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Evaluation of *In vitro* antioxidant activity of fungal pigments

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Abstract
Antioxidant and free radical scavenging ability of the six fungal pigments were assessed using different methods *viz.*, lipid peroxidation, reducing power, superoxide radical anion, hydroxyl radical, nitric oxide, metal chelation ability. The pigment extract exhibited a concentration-dependent radical scavenging activity in all the assays. The scavenging activity of pigment extract in the reducing power assay was found to be significantly higher than that in other methods. The scavenging effect of *Thermomyces* pigment extract on all the assays were very high followed by *Chaetomium* in all the concentrations.

Keywords: antioxidant, chaetomium, free radicals, fungal pigments, thermomyces

1. Introduction
Reactive oxygen species produced by ultraviolet light, ionizing radiation, chemical reactions, and metabolic processes have numerous pathological effects, such as causing lipid peroxidation, protein peroxidation, DNA damage, and cellular degeneration related to cardiovascular disease, ageing, cancer, inflammatory diseases, and a variety of other disorders. Free radicals are chemical entities characterized by a high reactivity. Free radical formation during the metabolism of xenobiotics is therefore an important mechanism employed by toxic agents in causing cellular damage. Reactive oxygen species (ROS) capable of damaging DNA, proteins, carbohydrates and lipids are generated in aerobic organisms. These ROS include superoxide anion radical, hydrogen peroxide, hydroxyl radical, and single molecular oxygen. The effects of these ROS are controlled by a system of enzymic and non- enzymic antioxidants. These antioxidants eliminate pro oxidants and scavenge free radicals [1].
Oxidative stress occurs as a result of an increase in oxidative metabolism, which produces a number of ROS. To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects [2]. High dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases such as heart diseases, cancers and cataracts. The fungal carotenoids are of considerable interest in nutrition because of their role as antioxidants and potential for preventing or delaying degenerative diseases and for enhancing immune responses in animals and humans [3]. Since carotenoids cannot be synthesized by animals, they must be supplied in their diet as colourants, as a source of vitamin A and their potential role as nutraceuticals [4]. A wide variety of fungi like *Penicillium roquefortii, Aspergillus candidus, Mortierella* sp., *Emericella falconensis* and *Acremonium* are known to produce fungal metabolites [5]. The present study was focused to assess the antioxidant potential of fungal pigments by different assays.

2. Materials and Methods
2.1 Preparation of extracts
Six extracellular pigment producing fungi namely *Penicillium purpuroscens, Aspergillus tamarii, Trichoderma sp. Thermomyces sp, Chaetomium sp* and *P. pupurogenum* were isolated from soil. The fungal cultures were inoculated on to potato dextrose broth and incubated at 35ºC for 5-7 days; supernatant was filtered through Whatman No1 filter paper, the filtrate were extracted with methanol and concentrated using vacuum rotary evaporator. The concentrated solutions were then lyophilized to get the dryness and stored at -20ºC until they were utilized for assays. The dried extract was used directly for analyses of antioxidant components or redissolved in deionised water to a concentration of 50 mg/ml and were then diluted to 5, 10, 15 and 20 mg/ml for further uses.

2.2 Lipid peroxidation activity
Antioxidant activity was determined by the conjugated diene method of Lingnert [6]. Each extract (5- 20 mg/ml, 100 µl), in deioinised water was mixed with 2 ml of 10 mmol/l linoleic acid emulsion (pH 6.6) in test-tubes and placed in darkness at 37 ºC to accelerate oxidation. After incubation for 0 h or 15 h, 0.1 ml of each tube was mixed with 7 ml of 800 ml/l methanol in deionised water and the absorbance of the mixture was measured at 234 nm, against a blank in a Varian spectrophotometer.
A value of 100% indicates the strongest antioxidant activity.

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

C – Control consist of solvent and the reagent solution
S - Pigment extract and the reagent solution

2.3 Reducing power assay
The reducing power was determined according to the method of Oyaizu [7]. Each extract (5–20 mg/ml) in methanol (2.5 ml) was mixed with 2.5 ml of 200 m mol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 10 mg/ml potassium ferri cyanide, and the mixture was incubated at 50º C for 20 min. After 2.5 ml of 100 mg/ml trichloroacetic acid were added, the mixture was centrifuged at 200g for10 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1ml of 1 mg/ml ferric chloride and the absorbance was measured at 700 nm against a blank. A higher absorbance indicates a higher reducing power. BHA is used for comparison.

