

Original article

Evaluation of antioxidant property of some fungal pigments by DNA protection assay

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Received March 3, 2018; Revised April 25, 2018; Accepted April 30, 2018; Published online June 30, 2018

Abstract

Reactive oxygen species (ROS) can damage DNA, RNA and proteins which subsequently contribute to the physiology of ageing. As compared to synthetic drugs, antioxidants from various biological sources have shown to protect the cell from these effects and are relatively safer. In the present study, the *in vitro* antioxidant potential of methanol-extracted pigments of six fungal isolates from forestry soil was evaluated. The pigments were assayed for their 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) reduction, ferric ion reducing antioxidant power (FRAP) and DNA protecting capability (supercoiled pUC18 plasmid DNA) against ROS inducing agents like ferrous chloride, stannous chloride, hydrogen peroxide (H₂O₂) and UV rays. The thermomyces pigments showed greater DPPH reduction (69 %) and FRAP (7.14 μM of Fe II) as compared to other pigments and, thus confirmed its ability to donate electrons. All the fungal pigments showed significant DNA protection against H₂O₂ and UV rays while ferrous chloride-induced DNA damage was protected only by the pigments of *Thermomyces* sp. and *Aspergillus tamarii*. This study opens the possibility of using the fungal pigments as natural antioxidants for food industry.

Keywords: Antioxidants, fungal pigments, reactive oxygen species, thermomyces, UV rays

1. Introduction

Free radicals are generated during normal bodily process. Exposure to UV light, cigarette smoke and other environmental pollutants also increase the body's free radical burden. Free radicals play an important role in pathogenesis of serious diseases (Kumar *et al.*, 2012). They cause tissue damage by reacting with poly-unsaturated fatty acids, found in cellular membranes, nucleotides in DNA and sulfhydryl groups in protein, resulting in chronic diseases, such as neurodegenerative disorders, cancer, liver cirrhosis, cardiovascular diseases, atherosclerosis, cataracts, diabetes and inflammation (Domínguez-Bocanegr, 2012). However, the compounds that can scavenge free radicals have great potential in ameliorating these diseases (Lin *et al.*, 2012; Balmani *et al.*, 2015).

In recent decades, natural antioxidants have attracted great attention from consumers over the world due to their being safer than synthetic antioxidants. The fungal carotenoids are of considerable interest in nutrition because of their role as antioxidants and their potential for preventing or delaying degenerative diseases apart from enhancing immune responses in animals and humans (Watgen *et al.*, 2002). The exploration of naturally occurring antioxidants, colourants and antimicrobials for food application has received increasing attention due to consumer awareness of natural food products and growing concern towards conventional food preservatives (Kirakosyan *et al.*, 2003). A wide variety of fungi

including *Penicillium roquefortii*, *Aspergillus candidus*, *Mortierella* sp., *Emericella falconensis* and *Acremonium* produce novel antioxidants have been earlier reported (Rios *et al.*, 2006 ; Mapari *et al.*, 2009). The present study, we have investigated the *in vitro* antioxidant property of six different colourants extracted from the fungi against the DNA damage induced by chemicals and UV rays demonstrated the ability of fungal colourants *in vitro* protecting the DNA against ROS.

2. Materials and Methods

2.1 Fungal pigments

Six different fungal isolates obtained from diversified soils of Tamil Nadu, India were extracted for pigments using methanol. The solvent was vacuum evaporated at room temperature and the pigment was concentrated to dryness using lyophilization and stored at 4°C until further assay (Frisvad and Thrane, 2004). The fungal species and the pigment characters are presented in Table 1.

2.2 *In vitro* antioxidant activity of fungal pigments

2.2.1 Determination of DPPH scavenging activity

The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time. The antioxidant activity has been evaluated by the ability of investigated extract to reduce the stable DPPH free radicals. Due to its unpaired electron, DPPH radical gives a strong absorption band at 517 nm (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The free radical scavenging activity of each extract was assayed using a stable 1,1-

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diphenyl-2-picrylhydrazyl (DPPH), following a standard method (Blois, 1958). The hydrogen atom or electron donating ability of the extracts was measured from bleaching of purple coloured EtOH solution of DPPH. An aliquot of 1 ml of pigment extract and 3 ml of 0.1 mM DPPH solution was added. After 30 min incubation period

at room temperature, the absorbance was read against a blank at 517 nm.

$$\text{Inhibition percentage (\%)} = \left(\frac{C - S}{S} \right) \times 100$$

C is absorbance of control and S is absorbance of sample solution.

