Research Article

Production of Two Novel Polyketide Pigments by *Fusarium chlamydosporum*

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ABSTRACT

Aim: The aim of the present study was to evaluate the pigment production by the fungus *Fusarium chlamydosporum* in Glucose Peptone Yeast Extract medium and further to assess its toxicity by *in vitro* methods. **Materials and Methods:** The coloured crude extract yielded 4 fractions when subjected to column chromatography, upon which Fraction 2 and 4 were subjected to characterization procedures by UV-Visible spectrophotometry, Fourier Transform Infrared Spectroscopy and Molecular weight determination by Liquid Chromatography Mass Spectroscopy (LCMS). The toxicity of the pigments was evaluated *in vitro* on L-929 Mouse Fibroblast cell lines by MTT Assay. **Results:** The fungus produced dark red pigment in Glucose Peptone Yeast Extract medium on extraction with ice cold chloroform. The results summarized Fraction 2 and 4 to be the polyketide derivatives, Rubrofusarin and 8-O-methyl fusarubin respectively wherein the IC_{50} values of rubrofusarin and 8-O-methyl fusarubin was 183.85 and 82.366 µg/ml respectively. **Conclusion:** The significance of the present study lies in the characterization of 2 novel polyketides, rubrofusarin and 8-O-methyl fusarubin from *F. chlamydosporum* and there have been no literature reviews on the production of these pigments from the fungus.

Keywords: *Fusarium chlamydosporum*, L929 Mouse fibroblast cell line, Rubrofusarin, 8-O-methyl fusarubin.

INTRODUCTION

A large number of bacteria, molds, yeast, algae are known to produce pigments; however only few are considered to be suitable for production. They must satisfy certain criteria such as (i) ability to use wide range of carbon and nitrogen sources (ii) should possess moderate growth conditions (iii) should be non-toxic and non-pathogenic (iv) should have reasonable color yield and (v) should be easily separable from cell mass.[1] Various genera of filamentous fungi such as *Pencillium, Monascus* and *Fusarium* are good source of pigments. In addition of imparting colour to the substrate, some pigments also have antimicrobial, antioxidant and cholesterol lowering effects.

Fungal pigments can be broadly classified chemically as polyketides and carotenoids. Fungal pigments have advantages over other pigments. Some of the attributes worth mentioning are as follows: They are stable at high range of temperature (pigments from *Monascus purpureus, Fusarium spp., Penicillium spp., Emericella spp.,*) stable and non-toxic, and are able to utilize low-cost substrate and industrial wastes for their growth and metabolite production (corn steep liquor, grape waste).[2]

Fusarium chlamydosporum belongs to the Genus, *Fusarium* which is the large filamentous fungi group widely found in plants and soil. The fungus can be soil borne, airborne, or carried in plant residue, and can be recovered from any part of a plant from the deepest root to the higher plant. The isolation of *F. chlamydosporum* from American long grain rice extract,[3] river water of South Spain,[4] maize and Egyptian clover[5] are some of the supporting

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literatures emphasizing the association of the fungus with plants and rhizosphere. The production of some potent mycotoxins, chlamydosporol,[3] isochlamydosporol and chlamydospordiol[6] and the production of a carotenoid[5] are the only available studies obtained in the production of secondary metabolites by the fungus. In this regard, the primary objective of the present study was to research the production of any secondary metabolites from *F. chlamydosporum* by culturing them on unexplored media.

MATERIALS AND METHODS

Growth and Pigment Production of *F. chlamydosporum*

F. chlamydosporum was procured from National Fungal Culture Collection Centre Institute (NFCCI), Pune with the Accession No. 3020 and its pigment production was evaluated by its growth on a non-defined Glucose Peptone Yeast Extract medium (GPY) (Glucose – 2%, Peptone and Yeast extract – 0.05%, $MgSO_4$ – 0.05%, K_2HPO_4 – 0.2% and KH_2PO_4 – 0.1%) maintained at pH 6.0 at 28±2°C. The extraction of pigments was carried out by the mycelial homogenization method as described by Soumya *et al*. 2014.[7] The extracted crude extract was evaporated to dryness and the units were expressed in mg/dry g weight of biomass.

