

Original Research Article

Antioxidant and Antimicrobial Evaluation of Bioactive Pigment from *Fusarium* sp Isolated from Stressed Environment

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ABSTRACT

Majorly fungal pigments were found to be produced from 19th century onwards and it has many potential applications in different fields such as medical, pharmaceutical and food industries. The pigments produced majorly by fungi such as *Aspergillus niger* and *Monascus purpureus*. They have been used as a natural colorant and as food additives in East Asia. In this study, we aimed to produce potential bioactive fungal pigments isolated from stressed environment which having the highest antimicrobial profile and antioxidant properties. Further, it can be taken for food and pharma industries to prevent or cure heart related diseases. The isolated soil contains stressed conditions with microbial population and this environment makes the microbes to produce different metabolites for their survival between their communities. About nineteen fungal strains were isolated from the steel mills near Coimbatore in that 5 were pigmented fungi. The pigmented five fungal strains were taken for screening of antagonist activity and in that FC1-3 strain was found to be the potential. The strain was identified as *Fusarium* sp FC1-3 through morphological, biochemical and physiological characterization. The organism produced reddish orange pigments and the total pigments were extracted using methanol. The crude pigment extract was subjected to TLC and from that four bands were obtained and the R_f value was noted. The radical scavenging activity for the crude methanolic extract was determined through DPPH, metal chelation, reductive power and total antioxidant activities. From this the crude pigment showed the maximum antioxidant activity at least concentrations. Further this pigment will be taken for purification and bioprospecting studies.

Keywords

Antagonism, antioxidant, antimicrobial, fungal pigments, TLC

Introduction

The pigments are used for different applications in different fields. The term pigment and color are usually applied to food color matters, sometimes distinctly. The color of each pigment is associated with absorption or reflection of light in

determined wavelengths, which is characteristic of the pigment molecule. The artificial colors are causing serious health effects to plants, animals and humans. Most of the pigments are toxic to various extents and some are potentially carcinogenic. With

understanding the harmfulness of the synthetic colors, natural pigments are being increasingly emphasized. By their natural character, safety and use as additives, natural pigments have reached commercial potential (Pszczolla, 1998). There are numerous natural pigments in the world. They are collected from the sources such as plants, animals and micro organisms. The uses of natural pigments are increasing world-. The natural pigments have several affecting factors like temperature, pH, availability and cost. 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 (Dufosse, 2009). 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. The pigments producing micro-organisms will produce the antibiotic and inhibit the disease causing pathogens. They cannot however treat viral infection such as common cold or non bacterial inflammation. Different antibiotics work effectively on different bacteria. Indiscriminate use of antibiotics should be identified for a particular disease. Majorly fungal pigments were found to be produced from 19th century onwards and it has many potential applications in different fields such as medical, pharmaceutical and food industries. The pigments produced majorly by fungi such as *Aspergillus niger* and *Monascus purpureus*. They have been used as a natural colorant and as food additives in East Asia. In this study, we aimed focused on the pigment producing fungi isolated from stressed environmental conditions and to check the antimicrobial and antioxidant properties of the fungal pigment.

Materials and Methods

Sample collection and isolation

Two different soil samples were collected from a Steel Rolling Mills, Coimbatore, TN, India (latitude: 11.0183° N & longitude: 76.9725° E). they were serially diluted from 10⁻² to 10⁻⁸ in sterile water and about 100µL of sample from each dilution was spread on to Potato Dextrose Agar (PDA) and Sabouraud's Dextrose Agar (SDA) and the plates were incubated at 28±2°C for 7 -21 days.

Screening of antagonistic activity

The isolated pigmented colonies were cultured to obtain pure cultures. The pigmented strains were screened for antagonistic activity against clinical pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus vulgaris*, *Staphylococcus epidermidis* and *Shigella* sp by cross streaking method (Rahman et al, 2011). After incubation at 28±2°C for 7 days, 12 hrs cultures of clinical pathogens were streaked perpendicular to the central strip of fungal culture and again incubated at 37°C for 24 hrs and zone of inhibition was measured.