Table 1: Fungal pigments used in this study

Fungal species	Source of isolation	Pigment yield (g/l)	Pigment characters	
			Colour	Solubility
<i>Penicillium purpuroscens</i>	Forestry soil (Ooty, India)	4.0	Brown	Water, methanol and ethanol
<i>Aspergillus tamarii</i>	Forestry soil (Topslip, India)	6.0	Orange	Water, methanol and ethanol
<i>Trichoderma</i> sp.	TNAU field soil (India)	1.5	Yellow	Water, methanol and ethanol
<i>Thermomyces</i> sp.	Forestry soil (Thottabetta, India)	14.0	Yellowish orange	Water, methanol and ethanol
<i>Chaetomium</i> sp.	Forestry soil (Kodaikanal, India)	6.5	Magenta pink	Water, methanol and ethanol
<i>P. purpurogenum</i>	Forestry soil (Ooty, India)	8.0	Red	Water, methanol and ethanol

2.2.2 Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed for all the six fungal pigments by following the procedure as described by Benzie and Strain (1996). This method uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. A quantity of 300 µl of freshly prepared FRAP reagent (redox indicator) was warmed to 37°C to which 40 µl distilled water was added and served as blank. In another cuvette, same amount of FRAP reagent was taken and added with 10 µl sample extract and 30 µl of water. Hence, the final dilution of the sample in the reaction mixture was 1/34. An absorbance at 593 nm was taken from 1 min till completion of reaction, i.e., up to 8 min. The change in the absorbance between final and initial readings was noted for each sample and related to the change in absorbance of the ascorbic acid as standard.

2.3 DNA protection assay

The ability of six different fungal colourants to protect the DNA damage by both chemicals and UV rays-induced ROS was determined by following the procedures described previously (Kwon *et al.*, 2006; Babbs, 1992). In brief, the double-strand supercoiled circular DNA (pUC 18) (Bangalore Genei, Bangalore, India) was diluted to 500 ng/µl using TE buffer (pH 8.0) and incubated with different chemical agents such as ferric chloride (250 mM), stannous chloride (20 mg/ml) and H₂O₂ (10%) separately in the presence of fungal pigments (100 µg/ml-dissolved in methanol) at 37°C for 30 min. Respective controls (solvents without chemicals agents and colourants) were also maintained.

For UV protection assay, the DNA (pUC 18) in the presence or absence of fungal pigments was exposed to low wavelength UV light (240 nm) for 90 min at room temperature. All the sample were then resolved by electrophoresis on 1% agarose gels stained with ethidium bromide and documented in Alpha Imager TM 1200 documentation and analysis system. The band intensity of each

gel was assayed and compared with untreated control and per cent reduction, due to chemical/UV treatment in presence or absence of pigments was quantified using GelQuant.NET software (Biochemlabsolutions).

2.4 Statistical analysis

All the data presented in Figures were expressed as Mean ± standard deviation of the three replicates. All the treatments were compared at $p \leq 0.01$ and 0.05 level of significance, using the Critical Difference (CD) test which was performed by Excel-2000. The analysis of variance (ANOVA), standard error (SE) and critical difference (CD) for dependent parameters and the level of significance was reported.

3. Results

3.1 DPPH radical scavenging activity

The DPPH scavenging activity of different concentrations of methanolic extracts of fungal pigments and BHA are shown in Figure 1. The *Thermomyces* sp. and *Chaetomium* sp. pigments showed significant scavenging effects against the DPPH radical, which increased with increasing concentration from 5-20 mg/ml.