Purification and Characterization of the Pigments

Thin Layer Chromatography

Analytical TLC was performed on TLC plate 20 cm × 20 cm (GF 254 60; Merck 250 mm thick).[8] Different solvent systems were experimented for the elution of the compounds and the best solvent system which resulted in a good separation of the compounds was selected for TLC.

Column Chromatography

The separation of compounds from the crude pigment extract from GPY medium was performed by Column Chromatography using gradient elution technique using a binary solvent system; chloroform: methanol (9.5:0.5) initially and thereby gradually increasing the polarity from chloroform: methanol (4:6) and then 100% methanol. Each of the separated compounds were then quantified (mg/g of crude extract) followed by TLC to confirm the presence of single compounds. The fractionated compound with the maximum yield was subjected for characterization.

UV-Visible Spectrophotometry

The fractionated compound with the maximum yield was subjected to UV-Visible Spectrophotometer (Schimadzu UV-1700, pharmaspec) to measure the absorption maxima.

Fourier Transform Infrared Spectroscopy

Analysis of the functional groups of the purified compounds was performed using the FT-IR spectrometer Spectrum Two (Perkin-Elmer, USA) wherein the absorbance spectra were acquired over the range 450-4000 cm^{-1} at a resolution of 16 cm^{-1} with 32 scans per spectrum.

Liquid Chromatography-Mass Spectroscopy analysis of the compound (LC-MS analysis): Chromatographic separation was achieved using Reverse phase C_{18} ZORBAX analytical column. The mobile phases used were eluent A containing formic acid in water, and eluent B with formic acid in methanol. The mobile phase was delivered into the column at a flow rate of 1 mL min^{-1} and the run time was about 40 min. The mass spectrometer performed different runs in positive and negative ionization modes over a mass range from 50 to 1000 Da at 4 GHz high resolution mode with a scan duration of two spectra/s in centroid and profile mode in. Data was collected using Data acquisition and analyses control was carried out by Labsolutions LCMS software (Shimadzu).

In vitro Cytotoxicity Determination by MTT Assay

The toxicity of the purified pigments was evaluated *in vitro* on L-929 Mouse fibroblast cell lines procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were seeded at 5000 cells/well in 96-well plates incubated for 48 hr. The samples were then placed in a new medium containing 50 µl of MTT solution (5 mg/ml).[9]

Statistical analysis

The experiments were performed in triplicates and the results are expressed as Mean ± SD (n=3).

RESULTS

Pigment Production by *F. chlamydosporum* on GPY medium

The colony morphology of the fungus grown on GPY medium exhibited deep red pigment interspersed with off-white pigmentation. The reverse side of the plate showed intense red pigment production when extracted with chloroform.

Purification and Characterization of Pigment

Thin layer Chromatography

The crude coloured extract from *F. chlamydosporum* in GPY were separated into 4 bands on a silica gel TLC plate with standardized mobile phases Chloroform: Methanol (9.5: 0.5) (Figure 1).

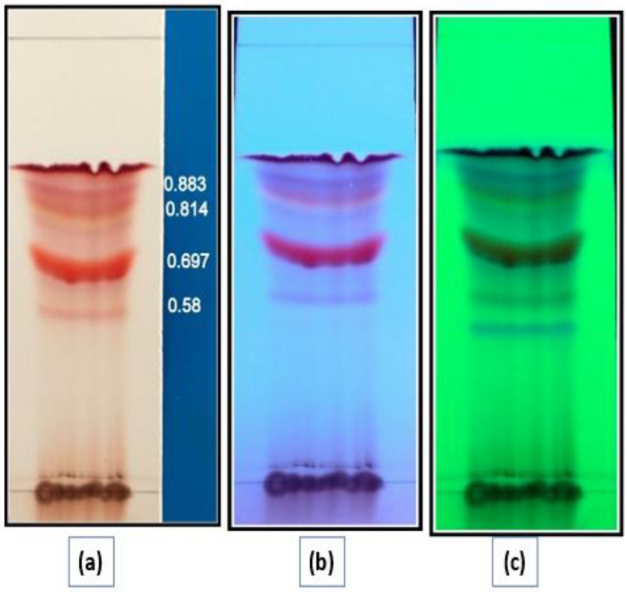

Figure 1: TLC of the crude red pigment extract of *F. chlamydosporum* in GPY medium visualized under (a) visible light (b) UV long wavelength (c) UV short wavelength.

