterial activity of partially purified fungal pigment against eight human pathogens

Organism/Concentration	200µg/mL	300µg/mL	400µg/mL	500µg/mL	Antibiotic (1mg/mL)
<i>Escherichia coli</i> MTCC 1687	-	-	-	-	3±0.2cm
<i>Bacillus subtilis</i> ATCC14579	-	1.2±0.2cm	1.4±0.1cm	1.5±0.2cm	3.2±0.1cm
<i>Staphylococcus aureus</i> MTCC 7443	-	-	1.2±0.2cm	1.3±0.1cm	4±0.1cm
<i>Salmonella typhi</i> A MTCC 733	-	-	1.2±0.1cm	1.3±0.1cm	3.2±0.3cm
<i>Klebsiella pneumoniae</i> MTCC 4031	-	-	-	-	3.2±0.2cm
<i>Leucobacter</i> AA7	1.2±0.1cm	1.2±0.2cm	1.3±0.1cm	1.3±0.1cm	2.3±0.1cm
<i>Pseudomonas aeruginosa</i> MTCC 1688	-	-	-	-	3.2±0.2cm
<i>Enterobacter cloacae</i> GS1	-	-	-	-	3.3±0.2cm

Table.2 IC50 values of Partially purified fungal pigment along with standard for antioxidant assay

	Partially purified fungal pigment	Standard
DPPH Assay	34.195±2.33	Ascorbic acid (10.21±3.24
Reducing power assay	46.515±3.57	Gallic acid (20.72±2.16)

Table. 3 GCMS analysis of partially purified fungal pigment

PEAK	RT	NAME	MOLECULAR FORMULA	MOLECULAR WEIGHT	AREA%
1	5.553	Hexanoic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	C ₁₆ H ₂₆ O ₂	250.38g/mol	1.79
2	7.764	Benzenemethanol, .alpha.-(phosphinomethyl)-	C ₈ H ₁₁ OP	154.15g/mol	1.95
3	8.953	2-Methyl-2-vinyloxirane	C ₅ H ₈ O	84.12g/mol	1.25
4	10.597	2-Butenenitrile, 2-chloro-3-(4-methoxyphenyl)-	C ₁₁ H ₁₀ ClNO	207.65g/mol	1.30
5	11.553	o-Methoxy-.alpha.,.alpha.-dimethylbenzyl alcohol	C ₁₀ H ₁₄ O ₂	166.22g/mol	10.68
6	11.853	Phenol, 2,4-bis(1,1-dimethylethyl)	C ₂₂ H ₃₀ O	310.5g/mol	3.67
7	12.064	Benzoic acid, 4-ethoxy-, ethyl ester	C ₁₁ H ₁₄ O ₃	194.23g/mol	18.79
8	12.608	2,4-Dimethoxyphenyl isocyanate	C ₉ H ₉ NO ₃	179.17g/mol	1.82
9	15.419	Acetamide, 2-(3,4-dimethoxyphenyl)-N-(9-oxo-9H-fluoren-2-yl)-	C ₂₃ H ₁₉ NO ₄	373.4g/mol	1.36
10	15.619	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	C ₁₇ H ₂₄ O ₃	276.4g/mol	4.43
11	15.797	1H-Indole, 1-(trimethylsilyl)-5-[(trimethylsilyl)oxy]-	C ₁₄ H ₂₃ NOSi ₂	277.51g/mol	1.64
12	16.008	Phthalic acid, 2,7-dimethyloct-7-en-5-yn-4-yl isobutyl ester	C ₂₂ H ₂₈ O ₄	356.5g/mol	4.84
13	17.341	Octadecanoic acid, 4,8,12-trimethyl-, methyl ester	C ₂₂ H ₄₄ O ₂	340.6	1.44
14	17.541	1H-Indole, 5-methyl-2-phenyl-	C ₁₅ H ₁₃ N	207.27g/mol	1.48
15	19.807	5-Methyl-2-phenylindolizine	C ₁₅ H ₁₃ N	207.27g/mol	2.20
16	19.918	Trimethyl[4-(1,1,3,3-tetramethylbutyl)phenoxy]silane	C ₁₇ H ₃₀ OSi	278.5g/mol	2.62
17	20.074	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄	330.5g/mol	23.73
18	21.107	Tetrasiloxane, decamethyl-	C ₁₀ H ₃₀ O ₃ Si ₄	310.68g/mol	2.45
19	21.207	1,1,1,3,5,5,5-Heptamethyltrisiloxane	C ₇ H ₂₁ O ₂ Si ₃	221.5g/mol	4.41
20	21.385	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	C ₁₃ H ₂₂ OSi ₂	250.48g/mol	8.16

