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SCREENING OF RADICAL SCAVENGING EFFICACY OF MELANIN ISOLATED FROM *ASPERGILLUS FUMIGATUS*

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ABSTRACT

Production of melanin is one of the most universal, but at the same time enigmatic adaptations of living organisms to the variable conditions on the earth. The presence of various kinds of melanin in representatives of almost every large taxon suggests an evolutionary importance of melanogenesis. Melanin could be a cause to induce virulence as well as enhancing the survival capability of the fungus under stress, leading to its success as a cosmopolitan species. This study was designed to appraise the antioxidant potential of melanin from *Aspergillus fumigatus*. Melanin was isolated from its conidial forms by alkaline extraction and acid hydrolysis. An initial antioxidant assay with respect to DPPH and ABTS radicals was carried out following which a detailed antioxidant screening assays were performed that includes valuation of peroxides, hydroxyl radicals, oxides, superoxides etc.,. This report delineating the antioxidant potential shows that the melanin extracted gives scientific basis for its use as potent antioxidant and favours antioxidants from biological samples to have a better advantage of applications. The slight increase in the activity shown thus favours antioxidants from biological samples to have a better advantage of applications when compared to samples synthetically or chemically obtained. This in turn reveals that the lipid soluble defence antioxidant also may have a role to play in the survival of the fungus in immune-compromised hosts.

KEYWORDS: **Melanin; Antioxidant; DPPH; ABTS; fungi; Scavenging efficacy.**

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INTRODUCTION

Many diseases are caused or affected by free radicals. In such cases, the natural defence of the organism against these free radicals is seen to be insufficient, thus, explicating the important role played by antioxidants in the protection of the organism against these radicals. Synthetic antioxidants were seen to be highly used for scavenging purposes but due to their carcinogenicity a search for effective natural antioxidants is on the rise[1]. Natural antioxidants include a wide variety of molecules such as phenolic compounds, vitamin, terpenoids, nitrogenous compounds, and endogenous metabolites[2,3]. Melanin synthesis is associated with the virulence for a variety of pathogenic microbes[5] and as a pigment it is found to contribute to the reduction in the hosts susceptibility by reducing the host antimicrobial activity[4]. Ironically, the biggest problem is the lack of a validated assay that can reliably measure the antioxidant capacity of biological samples. Several reviews have been published, and the opinions vary considerably and there seems to be no consensus of opinions mainly due to the use one-dimensional method to evaluate multifunctional biological antioxidants. Also, antioxidant assays differ from each other in terms of substrates, probes, reaction conditions, and quantitation methods. It is extremely difficult to conclude the antioxidant potential by compare the results from different assays[8]. In the meantime, new assays claiming to measure antioxidant capacity continue to be reported[7]. A general testing protocol validates by properly choosing a biologically relevant substrate; test various oxidation conditions; measure both initial and secondary oxidation products; compare antioxidants at the same molar concentrations of active components; and quantify on the basis of induction period, percent inhibition, or rates of hydroperoxide formation or decomposition, or IC50 (antioxidant concentration to achieve 50% inhibition)[6].The present study is undertaken targeting the effect of melanin enhancing the survival of *Aspergillus fumigatus*, mainly due to its function as a redox buffer by neutralizing oxidants produced by the host system. Therefore, a detailed antioxidant validation was carried out so as to infer the antioxidant potential with respect to the melanin produced by its conidial forms.

METHODS AND MATERIALS

EXTRACTION OF MELANIN

A. fumigatus (NCBI Genebank Accession Number JX041523), grown for 5 days on SDA slants or plates, was taken and the conidia was collected by adding 5ml of sterile Phosphate Buffered Saline (PBS) of 1X concentration (8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g of KH2PO4, pH 7.4, Distilled Water 1000ml) and scraped gently using a sterile loop. The conidia were then subjected to centrifugation at 8000g for 30min followed by washing in PBS thrice. A final wash was done using 1M Sorbitol and 0.1M Sodium citrate (pH 5.5). 5 µl macerozyme, as the cell lysing enzyme (Himedia; from *Rhizopus spp*), was added at 10mg/ml and incubated overnight at 30ºC to generate protoplasts. The protoplasts were collected by centrifugation and washed thrice by PBS and left overnight in 4.0M Guanidine thiocyanate (Himedia) at room temperature. The dark particles were collected by centrifugation at 5000g for 10 min followed by washing thrice using PBS. These were further treated using the Reaction buffer (10.0mM Tris, 1.0mM CaCl2 and 0.5% SDS, pH 7.8) with 10µl of 10mg/ml Proteinase K and incubated at 37ºC. Debris was washed three times with PBS and boiled in 6.0 M HCl for 90 min. After treatment by boiling in acid, melanin particles were collected by filtration through Whatmann paper (Grade 2) and washed extensively with distilled water at 2 hour intervals for 2 days. The pH of distilled water that is used to wash the crude melanin was checked using Methyl orange for the pH comes close to 7 indicating complete removal of the acid, lyophilized and used as required.