Morphological characterization of fungi

The best antagonistic strain FC1-3 was taken for morphological identification using Lactophenol Cotton Blue staining (Parija and Prabhaka, 1995). The fungal spores were viewed under 400x and 1000x magnification using light microscope and phase contrast microscope. The spore morphology was studied through Scanning Electron Microscopy (SEM) by cover slip technique.

Culture media

Different culture media such as PDB, SDB, Czapek Dox Broth (CDB), Malt extract medium (MEA) were prepared and inoculated with FC1-3 to find out the production media for maximum growth of fungal mat and pigment production. The inoculated media was incubated at $28\pm 2^{\circ}\text{C}$ for 7-15 days.

Physiological characterization

The physiological characteristics such as pH, temperature and salt tolerance were determined. The effect of pH was determined by the preparation of Sabouraud's dextrose broths with different pH ranging from pH 3 to pH 9. FC1-3 was inoculated and incubated at $28\pm 2^{\circ}\text{C}$ for 7-15 days. After the incubation period, the pigments were extracted and observed for maximum pigment production. Similarly the effect of temperature was characterized by preparing Sabouraud's dextrose broth and inoculated with FC1-3 strain and incubated at different temperatures (15°C , 25°C , 35°C , 45°C and 55°C) for 7-15 days. The results were observed after incubation period. The effect of salt tolerance was also examined by the inoculation of FC1-3 in Sabouraud's dextrose agar in different concentrations of NaCl ranging from 1% to 10%. The flasks were incubated at $28\pm 2^{\circ}\text{C}$ for 7-15 days. After incubation the results were observed for pigment and fungal mat production.

Production and extraction of pigments

The strain FC1-3 fungus was inoculated in selective production media and incubated at $28\pm 2^{\circ}\text{C}$ for 7-15 days. After incubation period the fungal mat was harvested using Whatmann No. 1 filter paper and it was washed thrice with sterile distilled water and dried. The pigment was extracted using

various solvents using mortar and pestle and the solvent mixture was kept in water bath at 60°C for 30 mins for extracting all the pigments. The crude extract was concentrated and stored for further studies.

Preliminary qualitative phytochemicals screening

The crude methanolic extract was checked for the presence of the following phytochemicals such as alkaloids, phenols, flavonoids, saponins, steroids, cardiac glycosides and tannins by standard procedures.

Cardiac glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. The crude dry powder of the extract was treated with 1 mL of FeCl_3 reagent (mixture of 1 volume of 5% FeCl_3 solution and 99 volumes of glacial acetic acid). To this solution a few drops of conc. H_2SO_4 was added. Appearance of greenish blue color within a few minutes indicated the presence of cardiac glycosides.

Steroids

Liebermann-Burchard reaction was performed to assess the presence of steroids. A chloroform solution of the crude dry powder of the extract was treated with acetic anhydride and a few drops of concentrated H_2SO_4 were added down the sides of the test tube. A blue green ring indicated the presence of terpenoids.

Alkaloids

The crude extract was evaporated to dryness in a boiling water bath. The residue was dissolved in 2 N HCl. The mixture was filtered and the filtrate was divided into 3 equal portions. One portion was treated with

a few drops of Mayer's reagent; one portion was treated with equal amount of Dragondroff's reagent and the other portion was treated with equal amount of Wagner's reagent.

The creamish precipitate, orange precipitate and brown precipitate, indicated the presence of respective alkaloids.

Flavonoids

In a test tube containing 0.5 mL of crude pigment extract, 5-10 drops of diluted HCl and small piece of zinc or magnesium were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink or dirty brown colour was produced.

Phenols

The crude pigment extract was dissolved in 5 mL of distilled water. To this few drops of neutral 5% ferric chloride solution are added. A dark green color indicates the presence of phenolic compounds.

Tannins

The crude extract was treated with alcoholic FeCl₃ reagent. A bluish black colour, which disappears on addition of a little dilute H₂SO₄ was followed by the formation of yellowish brown precipitate.

Saponins

The presence of saponins was determined by Frothing test. The crude dry powder of fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 min. No froth indicates absence of saponins and stable froth more than 1.5 cm indicated the presence of saponins.