Table.4 Textile dyeing of partially purified fungal pigment on cotton fabric

Samples	Mordant	Temperature (Dying)	Colour after dyeing	Fastness to laundering	Fastness to rubbing	
					Wet	Dry
Sample-1	Alum	60 ⁰ C	Pink	-1-	-4-	4-5
Sample-2	Ferrous Sulphate	60 ⁰ C	Beige	-1-	-3-	4-5
Sample-3	Myrobalan	60 ⁰ C	Beige	1-2	2-3	3-4
Sample-4	Lemon	60 ⁰ C	DK Coral	2	-4-	4-5
Sample-5	Control (Without mordant)	60 ⁰ C	Coral	1-2	3-4	-4-

Figures

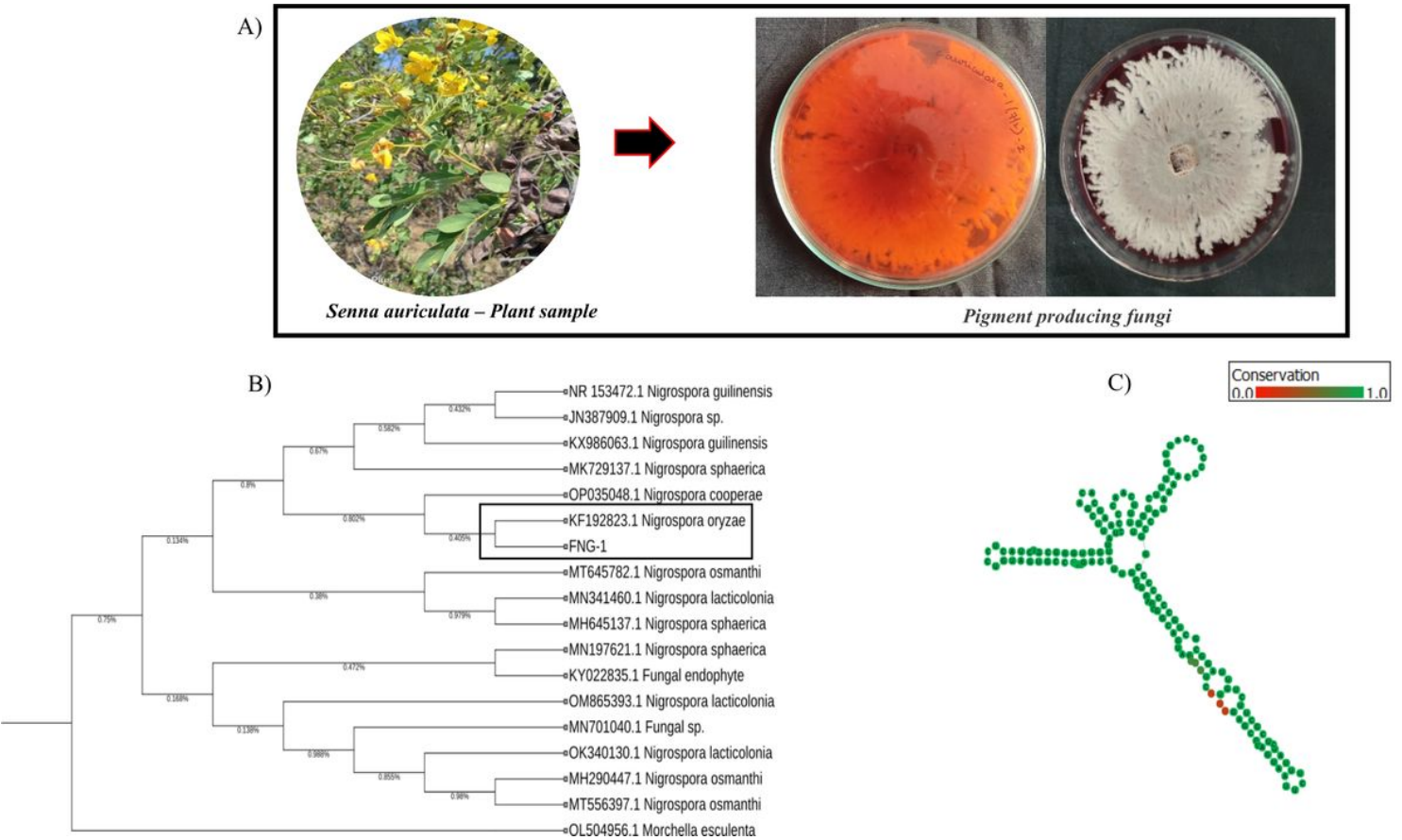


Figure 1

A) Plant sample along with the morphology of fungal pigment (*N. oryzae*) **B)** A neighbour-joining tree was constructed from the ITS and β -tubulin sequencing with *Morchella esculenta* as outgroup **C)** ITS2 Secondary structure of *N. oryzae*