2.4 Superoxide anion radical scavenging activity
The superoxide scavenging ability of the extract was assessed by the method of Nishimiki) [8]. The reaction mixture, containing pigment extract (5 -20 mg), PMS (30 µM), NADH (338 µM) and NBT (72 µM) in phosphate buffer (0.1 M pH 7.4), was incubated at room temperature for 5 min and the colour was read at 560 nm against a blank. The capability of scavenging the superoxide radical was calculated using the following equation:

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

2.5 Hydroxy radical scavenging activity
The assay was performed according to the method of Lioyd [9]. Various concentrations of extracts 0.6 mL in distilled water were taken in different test tubes. Iron-EDTA solution (0.8 mL; 0.021 % ferrous ammonium sulphate and 0.037 % ethylene diamine tetraacetic acid), ethylene diamine tetraacetic acid (EDTA, 0.4 mL; 0.022 %) and dimethyl sulphoxide (DMSO, 0.8 mL; 0.88% v/v in 0.1 mol/L phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.4 mL of 0.264 % ascorbic acid. Test tubes were capped tightly and heated in a water bath at 80–90 ºC for 15 min. The reaction was terminated by the addition of 1 mL of ice-cold TCA (17.5% w/v).
3 mL of Nash reagent (75.0 g of ammonium acetate, 3 mL of glacial acetic acid, and
2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for colour development. The intensity of the yellow colour formed was measured spectrophotometrically at 412 nm against reagent blank.

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

2.6 Nitric oxide radical scavenging activity
Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [10-11]. Three ml of 10 mM sodium nitroprusside in phosphate buffer was added into two ml of each extract and the reference compound in different concentrations (5, 10, 15 and 20 mg/ml). The resulting solutions were then incubated at 25 ºC for 60 min. The similar procedure was repeated with methanol as a blank which served as control. To 5 ml of the incubated sample, 5 ml Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was measured using spectrophotometer (Varian) at 546 nm. All tests were performed in triplicate. Percent inhibition of the nitricoxide generated was measured by comparing the absorbance values of control and test preparations.

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

2.7 Chelating ability on ferrous ions
Chelating ability was determined according to the method of Shimada [12]. To 2 ml of the mixture consisting of 30 mM hexamine, 30 mM potassium chloride and
9 mM ferrous sulphate were added each extract (5–20 mg /ml) in methanol (2 ml) and 200 µl of 1 mM tetramethyl murexide After 3 min at room temperature, the absorbance of the mixture was determined at 485 nm against a blank.

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

3. Results and Discussion
Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart diseases. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins; lipid or DNA to initiate regenerative diseases [13]. There are number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine could be the main factors for the observed efficacy of these foods in reducing heart diseases and sore cancer [14].
The methanolic pigment extract of *Thermomyces* sp expressed a significant ability to reduce Fe^{3+} (7.14 mM/µl) at 20 mg/ml concentration and thus confirmed 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 [15].
Lipid peroxidation is the most commonly used method for determining antioxidant activity to measure the inhibiton of linoleic acid. The antioxidant activities of the fungal pigments

increased with increased concentration of the pigment extracts of *Thermomyces* sp. and *Chaetomium* sp., which had exhibited higher antioxidant activities than other fungal pigments (Table 1). At 5 mg/ml, *Thermomyces* sp. and *Chaetomium* sp. exhibited 16.34 % and 12.13 % of antioxidant activity respectively, whereas *A. tamarii* showed antioxidant activity of 8.31 % at 5 mg/ml. *Trichoderma* sp., *P. purpuroscens* and *P. purpurogenum* pigment extracts recorded low antioxidant activities. However, antioxidant activity of BHA was 43.57, 45.39, 64.49 and 72.35 % at 5, 10, 15 and 20 mg/ml respectively.