Separation of the bioactive compound

TLC

The crude extract was subjected to TLC (Thin Layer Chromatography) (Geiss F, 1997). This is done to find out the separation of bioactive compounds from the crude methanolic extract using different solvent such as chloroform: methanol; n-Hexane: methanol; petroleum ether: ethyl acetate. The solvent system which separated maximum number of bioactive compounds from the crude extract was taken for further purification process. The solvent front was marked and R_f value was calculated.

Biological activity of the crude extract

Antimicrobial activity

The antimicrobial activity was done by well diffusion method (Bauer et al, 1966) against the test organisms such as *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, *Klebsiella* sp, *S. typhi*, *P. vulgaris*, *Shigella* sp, *Candida albicans* and *Aspergillus niger*.

The Mueller Hinton agar (MHA) plates were prepared and previously seeded with the test organism in each plate. The wells were cut by using a sterile cork borer. Forty microliter of culture filtrate and crude extract of the strain FC1-3 was added in each well. The diameters of inhibition were determined after 24 hr of incubation at 37°C for bacteria.

Antioxidant activities

DPPH radical scavenging activity

The crude methanolic pigment extracts were aliquoted into different concentrations (50-200µl) for determining its ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl

(DPPH) radicals using the method of Yildirim et al, 2009. DPPH solution (1mM DPPH radical solution in 95% methanol) of 800µl was mixed with 200µl of crude extract, vortexed well and then incubated for 30 min at room temperature in dark. After 30 min incubation, the samples were centrifuged for 5 min at 13,500 rpm (at room temperature). Then, the absorbance of each supernatant sample was measured at $\lambda=517$ nm and 1ml of 95% methanol was used as a control. Ascorbic acid was used as a reference compound. The antioxidant activity was given as percent (%) DPPH scavenging, calculated by the following formula:

DPPH radical scavenging activity (%) =

$$\frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{(\text{Abs}_{\text{control}})} \times 100$$

Reducing power

The reducing power was based on Fe (III) to Fe (II) transformation in the presence of the solvent fractions (Fejes et al, 2000). The Fe (II) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Various concentrations of the sample (50-200µl) were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50°C for 20 min followed by addition of 2 ml of Tri Chloroacetic acid (TCA) (100 mg/L).

The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution. A volume of 2 ml from each of the mixture earlier mentioned was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates a higher reducing power.

Metal chelating activity

The metal chelating activity was estimated by the method of Dinis et al, 1994. The reaction was performed in HEPES buffer (20mM, pH 7.2).

Various concentrations (100-200µl) of samples were mixed with solution of 12.5µM ferrous sulphate solution. The reaction was initiated by the addition of 75µM ferrozine and the mixture was shaken vigorously and incubated for 20 min at room temperature. After incubation the absorbance was measured at 562 nm. EDTA was used as the reference compound and the percentage chelating capacity was calculated as;

$$\text{Chelating effect (\%)} = \frac{[(A_{\text{control}} - A_{\text{sample}})]}{A_{\text{control}}} \times 100$$

Total Antioxidant activity

The total antioxidant capacity of the sample was determined by phosphomolybdate method using ascorbic acid as a standard (Umamakeshwari et al, 2008). An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate).

The tubes were capped and incubated in a water bath at 95°C for 90 min. After incubation period, the samples were cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank.

A typical blank contained 1 ml of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. Ascorbic acid was used as standard.

Results and Discussion

Isolation of pigmented fungi

The study has aimed to isolate the pigment producing fungi from stressed terrestrial environmental soil samples. Two soil samples were taken in which 19 fungal strains (FC1-1 to FC1-6, FC2-1 to FC2-13) were isolated. Out of 19 isolates, 5 were pigmented fungal strains (FC1-1, FC1-2, FC1-3, FC2-1 and FC2-2). From this only one potential strain which has the antimicrobial activity will be taken for the further studies. Due to the stressed condition the microbial communities were disturbed and only a fewer were found to be pigmented strains. This may be due to the variation in pH and temperature in the particular environment.

Antagonist activity

The isolated 5 pigmented strains were screened for antagonistic activity. This was done by cross streak method, in which one strain FC1-3 which showed antagonist activity for maximum of 7 pathogens and it was taken for further studies. The highest activity was found against *K. pneumoniae*, *Shigella* sp and *P. aeruginosa*. This means that this FC1-3 strain was found to have the highest antimicrobial potentiality when compared to other strains.