3.2 FRAP assay

The antioxidant power of extracts was compared with ascorbic acid as a reference standard. The FRAP values of extracts ranged from 0.24 to 7.14 µM at a concentration of 5, 10, 15 and 20 mg/ml against BHA (7.83 to 13.53 µM). The pigment extract of *Thermomyces* sp. showed a significant ability to reduce Fe³⁺ (7.14 µM) at 20 mg/ml concentration and, thus ability to donate electrons. This property suggested that *Thermomyces* sp. pigment extract might act as a free radical scavenger, capable of transforming reactive free radical species into stable non-radical products (Figure 1).

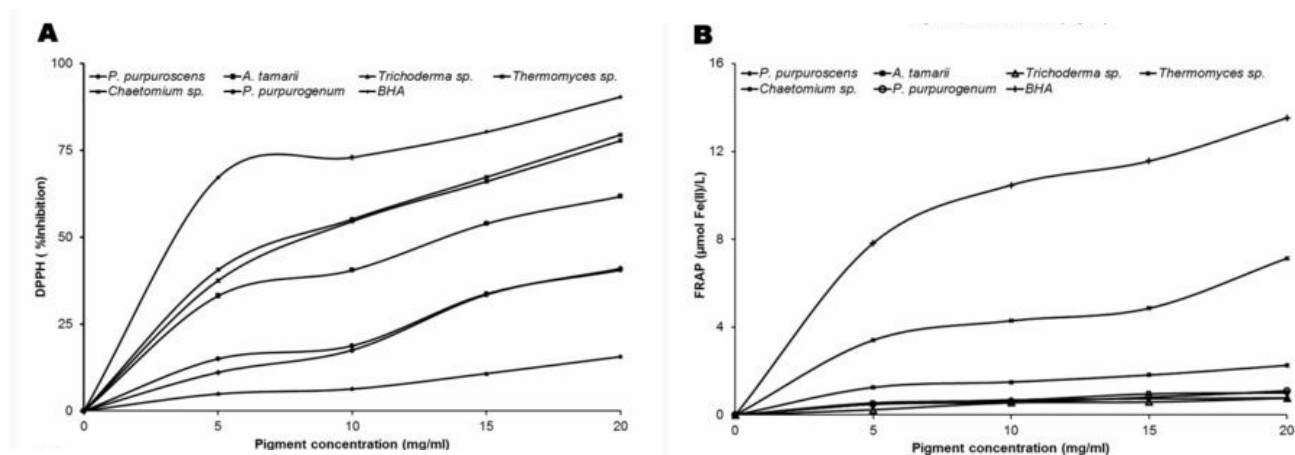


Figure 1: DPPH scavenging activity (A) and Ferric ion reducing antioxidant power and (B) of fungal pigments.

3.3 Effect of fungal pigments on FeCl_2 induced DNA damage

The double-strand circular plasmid DNA, pUC 18 was incubated with and without six different fungal pigments (100 $\mu\text{g/ml}$), followed by exposure to 250 mM FeCl_2 for 60 min at 37°C. The FeCl_2 significantly damaged the double - stranded DNA (Figure 2a) in the presence or absence of antioxidants. The synthetic antioxidant BHA also failed to protect the DNA from FeCl_2 - induced DNA damage. The fungal pigment from *Thermomyces* and *Aspergillus tamarii* protected the DNA from FeCl_2 to some extent (Figure 2a, Lanes Th and At). However, in these treatments, the concentration of supercoiled DNA was significantly reduced and linearized DNA was increased due to nicking by FeCl_2 . The nicking of DNA could not be protected even by these two extracts. The band intensity of FeCl_2 induced DNA nicking assay was analysed using Gel Quant software. The *A. tamarii* and *Thermomyces sp.* fungal pigments protected the plasmid DNA 35 and 54 %, respectively.

