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**Research Article**

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# Antimicrobial activity of pigments produced by fungi from Western Ghats

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**ABSTRACT**

*Maximum antibiotics are produced by filamentous micro-organisms like, actinobacteria, fungi. In this study totally 15 fungi isolates were isolated from soil sample from Western Ghats forest. Out of 15 isolates 6 strains produce pigment and identified upto genus level. The strain MF5 showed best activity among other strains against test pathogens. The pigment production from MF5 strain was produced by solid state fermentation and pigment was extracted by ethyl acetate. The extracted crude pigment compound was purified by thin layer chromatography and active spot was identified by bioautography. The minimum inhibitory concentration of purified compound was 12.5µg/ml for Bacillus subtilis*

**Keywords:** Pigment, activity, fungi, extracts bioautography.

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## INTRODUCTION

There is worldwide interest in process development for the production of pigments from natural sources due to a serious safety problem with many artificial synthetic colorants, which have widely been used in foodstuff, cosmetic and pharmaceutical manufacturing processes [1]. Humans have traditionally preferred natural sources to add colors to food, clothing, cosmetics and medicines. The new found interest in human safety and environmental conservation has kindled fresh enthusiasm for natural sources of colors. Microbes have advantages of versatility and productivity over higher forms of life in the industrial-scale production of natural pigments and dyes. Microbial pigments have two meaningful advantages over artificial and inorganic colors. One relates to fermentation, which is an inherently faster and more productive production compared to any other chemical process. The other enduring strength of microbes is their relatively large and easily manipulated strands of genes. [2].

Many fungi have been reported to produce non-carotenoid pigments but only a few of those have been explored as possible food colorants [3]. Soil provides a heterogeneous and complex environment for all soil inhabitants. Soil is also known to harbor different microorganisms including diverse group of fungi. Western Ghats are considered as one of the hot spot locations for biodiversity including microbial diversity. Hence the soils from Western Ghats can be a source of fungi of industrial importance [4]. The vast microbial biodiversity of the Western Ghats is yet to be exploited so that the indigenous soils can be screened for the isolation of other novel fungi with the ability of production of some other important enzymes, antibiotics and other bioactive compounds.

Recently, among the microorganisms, filamentous organisms like actinobacteria, fungi have been recognized as one of the last barely tapped resources for new biologically active secondary metabolites including antitumor,

antibacterial, antiviral, antifungal and enzyme inhibitor compounds. Overall research on marine derived fungi has lead to discovery of some 272 new natural products until 2002 and another 240 new structures from 2002 until 2004, thus, providing evidence that marine derived fungi have a potential to be a rich source of pharmaceutical leads.

Microbes can produce a large amount of stable pigments such as anthraquinones, carotenoids, flavonoids, quinines and rubramines. Fungi are more ecological and interesting source of pigments, as they produce stable colourants [5].

## EXPERIMENTAL SECTION

**Isolation of Pigment producing fungi:**
The soil sample was collected from the Kalakad Mundanthurai forest, Western ghats, Tamilnadu, India and serially diluted (upto $10^5$ dilutions) using sterile distilled water. About 0.1 ml of aliquot from $10^3$ to $10^5$ dilutions was plated on Sabaroud’s Dextrose (SDA) Agar. Plating was done in duplicate and all the plates were incubated at $28^0$C for 14 days. Morphologically different pigment producing fungal colonies were selected and maintained on SDA slants [6].

**Characteristics study of fungi**
All the fungal isolates were inoculated into SDA plates and incubated at $28^0$Cfor 14 days. Growth rate, mycelial colour and soluble pigmentation were noted. Microscopic characteristic was studied by slide culture technique. The mycelial structure was observed under light microscope after lactophenol cotton blue staining. Microscopic characteristics recorded include mycelial structure, conidia and its arrangement, sporangia, mycelial fragmentation. Results were recorded after 21 days of incubation. All the fungal isolates were identified at genus level based on their cultural and microscopic characteristics [6].