Morphological characterization of fungi

The fungal strain FC1-3 (Fig 1) which showed the highest antagonist activity was taken and subjected to Lactophenol Cotton Blue staining for the morphological identification. It produced oval shaped spores and it was found to be *Fusarium* sp (Fig 2). Scanning electron microscopy image of the fungal strain FC1-3 mycelium showed that the aerial mycelium formed

chains of basidiospores with numerous spores in it. The spores were oval to cylindrical in shape with smooth surfaces.

Culture growth in different media

The strain FC1-3 fungus was inoculated in different media and the strain produced reddish biomass and it was found to grow well in PDB and CDB. The pigment and biomass production was more in SDB at stationary condition when compared to other media. The fungal mat (biomass) produced by the strain was measured at an interval of 3 days from 3rd day to 15th day. The weight was calculated and it was found that the biomass weight increased from 3rd day to 13th day. The fungal mat (biomass) weight of the isolate FC1-3 was maximum of 50g/L on 13th day in SDB medium. Hence 13th day was taken as the incubation period required for maximum biomass production. This fungus produced intracellular orangish red pigment from biomass and the strain does not produce extracellular pigments.

Physiological characterization

The strain FC1-3 (*Fusarium* sp) was found to grow well in pH ranging from 3 to 9. The maximum pigment production was found from pH 6 to 8. The culture was found to produce more fungal biomass and maximum pigment at pH 8. The biomass was found to be 54.3g/L at pH 8. This indicates that the strain is tolerable to neutral pH range and pH was found to be an important source for the growth and production of pigments by the organism. It is a significant factor which affects the physiology of the organism and makes it to uptake the nutrients for its growth. The culture growth was good from the temperature range 15°C to 45°C. The strain *Fusarium* sp was found to produce maximum pigment production and biomass of about 57.6g/L at 25°C. The temperature

was an important characteristic for the production of metabolites for the organism's growth and it influences the metabolic process inside the organism. The FC1-3 strain was inoculated in SDA in different NaCl concentrations ranging from 1% to 10%. It was incubated at $28\pm 2^{\circ}\text{C}$ for 13 days. After incubation the results were observed. The strain FC1-3 was found to tolerate the salt concentration at 4%. These characteristics were similar to the sampled terrestrial soil environment.

Production and extraction of pigments

The strain FC1-3 was inoculated in SDB and incubated for 11 days without any disturbance at stationary condition for fungal mycelia mat formation. The filtered biomass was washed thrice with sterile distilled water to remove the debris and dried.

The maximum pigment was extracted using polar solvents like methanol, ethyl acetate and acetone by homogenization method. The crude pigment was reddish orange color and it was collected and concentrated in rotary vacuum evaporator. This crude methanolic extract was taken for further biological studies.

Qualitative phytochemical analysis

Preliminary phytochemicals screening of the crude extract in methanol, ethyl acetate, acetone and n-hexane of the FC1-3 strain pigments was carried out by standard protocols and the results were given in Table. 1. Alkaloids, phenols, flavonoids and tannins were present in the total pigment extract and when it was taken to purification process we can separate the particular phytochemical constituents which can be taken for further studies.

Partial purification of the crude pigment

TLC

Thin layer chromatography was performed using different solvent systems. The best solvent system for movement of the pigment was found to be with petroleum ether: ethyl acetate (65%) (Fig 3). The solvent front was marked and R_f value was calculated. Four bands were obtained through TLC.

Biological activity of the methanolic pigment

Antimicrobial activity

The antimicrobial activity was done by well diffusion method (Bauer et al, 1966). The antimicrobial activity was checked for crude pigment extract and culture filtrate but, the culture filtrate does not have any antimicrobial activity. *E. coli* was found to be highly susceptible to methanolic extract with the zone of inhibition at 2.9 cm. The methanolic extract was able to inhibit *Shigella* sp with the inhibition zone of 2.1 cm.