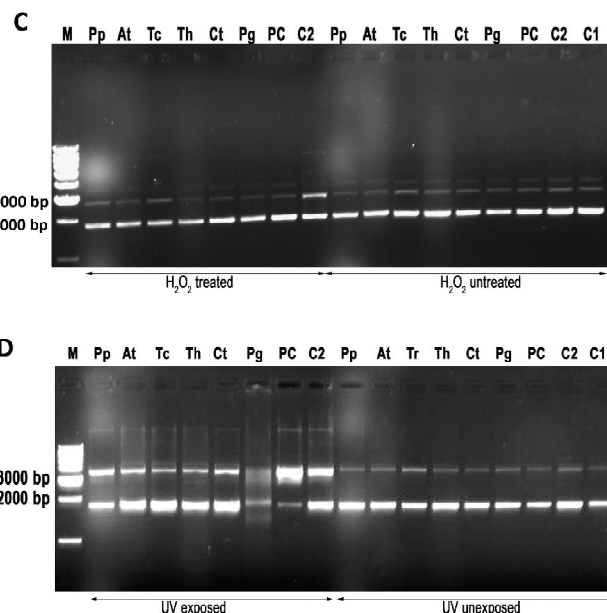
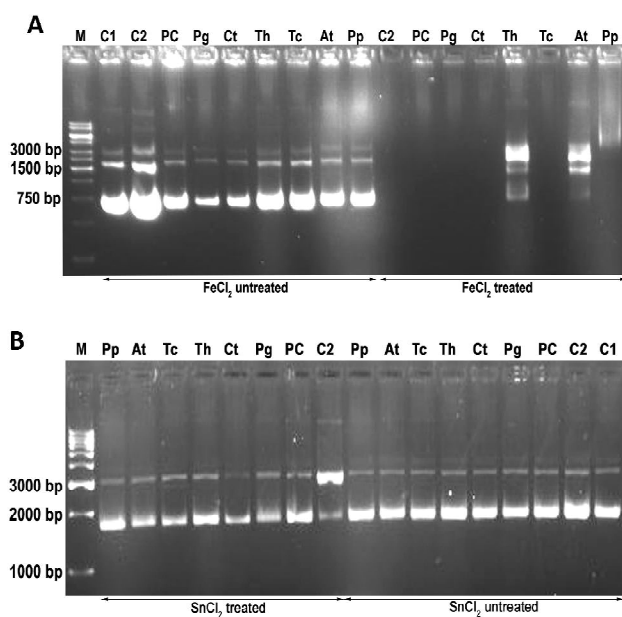


Figure 2: Protection of ferrous chloride (A), stannous chloride (B), H_2O_2 (C) and UV (D) induced DNA damage by different fungal pigments [M, 1kb DNA marker; Pp, *P. purpuroscens*; At, *Aspergillus tamarii*; Tc, *Trichoderma sp.*; Th, *Thermomyces sp.*; Ch, *Chaetomium sp.*; Pg, *P. purpurogenum*; Pc, positive control (BHA, synthetic antioxidant); C2, pUC 18 DNA (Control); C1, pUC 18 DNA with methanol].

3.4 Effect of fungal pigments on SnCl_2 - induced DNA damage

When plasmid DNA was treated with SnCl_2 , the intact supercoiled DNA was broken into linearised form compared with the untreated DNA (Figure 2b, lane C1). It was also noticed that the SnCl_2 -induced DNA damage was much more, though less than FeCl_2 (Fig 2b, Lane

C1 and Figure 2b, Lane C1). The SnCl_2 caused the nicking of DNA, leading to linear DNA formation (Figure 2b, Lane C2). All the fungal extracts significantly reduced the SnCl_2 -induced DNA nicking equally. The DNA was protected from the SnCl_2 -induced DNA damage by most of the fungal pigments. The scavenging activity of all the fungal pigments significantly increased. *Thermomyces* and *Chaetomium* exhibited 75% and 70% protection over control, respectively.

3.5 Effect of fungal pigments on H_2O_2 -induced DNA damage

The H_2O_2 also caused less damage to DNA, when exposed for 60 min at 37°C (Figure 2c). It formed nicking of supercoiled circular DNA molecule, leading to linearization of DNA (Figure 2c, lane C2). The pretreatment with the fungal pigments protected the nicking of DNA by H_2O_2 . The densitometric analysis revealed that all the fungal pigments protected the DNA from H_2O_2 -induced damage especially

Thermomyces and *A. tamarii* pigments with 63% and 38%, respectively.