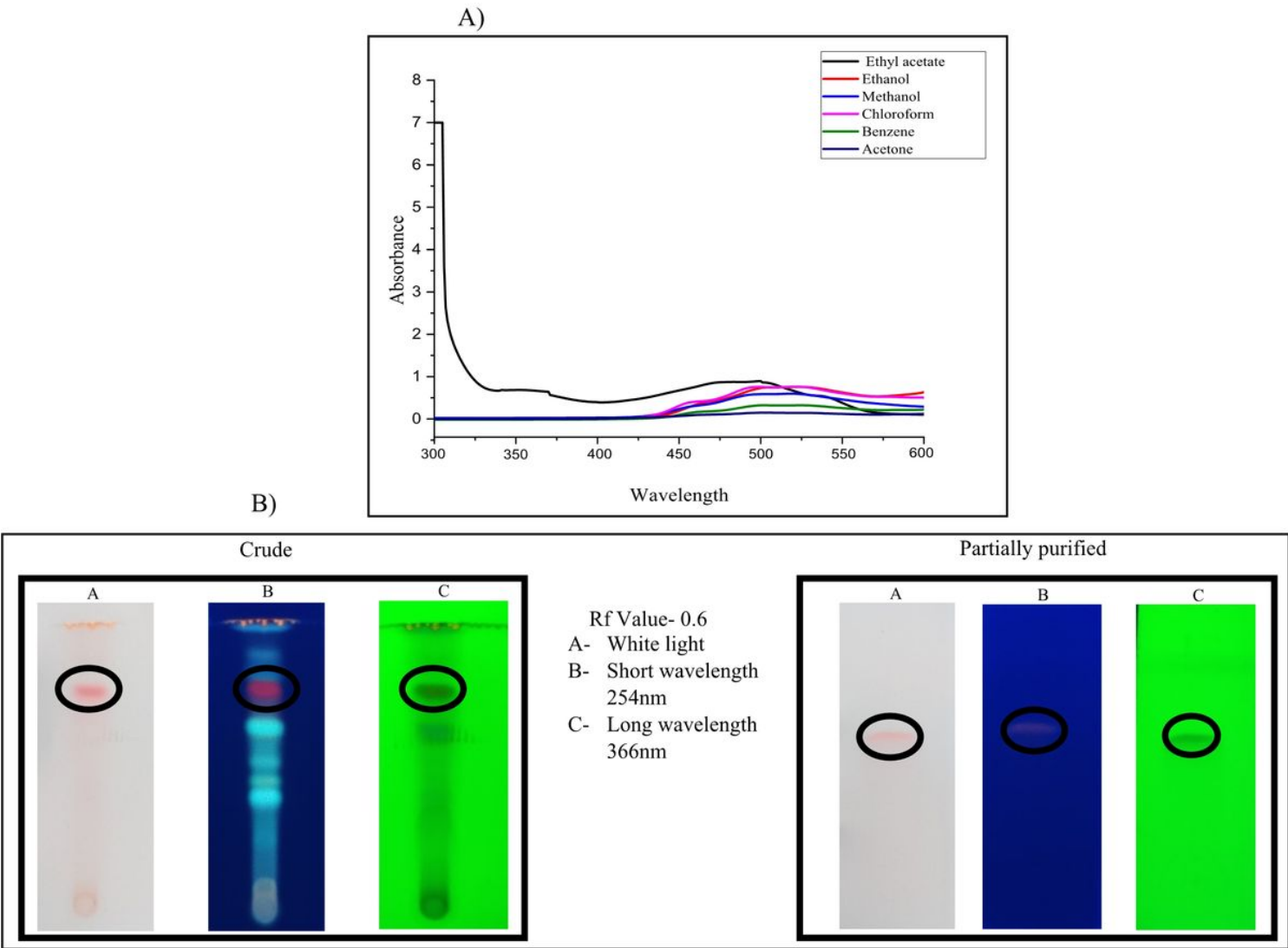
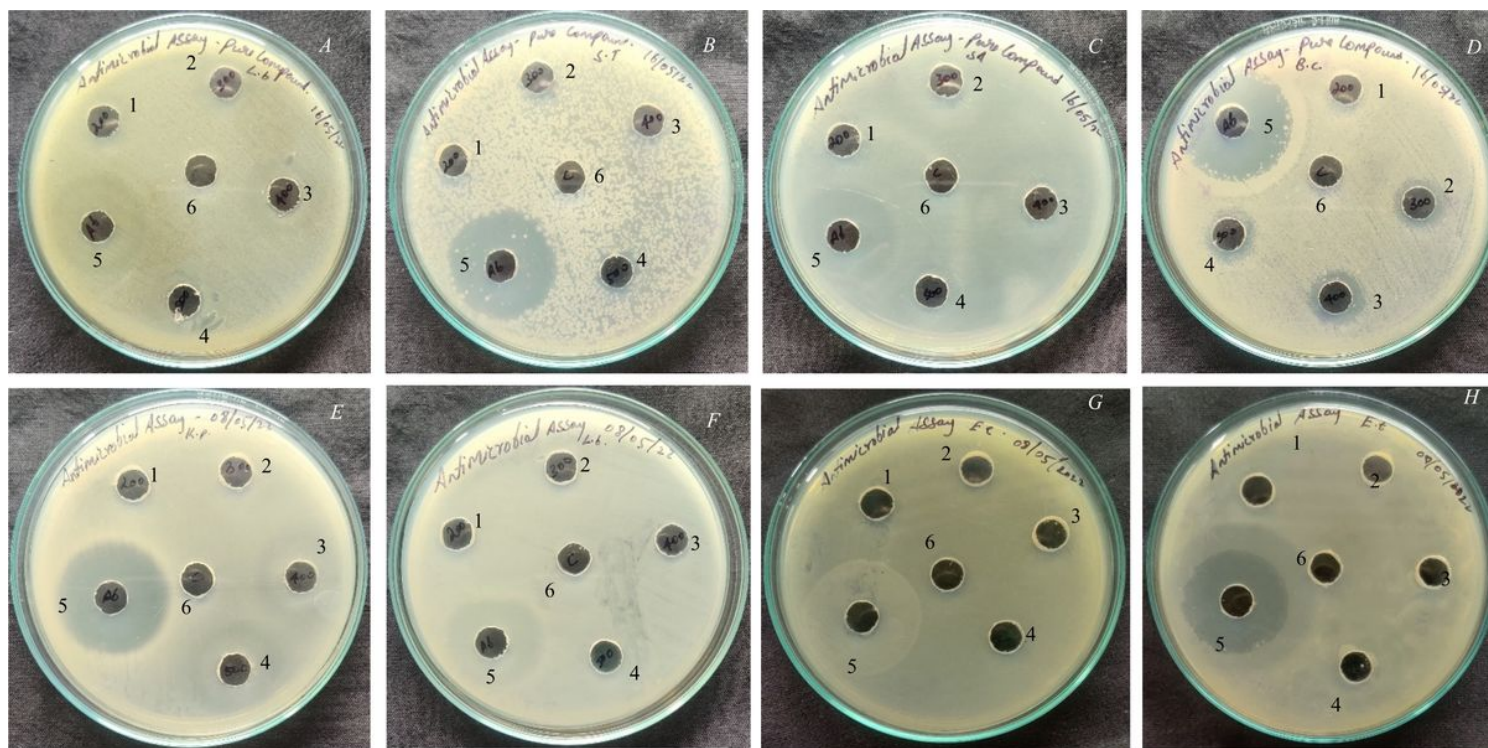


Figure 2

A) UV-VIS spectrometer (300-600nm) analysis of solubility of crude pigment in various solvent.
B)Chromatogram of crude and partially purified fungal pigment.