The good zone of inhibition of 1.6 cm and 1.2 cm were also observed against *K. pneumoniae* and *S. typhi* respectively. The methanolic extract of the strain FC1-3 also showed good antifungal activity against *Candida albicans* and *Aspergillus* sp with an inhibiting zone of 1.9 cm and 2.1 cm respectively. The results of antimicrobial activity of crude methanolic extract are tabulated in Table 2.

Antioxidant Activity

DPPH radical scavenging assay

The free radical-scavenging activity of fungus, *Fusarium* sp along with reference

standard ascorbic acid was determined by the DPPH assay. The fungal extracts of the present study showed a concentration dependent antiradical activity by inhibiting DPPH radical. Significant DPPH scavenging potential of methanolic crude pigment extract of the fungal strain FCO1-3 may be due to hydrogen groups present in the extracts (Nithya et al, 2009). The IC₅₀ value of fungal methanolic extract of FC1-3 was seen in 162µl concentration (1.62mg/mL) (Fig. 4). Scavenging of DPPH represents the free radical reducing activity of antioxidants based on a one electron reduction.

Metal chelating activity

Metal chelating activity of the fungal pigment crude methanolic extract at various concentrations was depicted in Fig. 4. FC1-3 showed the metal chelating activity of IC₅₀ at 153µl concentration (1.53mg/ml) and it represents the greatest inhibition activity when compared to ascorbic acid. From this it is evident that the crude pigment extract has the highest metal chelation when compared to the standard ascorbic acid.

Reductive ability

The reducing capacity of the compound may serve as a significant indicator of potential antioxidant activity. The reduction of ferrous ion (Fe³⁺) to ferric ion (Fe²⁺) is measured by the intensity of the resultant Persian-blue solution which absorbs at 700 nm. The reducing properties are generally associated with the presence of reductones. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom (Gordon, 1990). Similar to the antioxidant activity, the reducing power of crude methanolic extract of the fungal isolate FC1-3 increased with increasing dosage. The result showed that methanolic extract consists of hydrophilic poly phenolic compounds that cause the

greater reducing power. As the OD value increases the reducing capacity also increases which is depicted in table 3.

Total antioxidant activity

Phenolic and flavonoid compounds seem to be the important compounds that are associated with the antioxidant activity; they exhibit in providing an important role in stabilizing the oxidation of lipids. Phenols are important because their hydroxyl groups confer scavenging ability. The total antioxidant activity of methanolic crude fungal pigment extract was evaluated and was found to be 1.72 mg/ml concentration which was equivalent to micrograms of the standard ascorbic acid.

The current investigation implied on isolating a potential fungal strain from stressed environmental conditions and extracted the bioactive secondary metabolite for biological activities. The microbial communities were prevalent in the samples which were very efficient and potent from the stressed conditions and they produce bioactive metabolites for their survival which are found to be useful to mankind. The isolated potential fungus was *Fusarium* sp which had a potency to produce maximum pigmented metabolites SDB media and the presence of dextrose influences the growth of the organism. Whereas media without dextrose produced lighter pigmentation and the growth of the organism was also lesser when compared to other studied media. The same study of different growth media for fungi was reported by Stanley et al (2013). The strain FC1-3 produced maximum pigments at pH 8 and from this we can conclude that the organism can better sustain well in alkaline state at medium range and this may be due to the adaptability of the organism to the environmental conditions.

Table.1 Preliminary qualitative phytochemical screening

Phytochemicals	FC1-3			
	Methanol	Ethyl acetate	Acetone	n-Hexane
Alkaloids	+	+	-	-
Phenols	+	+	-	-
Steroids	-	-	-	-
Flavanoids	+	-	-	+
Saponins	-	-	-	-
Tannins	-	-	-	+
Carbohydrates	+	-	+	-
Glycosides	+	-	-	-

(+) Presence (-) Absence

Table.2 Antimicrobial activity of the crude extract of FC1-3

S. No.	Pathogens	Methanolic extract Zone of inhibition (in cm)
1	<i>Staphylococcus aureus</i>	-
2	<i>Klebsiella pneumoniae</i>	1.6
3	<i>Salmonella typhi</i>	-
4	<i>Bacillus subtilis</i>	-
5	<i>Escherichia coli</i>	2.9
6	<i>Proteus vulgaris</i>	-
7	<i>Shigella sp</i>	2.1
8	<i>Aspergillus niger</i>	2.1
9	<i>Candida albicans</i>	1.9