3.6 Effect of fungal pigments extract on UV-induced DNA damage

UV rays - induced DNA damage was relatively higher than FeCl_2 and less than SnCl_2 and H_2O_2 (Figure 2d). Exposing the pUC 18 DNA for 60 min caused linearization of circular supercoiled DNA. The relative ratio of linear DNA to circular was significantly increased due to UV damage (Figure 2d, lane C2). Difference in DNA concentration was noticed between UV exposed and unexposed samples which were mainly due to drying of sample for long-term UV exposure. Among the six fungal pigments, *A. tamarii*, *Trichoderma* sp., *Thermomyces* sp. and *Chaetomium* sp. protected the DNA more efficiently than other two. The pigment from *P. purpurogenum* and positive control antioxidant (BHA) had lowest protection efficiency. The densitometry analysis of DNA bands run in agarose gel electrophoresis gels after exposed to UV rays revealed that the pigments of

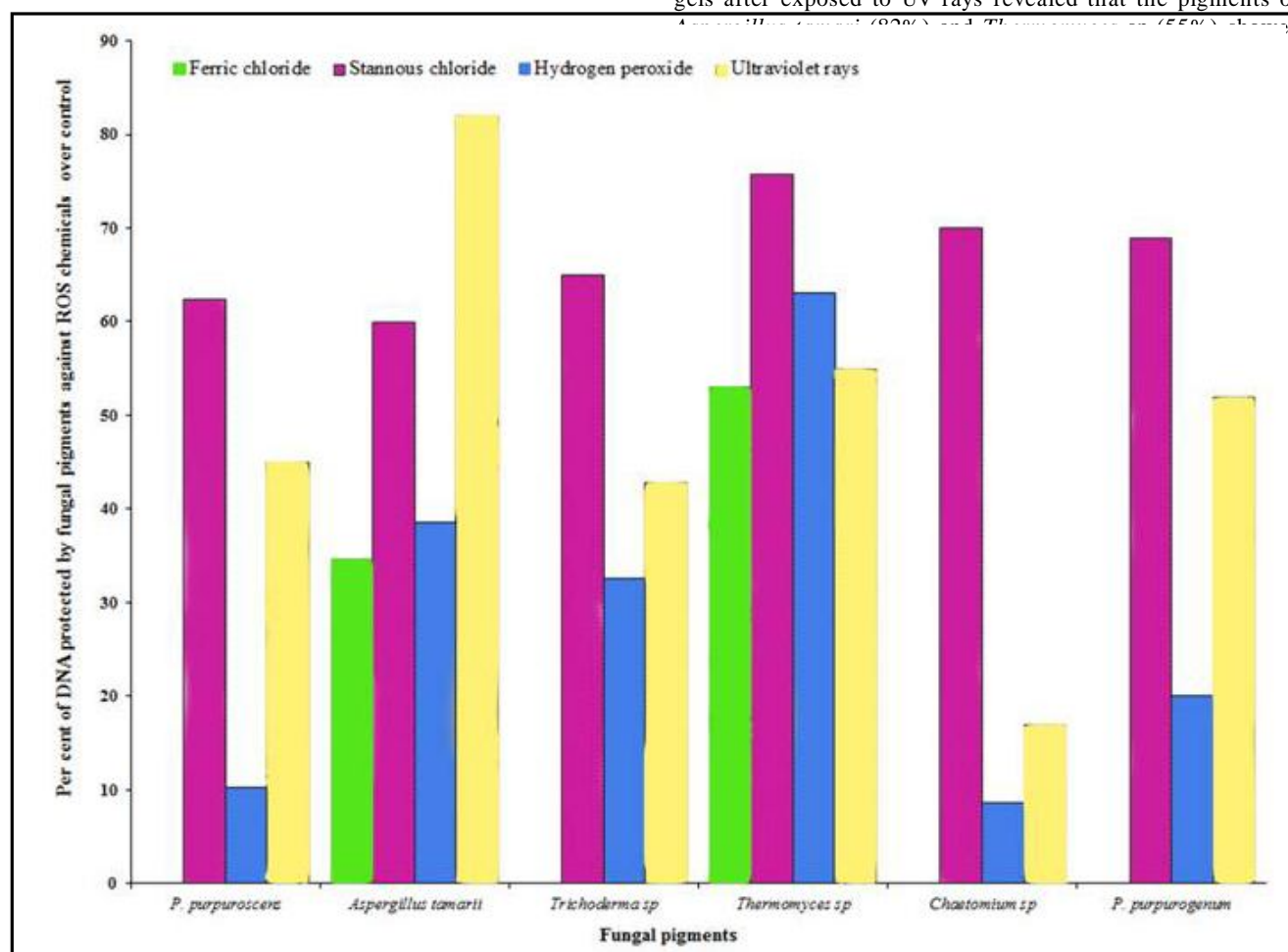


Figure 3: Percent inhibition of DNA damage by fungal pigments as quantified by densitometry of agarose gel.

4. Discussion

The present study was aimed to assess the feasibility of some of the fungal pigments for antioxidant properties against ROS. ROS are being produced in the human body during a variety of metabolic processes or penetrated directly into the body from the environment. During metabolism, oxygen consumption involves

the constant generation of the ROS and there are many enzymatic and non-enzymatic defense systems in the body to remove these toxic substances (Vinay Kumar *et al.*, 2013). It is essential to scavenge these ROS from the body to prevent the free radical-mediated diseases. Many dietary foods contain a wide variety of ROS-scavenging antioxidants. Search for natural antioxidants from

food, plant and microbial resources is the gained considerable attention in recent years as the synthetic antioxidant compounds have shown toxic and/or mutagenic effects (Mielnik *et al.*, 2003; Moreno *et al.*, 2006; Kuo *et al.*, 2006). Studies on numerous plant species have shown free radical scavenging or antioxidant activity due to the presence of flavanoids and other phenolic compounds as well as pigments like astaxanthin, zeaxanthin and lutein. However, the microbial resources for antioxidants have been less exploited (Arouma and Cuppett, 1997). The ascomycetous fungus, *Monascus pilosus* is the only fungus that has been exploited for antioxidant property (Kuo *et al.*, 2006).