**Screening of antibacterial activity of pigmented fungi**
Antibacterial activity of fungal isolates was studied by adopting agar plug method [7]. For the preparation of agar plug, the fungal cultures were inoculated into SDA plates. After 14 days of incubation at $28^0$C, the mycelial growth was removed from the agar surface using sterile spatula. Agar plugs (5 mm in diameter) which contain the secreted bioactive compounds was cut using sterile gel puncture. Test organisms used in this study include *Staphylococcus aureus* resistant to methicillin (MRSA), extended spectrum beta lactamase (ESBL) producing *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Suspensions of test organism’s equivalent to 0.5 McFarland standards were prepared using nutrient broth and inoculated into Muller Hinton Agar (MHA) plates using sterile cotton swabs. Then the agar plugs were aseptically placed over test organisms inoculated on MHA plates. Zone of inhibition was measured after 24 hours of incubation at $37^0$C. Fungal isolates which showed good antibacterial activity in primary screening was selected for further investigation.

**Production and extraction of extracellular pigment**
Pigment from the fungal isolates was produced by adopting agar plate method. The fungal growth was inoculated into each five SDA plates and incubated at $28^0$C for 14 days. Then the whole medium was cut into pieces and extracted using n-hexane, ethyl acetate, chloroform and methanol for 24 hours. The crude extracts were concentrated at $45^0$C. The extracted pigments were tested for further studies [6].

**Antibacterial activity crude extracts**
The antibacterial activity of extracted pigment was studied by paper disc diffusion method100µg/disc concentration [8]. Test organisms used in this study include Methicillin resistant *Staphylococcus* (MRSA), *Bacillus subtilis,* extended spectrum beta lactamase (ESBL) producing *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* Suspensions of test organism’s equivalent to 0.5 McFarland standards were prepared using nutrient broth and inoculated into Muller Hinton Agar (MHA) plates using sterile cotton swabs. Then the paper disc impregnated with pigment extract was aseptically placed over test organisms inoculated on MHA plates. Zone of inhibition was measured after 24 hours of incubation at $37^{\circ}$C.

**Purification of crude pigment**
The number of compounds present in the crude pigmented extract was analysed by analytical thin layer chromatography using ready-made silica gel coated plates. The crude extract was dissolved in small volume of solvent and spotted at the bottom of the TLC sheet using glass capillary tube. Chromatogram was run by using different solvent systems such as chloroform: methanol; dichloromethane: methanol; hexane: ethyl acetate and ethyl acetate; acetone in different ratio. The separated spots were observed under naked eye, under UV light and also by

using iodine, anisaldehyde-sulphuric acid. Rf values of the separated compounds were calculated by adopting standard formula [9].

### Bioautography

The bioautography procedure described by Saravanan [10] was followed for the detection of active compound separated in TLC. Chromatogram developed as described above was placed in a sterile bioassay petridish and overlaid with 10 ml of molten nutrient agar seeded with 0.2ml of *S. aureus* and incubated at 37°C for 24 hours. Reference chromatogram was also prepared. The Rf value of the inhibition zones on test chromatogram was compared with the Rf of reference chromatogram. The corresponding spots that showed antibacterial activity were collected and used for further studies.

### MIC of partially purified compound

Preparative TLC was performed to separate the active compound from the crude extract. The active fraction separated in TLC was scrapped and its minimum inhibitory concentration was determined by adopting broth dilution method [11].

## RESULTS AND DISCUSSION

### Isolation and identification of pigmented fungi

Fungi contain several anthraquinone compounds and pigments such as delphinidin, melanin and volatile organic compounds (VOC's) which have been identified as their secondary metabolites. In this present study totally 15 morphologically different fungal colonies were isolated after incubation from soil samples. Out of 15 isolates 6 pigmented producing fungi were recovered, purified and sub cultured for further studies. Among 15 fungal colonies 6 pigment colonies were recovered and identified to genus level [8]. The selected six fungal colonies were identified at generic level (Table 1).