Table.3 Reductive power ability of FC1-3

Concentration (µg)	OD Value of FC1-3
120	0.17±0.02
140	0.24±0.67
160	0.35±0.51
180	0.45±0.67
200	0.51±0.0

Fig.1 FC1-3 strain



Fig.2 LCB staining of FC1-3 at 40x



Fig.3 TLC of crude methanolic pigment

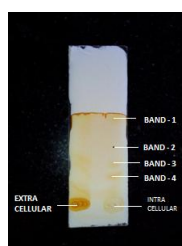
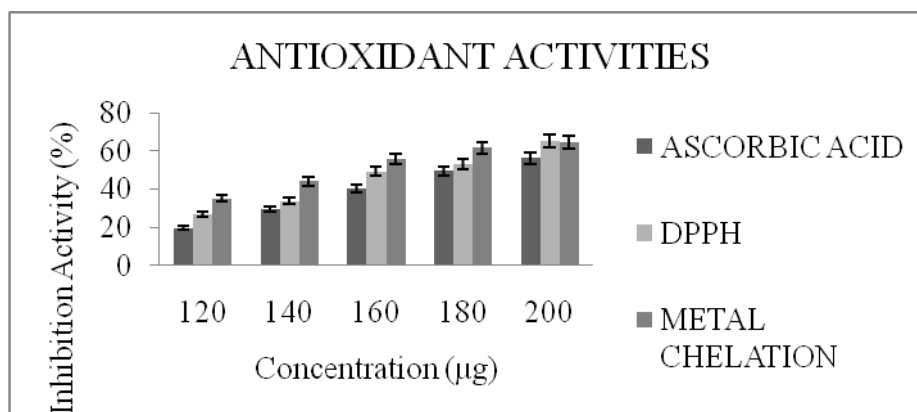


Fig.4 Antioxidant activities of DPPH and Metal chelation assay



Also the strain survived at the temperature range of 45°C and temperature is an important characteristic for the fungal growth and pigmentation. This isolated fungus *Fusarium* sp produced reddish pigment on extraction with ethyl acetate a polar solvent. Most of the fungal pigments/ or metabolites were extracted using ethyl

acetate as it a polar solvent and this was similar to the fragmentary reports stated already. The fungal pigment extract exhibited a promising antimicrobial activity at least concentrations and this proves the fungal extract can be taken for quorum quenching activity and medicinal fields. Apart from the antimicrobial profile, the

extract unveiled its potency in antioxidant properties. The scavenging effects were very efficient in least concentrations. Scavenging DPPH free radical determines the free radical scavenging capacity or antioxidant potential (AOD) of the sample, which shows its effectiveness prevention interception and repair mechanisms against injury in a biological system (Lee *et al.*, 2001). The reduction in the number of DPPH molecules can be correlated with the number of available hydrogen groups (Nithya *et al.*, 2011). The extract was also subjected to reducing power activity where it exhibited a potential activity at least concentration. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom (Gordon, 1990). Similar to the reducing power the metal chelation was also revealed the property at a range of concentration. Though Cu^{+2} and Fe^{+2} are required by the organisms for transport, protection against oxidative stress, cell growth and development they can catalyze hydroxyl radical formation by the Fenton reaction (Campos *et al.*, 2005). Therefore, the levels of Fe^{+2} and Cu^{+2} in cells should be carefully controlled and this was effectively done by the fungal extract. The results of this investigation suggested that the pigmented fungal extract can be taken for industrial purposes after purification and chemical characterization processes.

In conclusion, the environmental soil which has been stressed due to pollution but it contains majority of micro organisms which were prevalent in producing different metabolites for their survival between microbial communities. The isolated strain *Fusarium* sp produced reddish orange pigments and it possesses the antimicrobial as well as antioxidant properties. So we suggest that this pigment can be taken as natural colorant in medicinal, food and

pharma fields. Further the crude extract has to be purified and studied for characterization of the pigmented compound also to be done to make it a potential drug.

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