Recently, microbial sources have been exploited for extraction of natural colourants for food and other industrial usage (Mapari *et al.*, 2009). Many of the microbial originated food colourants have multiple benefits, apart from serving as colouring agent. β -carotene pigment obtained from the algae, *Dunaliella salina* also serve as source for antioxidant, which has also shown suppresses the effects of premature of ageing caused by UV rays (Duffosse *et al.*, 2005). Similarly, astaxanthin, a red pigment from *Haematococcus* alga is a powerful quencher of singlet oxygen. Depending on the type of the compound, they serve different functions varying from a protective action against lethal photo-oxidation (carotenoids) to environmental stress (melanins) and acting as cofactors in enzyme catalysis (flavins) (Santocono *et al.*, 2006; Yadav *et al.*, 2017). Like plants and algae, filamentous fungi are also known for their pigments. However, their utilization for industrial processes is still unexplored. In the present study, we focused mainly for search of food colourants from different fungi that are isolated from forest ecosystems. We could isolate six different pigmented fungi from Indian forestry soils, yielding very high extractable pigments ranging from yellow, orange, red and brown these food colourants are added for value addition and appearance, and may also have other beneficial property, such as antioxidants. To evaluate the antioxidant property of these six fungal pigments, *in vitro* DNA protection assay was carried out against physical and chemical agents.

DPPH is a free radical, stable at room temperature and produces a violet solution in ethanol in presence of antioxidant compounds; the DPPH is reduced producing a non colour ethanol solution. It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate further oxidation (Dufosse *et al.*, 2005). In the present study, the scavenging activity of DPPH is at highest activity of fungal pigments in *Thermomyces* sp. pigment extract at the concentration of 20 mg/ml exhibited 69.63% inhibition, whereas the standard BHA at the same concentration could exhibit 84.25% inhibition. Likewise, in the reducing power assay, *A. tamarii* and *Thermomyces* sp. increased as extract concentration increased, indicating that the compounds present in fungal cultures were both electron donors and could react with free radicals and convert them into more stable products to terminate radicals chain interaction. In this experiment, 20 mg/ml concentration of *Thermomyces* sp. pigment showed higher antioxidant activities than control. The FRAP assay showed the antioxidant activity ranging from 0.52 to 7.14 $\mu\text{mol Fe}^{2+}$ equivalents per μl of sample. The methanolic extract of *Thermomyces* sp. pigment showed a significant ability to reduce Fe^{3+} (7.14 μM) at 20 mg/ml concentration, suggesting its ability to donate electrons. Scavenging of free radicals has been known as an established phenomenon in inhibition of lipid peroxidation which otherwise can be deleterious to cellular function. In the present