Table 1: Inhibition of lipid peroxidation of methanolic extracts of fungal pigments (% Inhibition)

Pigment concentration (mg/ml)	*P. purpuroscens*	*M.ruber*	*M. purpureus*	*Thermomyces* sp	*Chaetomium* sp	*P. purpurogenum*	BHA
5	10.78 ± 0.31	8.31 ± 0.24	10.47 ± 0.07	16.34 ± 0.94	12.13 ± 0.35	3.72 ± 0.11	43.37 ± 1.25
10	11.43 ± 0.33	12.35 ± 0.36	15.23 ± 0.15	21.65 ± 0.19	14.15 ± 0.41	6.43 ± 0.19	45.39 ± 1.31
15	15.05 ± 0.43	14.60 ± 0.42	21.31 ± 0.24	37.69 ± 0.30	23.82 ± 0.69	15.12 ± 0.29	64.49 ± 1.86
20	17.07 ± 0.49	16.21 ± 0.47	32.17 ± 0.29	50.20 ± 0.53	28.19 ± 0.81	22.32 ± 0.36	72.35 ± 2.09

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

The rate of increase was found to be high for the pigment extract of *Thermomyces* sp and low for the pigment extract of *Trichoderma* sp. At 5 mg/ml concentration, reducing power of the pigment extracts of *Thermomyces* sp. was in the range of 0.08 to 1.01.

At 20 mg/ml, the reducing power of methanolic extracts were in the range of 0.34 to 1.64. However, reducing power of BHA were 1.41 at 5 mg/ml and as the concentration increases it followed the same pattern as methanolic extracts of *Thermomyces* sp. (Table 2). From this it could be inferred that antioxidant properties were associated with development of reducing power as described by Tanaka [16].

Table 2: Reducing power activity of methanolic extracts of fungal pigments

Pigment concentration (mg/ml)	*P. purpuroscens*	*M.ruber*	*M. purpureus*	*Thermomyces* sp	*Chaetomium* sp	*P.purpurogenum*	BHA
5	0.23 ± 0.05	0.10 ± 0.02	0.88 ± 0.16	1.01 ± 0.06	0.59 ± 0.10	0.33 ± 0.01	1.41 ± 0.02
10	0.29 ± 0.12	0.18 ± 0.01	0.95 ± 0.13	1.23 ± 0.10	0.52 ± 0.05	0.37 ± 0.12	1.45 ± 0.03
15	0.29 ± 0.02	0.24 ± 0.02	1.53 ± 0.12	1.43 ± 0.05	0.56 ± 0.01	0.45 ± 0.01	1.63 ± 0.01
20	0.34 ± 0.29	0.41 ± 0.03	1.64 ± 0.15	1.5 ± 0.07	0.67 ± 0.02	0.47 ± 0.14	1.74 ± 0.02

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

In superoxide radical assay, superoxide are produced from molecular oxygen due to oxidative enzymes [17] of body as well as *via* non enzymatic reaction such as autooxidation by catecholamines. The activity of *Thermomyces* sp. at the concentration of 20 mg/ml was 87.87 %. The other pigment extracts exhibited the inhibition in the range of 8.43 % to 73.94 % (Table 3). The present study revealed that *Thermomyces* sp showed highest scavenging activity of superoxide compared to other fungal cultures.

Table 3: Superoxide anion radical scavenging activity of methanolic extracts of fungal pigments (% inhibition)

Pigment concentration (mg/ml)	*P. purpuroscens*	*M.ruber*	*M. purpureus*	*Thermomyces* sp	*Chaetomium* sp	*P.purpurogenum*	BHA
5	16.43 ± 0.71	17.75 ± 0.85	21.43 ± 0.39	35.05 ± 0.63	27.64 ± 0.27	19.66 ± 0.80	66.83 ± 0.8
10	21.34 ± 0.61	29.51 ± 1.33	39.21 ± 0.58	48.54 ± 0.44	35.13 ± 1.33	25.53 ± 0.35	75.23 ± 0.29
15	24.89 ± 1.11	32.23 ± 1.48	45.41 ± 0.49	54.77 ± 0.32	56.43 ± 0.89	33.49 ± 0.77	81.79 ± 0.13
20	32.90 ± 0.98	33.94 ± 2.18	64.07 ± 1.63	67.87 ± 1.20	64.34 ± 1.72	44.41 ± 1.63	89.04 ± 0.59

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Hydroxyl radical produced may cause sugar fragmentation, base loss and leakage of DNA strand [15, 18]. Hydroxyl radicals are the major ROS causing lipid peroxidation and enormous biological damage [19]. *Thermomyces* sp methanolic extracts scavenge off these free radicals and hence inhibit cellular damage. It exhibited a maximum of 78.56 % activity at 20 mg/ml followed by *A. tamarii* (64.08%) > *Chaetomium* sp. (41.13 %) > *P. purpurogenum*
(33.81 %) > *P. purpuroscens* (14.66%) > *Trichoderma* sp. (13.46 %) (Table 4). It is apparent from the present study that the *Thermomyces* sp pigment not only scavenges off the free radical but also inhibits the generation of free radicals.