1- 200µg, 2, 300µg, 3- 400µg, 4- 500µg, 5- Antibiotic (Streptomycin), 6- Sterile Water

A- *Klebsiella aerogenes* MTCC 4031, B- *Salmonella typhi* A MTCC 733, C- *Staphylococcus aureus* MTCC 7443, D- *Bacillus cereus* ATCC14579, E- *Leucobacter* AA7, F- *Pseudomonas aeruginosa* MTCC 1688, G- *Escherichia coli* MTCC 1687, H- *Enterobacter cloacae* GS1

Figure 3

Assessment of antibacterial activity in partially purified fungal pigment

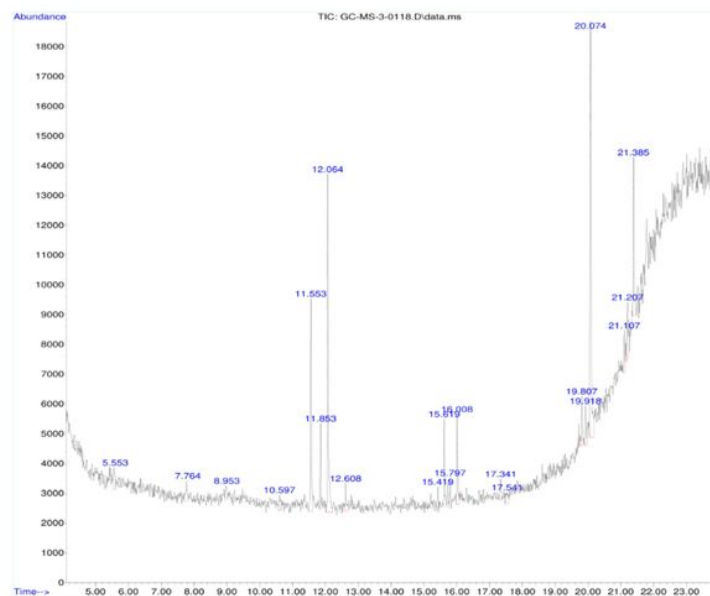
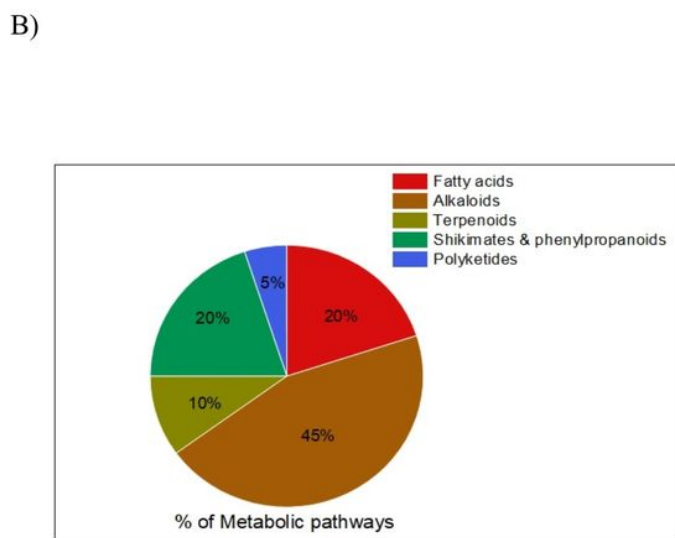
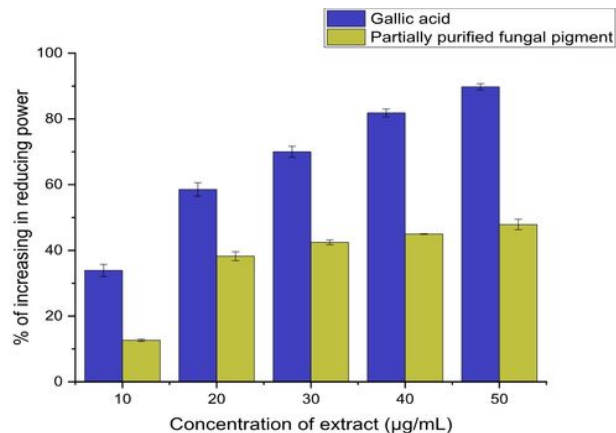
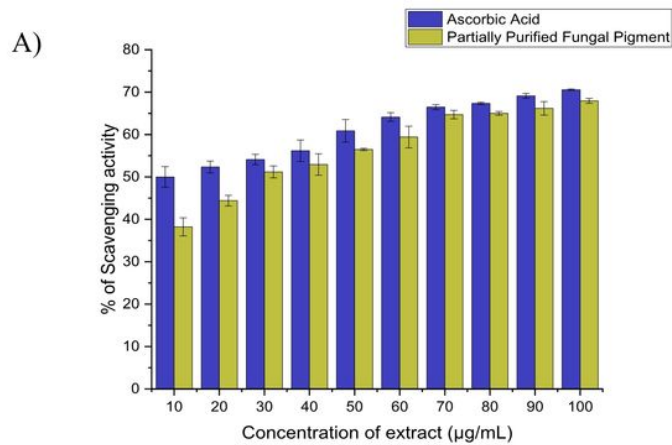