**Table 1. Identification of pigment producing fungal isolates**

| Strain name | Genus name | Pigment |
|---|---|---|
| MF2 | *Aspergillus sp.* | Black |
| MF5 | *Penicillium sp.* | Green |
| MF7 | *Penicillium sp.* | Yellow |
| MF10 | *Fusarium sp.* | Red |
| MF11 | *Aspergillus sp.* | Yellow |
| MF15 | *Aspergillus sp.* | Brown |

### Antimicrobial activity of fungal pigments

Antibacterial activity of fungal isolate was given in table 2. Only three out of 6 isolates showed antibacterial activity in which strain MF5 showed broad spectrum activity and strain MF2 showed inhibition against *Bacillus subtilis* and *E. coli* and MF3 showed inhibition only against *S. aureus.* The strain MF5 showed broad spectrum activity among other strains hence it was selected as potential strain for further studies. Antifungal producing actinomycets were isolated and screened by agar plug method [7].

**Table 2. Antibacterial activity of fungal isolates by agar plug method**

| Strain name | Test Pathogens (zone of inhibition in mm) | | | | |
|---|---|---|---|---|---|
| | MRSA | *B. subtilis* | *E. coli* (ESBL) | *K.pneumoniae* (ESBL) | *P. aeruginosa* (ESBL) |
| MF2 | - | - | - | - | - |
| MF5 | 12 | 16 | 13 | 12 | 12 |
| MF7 | - | 18 | 12 | 14 | - |
| MF10 | - | - | - | - | - |
| MF11 | 11 | 15 | - | 12 | - |
| MF15 | - | - | - | - | - |

### Production and extraction of pigment from potential strain

In agar surface fermentation, strain MF5 showed good growth with soluble yellow pigment production in SDA medium. Among the four different solvents tested for the extraction, the yellow pigment was extracted only in ethyl acetate. The ethyl acetate extracts showed maximum extraction of pigment when compared to methanol, chloroform and n-hexane. In general, filamentous organisms prefer solid medium for their growth and metabolite production.

There are many secondary metabolites and enzymes are produced from fungi by adopting solid state fermentation [6]. In this present study also crude extracts produced in solid state fermentation.

**Antibacterial activity by disc diffusion method**

Ethyl acetate extract of strain MF5 showed maximum of 22 and 17 mm of zone of inhibition against *Bacillus sp., and Staphylococcus aureus* respectively. The extracts also show antibacterial activity against other test pathogens (Table 3). The ethyl extracts showed activity against test pathogens; where as other solvent doesn't show any activity. The pigment compound was extracted by ethyl acetate and activity was tested by disc diffusion method [12].

**Table 3. Antibacterial activity of ethyl acetate extracts by disc diffusion method**

| Test Pathogens | Zone of inhibition in mm in diameter |
|---|---|
| MRSA | 17 |
| *B. subtilis* | 22 |
| *E. coli* (ESBL) | 16 |
| *K.pneumoniae* (ESBL) | 15 |
| *P. aeruginosa*(ESBL) | 16 |

**Purification and biautography of crude extracts**

In analytical TLC, one spot were got separated from crude ethyl acetate extract using methanol:n-butanol:water (75:10:15 ratio). Bioautography showed localized antibacterial activity of an extract on the chromatogram. In this present study activity of purified compound was detected by agar over lay method. The Rf value of bioactive spot was 0.6 and the active spot was identified by autobiography [9].

**MIC of partially purified compound**

The crude compound was purified and minimum inhibitory concentration (MIC) was tested. The MIC of crude compound was found to be 12.5µg/ml for *Bacillus subtilis.*

## CONCLUSION

Microorganisms from less/unexplored ecosystems are the promising source for novel molecules. In the present study antimicrobial activity was exhibited by fungi isolated from forest ecosystems. The pigment produced from strain MF3 show the promising source for bioactive metabolite isolated from forest ecosystems. Finding of the present work evidenced that the Western Ghats ecosystems investigated in this study deserves the potential for bioactivity.

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