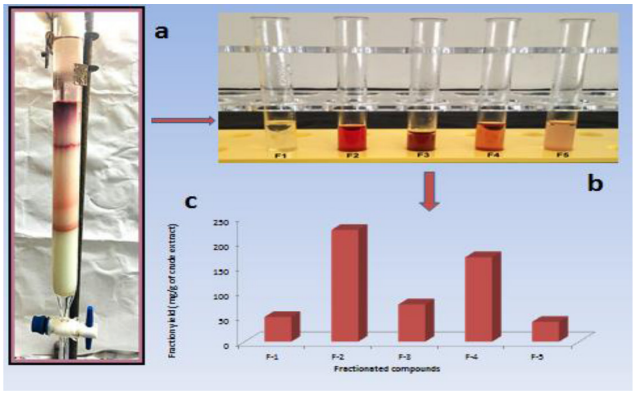

Figure 2: (a-c). Purification process of crude pigment of *F. chlamydosporum* in GYP medium.

Column Chromatography

The solvent system consisted of Chloroform

Methanol (9.5: 0.5), followed by gradually increasing the polarity of the binary solvent system and finally the compounds were eluted with 100% methanol. The crude pigment separated into 5 bands and was simultaneously collected in different test tubes. The whole of the purification procedure is depicted in Figure 2 (a-c). The separated compounds collected from the column were dried and weighed wherein the unit was expressed in mg / dry g weight of the crude extract. Fraction 2 and Fraction 4 yielded around 225 and 170 mg/ g of crude extract respectively which were used for further identification.

Column chromatography (b) collection of fractionated compounds (c) quantification of the fractionated compounds.

UV-visible Spectrophotometric Analysis

The absorption spectra of Fraction 2 and 4 on UV-Vis Spectrophotometry were analyzed to be 498 nm and 502 nm.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Based on the FTIR spectroscopic analysis of fraction 2 and fraction 4, the fingerprint regions were recorded located between 450 and 4000 cm^{-1} for the determination of the functional groups (Figure 3a and 3b respectively). The IR of fraction 2 and 4 interprets the presence of a benzene moiety with -OH, C=C, C-H and aromatic substitutions suggesting the compounds to be a naphthoquinone compound. Fraction 4 also possesses the presence of a CH_3 group suggesting the presence of a CH_3 substitution within the compound.

IR of fraction 2: 3419 (O-H); 2922, 2852 ($C\text{-}H_{str}$); 1744, 1646, 1852 (C=O); 1249 (C=C); 829, 658 cm^{-1}($C\text{-}H_{bending}$); 961 (aromatic ring substitutions); 1374, 978 ($C\text{-}C_{str}$ phenyl group); 720 (benzene derivative); 3005 ($CH_{3\,(str)}$); 1549, 1462 (C=C aromatic); 1164 ($C\text{-}H_{wagging}$)

IR of fraction 4: 3331 (O-H); 2923, 2853 ($C\text{-}H_{str}$); 1741,1838 (C=O); 1414, 1521 (C=C aromatic); 813, 756, 666 cm^{-1}($C\text{-}H_{bending}$); 1375, 704, 722 (benzene derivative), 3003 ($CH_{3\,(str)}$), 1163 ($C\text{-}H_{wagging}$); 1375, 1461 ($CH_{3\,bend}$)

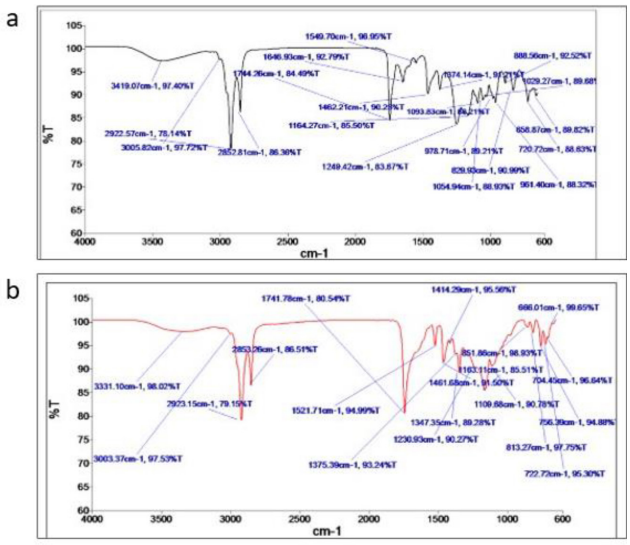

Figure 3: (a) FTIR spectrum of fraction 2; (b) FTIR spectrum of fraction 4.