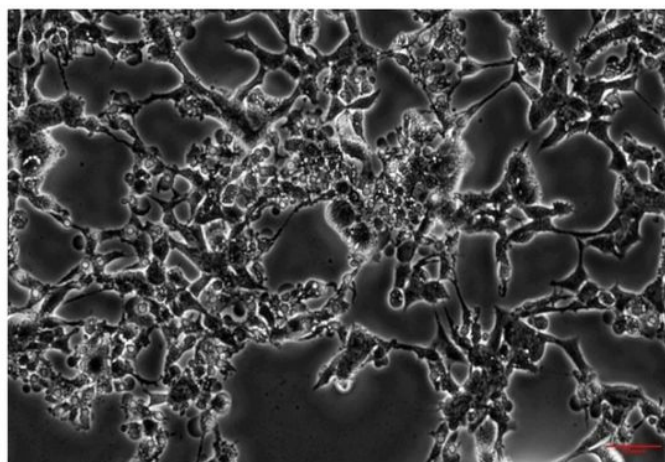
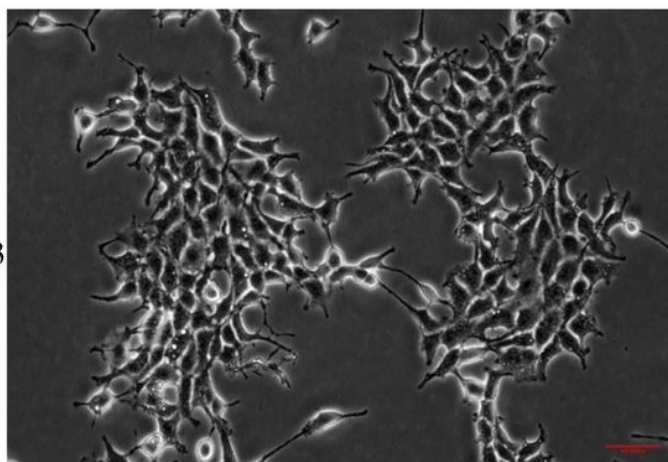
Figure 4

A) Graphical representation of free radical potential of partially purified fungal pigment extract. **B)** GCMS spectra of partially purified fungal pigment along with the total percentage of metabolites.