study, concentration- dependent scavenging activity was observed in all samples, with two fungal pigments, *viz.*, *Thermomyces* sp. (50.20%) and *Chaetomium* sp. (28.19%) showed a significant activity at a concentration of 20 mg/ml.

The chemicals like SnCl_2 , Fe^{2+} and H_2O_2 cause severe damage to DNA by induction of ROS, which has been demonstrated earlier in several studies either as whole cell (*E. coli*) or as circular DNA molecule (plasmids) (Dantas *et al.*, 1999; Felzenszwalb *et al.*, 1998; Reiniger *et al.*, 1999; Golla *et al.*, 2014). Similarly, the low wavelength UV rays also deform the DNA which could lead to alteration in DNA itself and also in nucleotides and bases. Further UV rays also induce oxidative stress because of production of ROS and modulate the level of antioxidants. The present study clearly revealed that the fungal pigments could also serve as potential antioxidants and could protect the DNA from damage by these agents (Perveen *et al.*, 2012; Radha and Padma, 2016). Among the four different agents namely SnCl_2 , H_2O_2 , Fe^{2+} and UV rays, Fe^{2+} induced damage was more severe than the other three agents. In presence of 250 mM of ferric chloride, the ROS completely degraded the DNA with trace observed in the respective lanes, whereas other three agents caused less damage to DNA by formation of nicking in the double strand. When these six different fungal pigments were pretreated with plasmid DNA, the degree of ROS induced DNA damage of plasmid DNA a vary significantly. Among the six different fungal pigments, yellow and orange pigments by *Thermomyces* sp. and *A. tamarii* other four pigments. The possible components responsible for DNA protection by *Thermomyces* are quinone and xanthone which were already reported. The fungal pigments present in the *A. tamarii* are riboflavin and asperxanthone. In the present study, *Aspergillus tamarii* and *Thermomyces* showed significant protection of DNA from damage caused by free radicals as revealed by densitometric analysis. The toxicological evaluation of yellow pigment from *Thermomyces* sp. in albino mice for 28 days has revealed no adverse effect due to intake of pigment (Poorniammal *et al.*, 2012). The pigments from *Monascus pilosus* has shown significant free radical scavenging activity when grown in garlic extract medium that could protect the calf thymus DNA in the presence of H_2O_2 and FeSO_4 (Kuo *et al.*, 2006). The pigments detoxify the ROS produced by $\text{FeSO}_4/\text{H}_2\text{O}_2$ complex. Similar studies conducted in different plant extracts, *i.e.*, *Chukrasia tabularis*, *Ginkgo biloba*, rosemary (*Rosmarinus officinalis* L.), oregano (*Origanum vulgare* L.), sage (*Salvia officinalis* L.) and echinacea (*Echinacea purpurea* L.), *Cedrela serrata*, *Koelreuteria paniculata* and *Brassica oleracea* also revealed the same (Moreno *et al.*, 2004 and Aherne *et al.*, 2007)

5. Conclusion

We have isolated five different pigments from six different fungi from forestry soil indicating that the soil fungi could be a potential resource for diversified pigments. The present investigation confirms that all the fungal pigments had antioxidant property and among them, *Thermomyces* (yellow) and *A. tamarii* (orange) pigments exhibited maximum free-radical scavenging potential. Further investigations are in progress in our laboratory to identify the active principle molecule involving in these antioxidants and exploring the pigments for food industry.

Conflict of interest

We declare that we have no conflict of interest.

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