Table 4: Hydroxyl radical scavenging activity of methanolic extracts of fungal pigments (% inhibition)

Pigment concentration (mg/ml)	*P. purpuroscens*	*M.ruber*	*M. purpureus*	*Thermomyces* sp	*Chaetomium* sp	*P.purpurogenum*	BHA
5	5.04 ± 0.26	5.69 ± 0.22	20.21 ± 0.28	24.72 ± 0.25	13.63 ± 0.23	11.63 ± 0.11	65.44 ± 0.19
10	6.93 ± 0.09	8.91 ± 0.18	28.61 ± 0.56	32.49 ± 0.19	21.78 ± 0.14	14.84 ± 0.07	70.80 ± 0.19
15	8.90 ± 0.11	12.02 ± 0.15	40.99 ± 0.12	53.59 ± 0.21	36.81 ± 0.15	27.83 ± 0.41	82.26 ± 0.51
20	14.66 ± 0.12	13.46 ± 0.19	54.08 ± 0.51	68.56 ± 0.23	41.13 ± 0.31	33.81 ± 0.89	96.40 ± 0.21

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological processes [20]. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals [17]. In the present study, the pigment extract competes with oxygen to react with NO and thus inhibited generation of the anions. At 20 mg/ml concentration the pigment extract of *Thermomyces* sp. exhibited 45.61%

inhibition where as BHA standard exhibited 81.58 % of inhibition (Table 5).

Table 5: Nitric oxide anion radical scavenging activity of methanolic extracts of fungal pigments (% inhibition)

Pigment concentration (mg/ml)	*P. purpuroscens*	*M.ruber*	*M. purpureus*	*Thermomyces* sp	*Chaetomium* sp	*P.purpurogenum*	BHA
5	13.97 ± 0.22	17.32 ± 0.20	27.95 ± 0.35	36.65 ± 0.13	18.74 ± 0.34	13.22 ± 0.19	57.41 ± 0.29
10	17.72 ± 0.17	17.80 ± 0.14	34.20 ± 0.25	38.26 ± 0.03	22.75 ±0.42	18.44 ± 0.19	60.21 ± 0.12
15	18.86 ± 0.16	18.92 ± 0.09	36.46 ± 0.37	39.16 ±0.36	25.49 ± 0.83	25.22 ± 0.12	78.44 ± 0.16
20	20.59 ± 0.10	20.12 ± 0.06	37.34 ± 0.30	45.61 ± 0.27	26.49 ± 0.26	29.40 ± 0.15	81.58 ± 0.10

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 6: Metal chelation activity of methanolic extracts of fungal pigments (% inhibition)

Pigment concentration (mg/ml)	*P. purpuroscens*	*M.ruber*	*M. purpureus*	*Thermomyces* sp	*Chaetomium* sp	*P.purpurogenum*	EDTA
5	4.72 ± 0.3	3.47 ± 0.2	14.25 ± 0.82	28.53 ± 1.6	12.46 ± 0.7	18.43 ± 1.1	62.12 ± 3.6
10	6.17 ± 0.4	3.89 ± 0.2	16.23 ± 0.90	36.23 ± 2.1	15.12 ± 0.9	10.12 ± 0.6	74.78 ± 4.3
15	6.78 ± 0.4	5.12 ± 0.3	21.01 ± 1.20	45.94 ± 2.7	23.91 ± 1.4	11.28 ± 0.7	82.43 ± 4.8
20	13.17 ± 0.8	8.27 ± 0.5	23.01 ± 1.3	56.37 ± 3.3	26.32 ± 1.5	16.47 ± 1.0	95.78 ± 5.5

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Iron and copper are essential transition metal elements in the human body required for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O_2 transport and redox reactions. Unfortunately, they contain unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions [9].

With this assay, *Thermomyces* sp showed strong Fe^{2+-} chelating activity even at the minimal concentration of 20 mg /ml. Its chelating rate was 56.3 %. Since ferrous ions are the most effective prooxidants in the food system, high chelating abilities of methanolic extracts from various monascal products would be beneficial [21].

4. Conclusion

The present study concludes that the fungal pigment extract exhibited antioxidant activity due to the presence of bioactive natural compounds. Furthermore, active compounds present in the pigment are being subjected to purification process for identification of active compound which may provide a better source for developing new pigment or colourants from fungal sources. These compounds have potential application as antioxidants and food colourants in food and beverage industries.

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