Liquid Chromatography Mass Spectroscopy (LCMS) analysis of Fraction 2 and 4

The LC-MS analysis of Fraction 2 and Fraction 4 was identified to be Rubrofusarin and 8-O-methyl fusarubin respectively. Rubrofusarin is an orange polyketide pigment with the molecular formula $C_{15}H_{12}O_5$ and molecular weight 272.253 $gmol^{-1}$. The compound exhibited good mass spectrometric response in the positive ion mode. The mass to charge spectral peak was observed at 274.30 $(M+H)^+$ with the retention time 11.233 min in comparison with the standard whose retention time was 11.088 min. Figure 4 (A-E) depicts the whole of the characterization analysis of rubrofusarion by LC-MS.

With respect to the characterization of fraction 4, the compound was characterized as 8-O-methyl fusarubin which is a polyketide of reddish hue with molecular formula $C_{16}H_{16}O_7$ and molecular weight 320.29 $gmol^{-1}$. The compound exhibited good mass spectrometric response in the positive ion mode. The mass to charge spectral peak was observed at 322.10 $(M+H)^+$ and 663.60 $(2M+Na)^+$ with the retention time 11.789 min in comparison with the standard whose retention time was 11.791 min. Figure 5 (A-E) depicts the whole of the characterization analysis of 8-O-methyl fusarubin by LC-MS.

Chromatogram of f-2 with retention time of 11.233 min; B- Chromatogram of the standard with retention time 11.088 min; C- Fragmentation pattern of f-2; D- Fragmentation pattern of the standard

Chromatogram of f-4 with retention time of 11.789 min; B- Chromatogram of the standard with retention time 11.791 min; C- Fragmentation pattern of f-4; D- Fragmentation pattern of the standard.

In vitro Cytotoxicity Determination

Upon MTT assay, the IC_{50} of rubrofusarin and 8-O-methyl-fusarubin was found to be 183.85 µg/mL and 82.36 µg/mL respectively when compared with the anti-cancer compound doxorubicin with IC_{50} value of 79.29 µg/mL (Figure 6). The changes in the cell morphology on pigment treatment are shown in the Figure 7.

A. Untreated control cells exhibiting spindle shaped morphology. Arrows (a) indicates elongation of cells (b) rounding of cells (c) vacuolization which are considered as a sign of cytotoxicity.

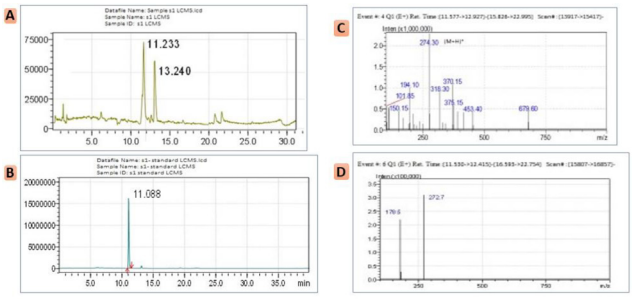

Figure 4: (A-D). Characterization of fraction 2 compound by LC-MS.

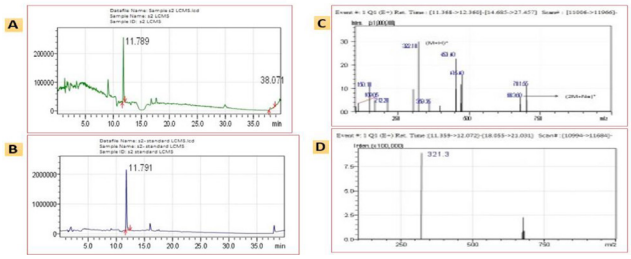

Figure 5: (A-D). Characterization of fraction 4 compound by LC-MS.