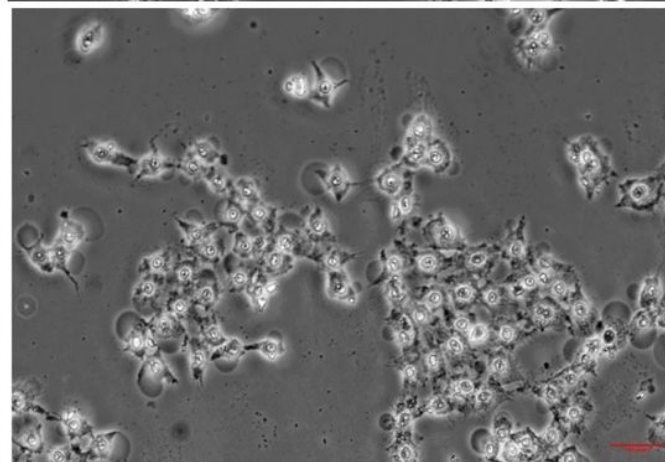
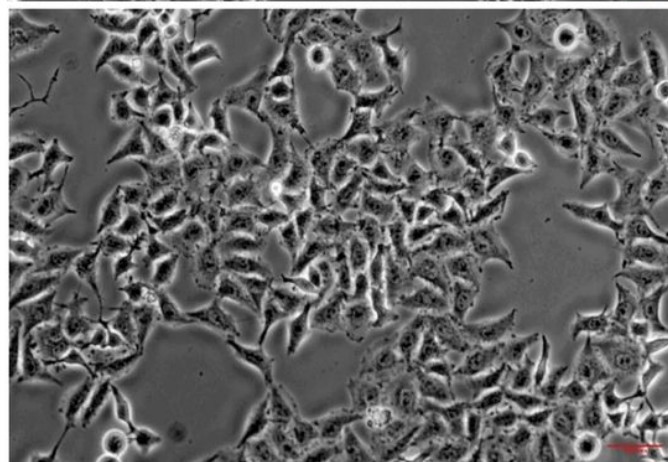
CONTROL CELL LINE

PARTIALLY PURIFIED- IC50 TREATED

HEK293



HeLa



A549

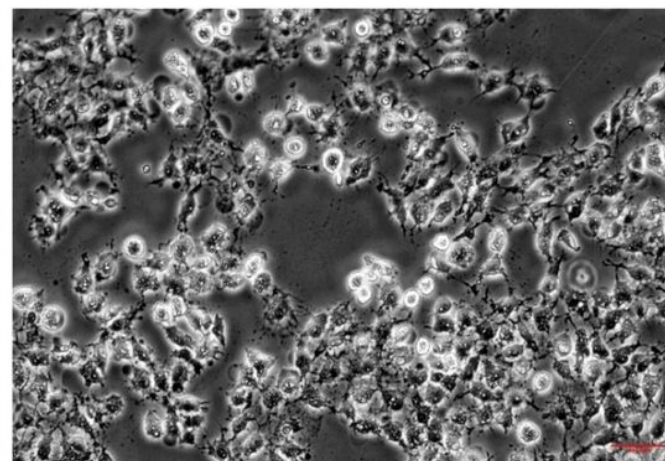
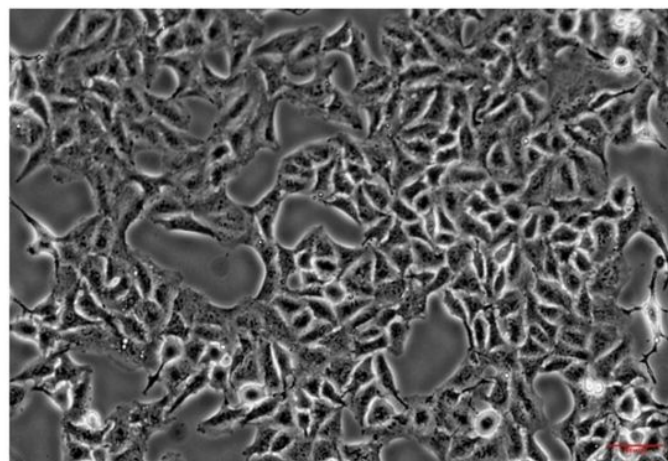


Figure 5

Morphological analysis on partially purified fungal pigment treated with IC50 concentration on HEK 293 T, HeLa and A549 cell lines.

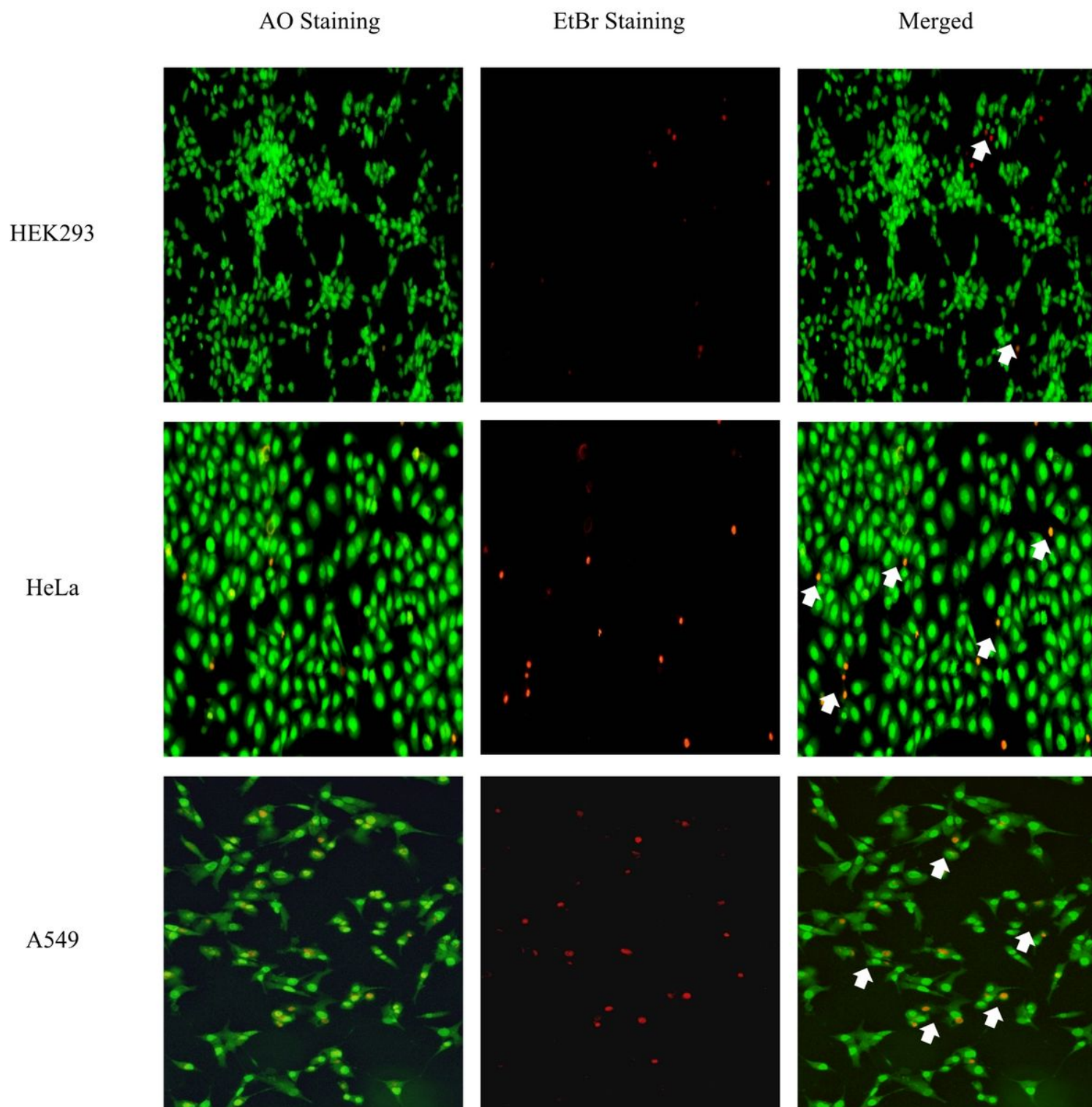


Figure 6

AO/EtBr staining on HEK293, HeLa and A549 cells treated with IC50 concentration of partially purified fungal pigment. Arrows indicating the dead cells

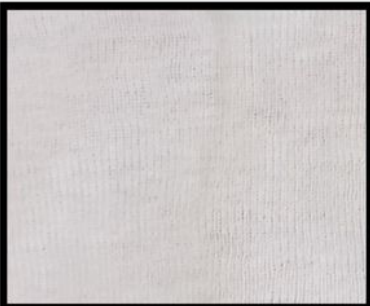
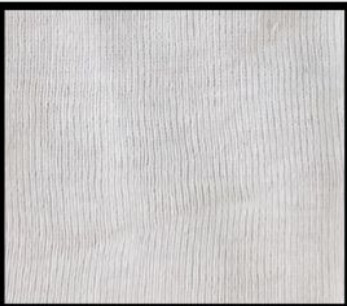
	Before Mordant	After Mordant	After Dyeing
Alum			
Ferrous			
Myrobalan			
Lemon			
Control			

Figure 7

Shades of cotton fabric before and after dyeing on fungal pigment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarydata.docx](#)