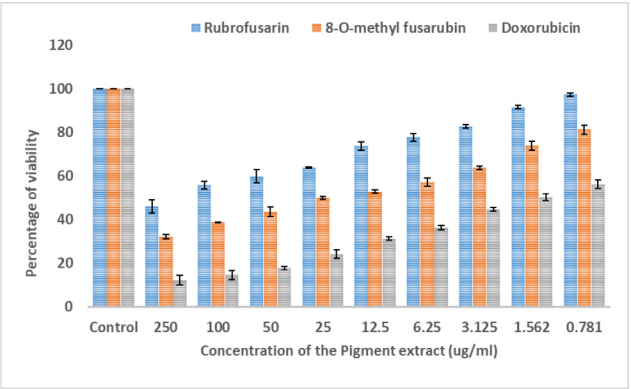

Figure 6: Cytotoxicity of rubrofusarin and 8-O-methyl fusarubin on L-929 Mouse fibroblast cell lines.

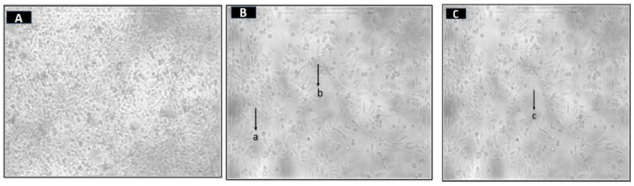

Figure 7: Microscopic examination of untreated and treated L929 mouse fibroblast cells with pigment extracts under 40x magnification of inverted phase contrast tissue culture microscope.

DISCUSSION

Fungi mainly produce carotenoids and polyketides as secondary metabolites. *Fusarium chlamydosporum* is reported to produce trichothecene, neosolaniol monoacetate, moniliformin and chlamydosporol. [3] In the present study, Rubrofusarin and 8-O-methyl fusarubin were identified from *F. chlamydosporum*. In

accordance with the earlier studies by Rao *et al.* 2017,[10] fungi are capable of producing diverse metabolites with respect to their physical environments. A study by Joshi *et al.* 2003,[1] reports that pigment production depends on method of fermentation employed. Our present study also holds well with these previous studies wherein we were able to identify 2 different pigments from Glucose Peptone yeast extract medium.

Rubrofusarin is a naphthopyrone derivative and has been reported previously for a number of biological activities, including hepatoprotective, anti-inflammatory and anti-microbial activities. Production of Rubrofusarin was carried out from *Aspergillus niger* GTS01-4, quoted by Megavati *et al.* 2017.[11] Production of 8-O-methyl fusarubin was reported by Vijitphan, 2019,[12] from fungus *Pestalotiopsis sp.* PSU-ES180 which displayed high potent cytotoxicity activity against cancer cells; this holds good with the present study as well.

Apart from the production of rubrofusarin and 8-O-methyl fusarubin, a recent study of ours have characterized the polyketide pigment fusarubin when grown on an undefined medium.[13] This is the first and foremost work characterizing these two polyketide pigments from *F. chlamydosporum*.

SUMMARY AND CONCLUSION

The aim of our project was to evaluate the pigment production by the fungus *Fusarium chlamydosporum* in Glucose peptone Yeast Extract medium followed by purification and characterization of the pigment. *F. chlamydosporum* produced dark red pigment on GPY medium extracted using ice cold chloroform by mycelial homogenization method. The purification of the crude extract from GPY was performed using Column Chromatography following which TLC was also performed to confirm the purity of the compounds with a single band. Fraction 2 and 4 accounted for the major portion of the crude pigment. Both the fractions were subjected to UV-Vis Spectrophotometry and LC-MS. The maximum absorption of F-2 using UV-Vis Spectrophotometer was 498 nm and on LC-MS the compound was revealed to be rubrofusarin. The maximum absorption of F-4 using UV-Vis Spectrophotometer was 502 nm and on LC-MS the compound was revealed to be 8-O-methyl fusarubin. The toxicity of the compound was also evaluated on L-929 Mouse Fibroblast cell lines by MTT Assay wherein the IC_{50} values of rubrofusarin and 8-O-methyl fusarubin was 183.85 and 82.366 µg/ml respectively.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **Da:** Dalton; **°C:** Degree Celsius; **FTIR:** Fourier Transform Infrared Spectroscopy; **GPY:** Glucose Peptone Yeast Extract; **LCMS:** Liquid Chromatography Mass Spectroscopy; **µg:** micro gram; **µL:** micro litre; **mg:** milli gram; **mL:** milli litre; **NFCCI:** National Fungal Culture Collection Centre Institute; **Rf:** Retention factor; **SD:** Standard Deviation; **TLC:** Thin Layer Chromatography.

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