SAMPLE PREPARATION

The synthetic melanin samples and the sample extracted from *Aspergillus fumigatus* were diluted to 25µg/ml, 50µg/ml and 100µg/ml respectively. Each of these samples was subjected to Standard Antioxidant assays

(DPPH Radical Scavenging Assay, ABTS Radical Scavenging Assay, Hydrogen Peroxide Scavenging Assay, Hydroxyl Radical Scavenging Assay, Determination of Nitric Oxide Generation, Determination of Superoxide Generation, Reduction Potential and Phosphomolybdenum Method). All the Standard Antioxidant Assays was checked for percentage inhibition or scavenging activity by the following formula:

$$Scavenging\ Activity = \frac{[Absorbance\ of\ Standand - Absorbance\ of\ Sample] \times 100}{Absorbance\ of\ Sample}$$

ANTIOXIDANT ACTIVITY

(i) DPPH Radical Scavenging Assay[9]

500µl of 1.0mM 2,2-diphenyl picryl hydrazyl (DPPH) in methanolic solution was added to 100µl of each of the concentrations of the melanin extracts. DPPH in methanolic solution was used as positive control and methanol was used as blank. After 30min, the discolouration from deep violet to yellow colour was measured at 515 nm in a spectrophotometer.

(ii) ABTS Radical Scavenging Assay[11]

2-2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) solution (2.45mM Ammonium persulphate in 7mM ABTS solution) was kept in the dark for 12-16 hours at room temperature. 50µl of melanin samples (Synthetic and Extracts) was added to 300µl ABTS solution and the final volume was made upto 1ml using ethanol. After 5 minutes the absorbance was read at 745 nm.

(iii) Hydrogen Peroxide Scavenging Assay[10]

40mM of H2O2 was prepared in Phosphate Buffer (pH 7.4). 50µl of each concentration of melanin sample was added to 100µl of H2O2 solution and made upto 3ml using 0.1M Phosphate Buffer. The solution containing Phosphate Buffer without H2O2 acted as blank.The absorbace of the reaction mixture was recorded at 230nm and percentage inhibition was calculated.

(iv) Hydroxyl Radical Scavenging Assay[13]

The reaction mixture contained 100µl each of 2.8mM Deoxyribose, 0.1mM EDTA, 1.0M H2O2, 0.1 mM Ascorbate and 20mM KH2PO4-KOH buffer of pH 7.4. 50µl of melanin samples was added such that the total volume was made upto 1ml. The reaction mixture was incubated for an hour at 37ºC after which 500µl each of 70% ethanol and 1% Thiobarbituric Acid was added. The mixture was kept in boiling water bath for 20 min and cooled. The pink colour developed was measured at 535nm.

(v) Determination of Nitric Oxide Generation[12]

Reaction mixture of 300µl of 100mM Sodium Nitroprusside,20µl of melanin samples was added to Phosphate Buffered Saline (pH 7.2) and made it upto 1ml. This was further incubated at 25ºC for 150min. Control was maintained without the melanin sample. After incubation 500µl of the reaction mixture was taken and added to an equal volume of Griess Reagent (1% Sulphanilamide, 2% Orthophosphoric Acid, 0.1% N-(1-naphthyl)-ethylene diamine hydrochloride). The absorbance was checked at 546nm.

(vi) Determination of Superoxide Generation[14]

The reaction mixture consisted of 200µl of 0.1M EDTA containing 1.5mg NaCN per 100ml,100µl of Nitroblue Tetrazolium, 50µl of 0.12mM Ribolflavin, 50µl of sample and made upto 3ml with Phosphate buffer. All the tubes were vortexed and the optical density was measured at 560nm. The tubes were then placed in area of uniform illumination for 30 min. The optical density was again measured at 560nm. The difference in optical density before and after illumination gives the generation of superoxide by the test sample and was calculated by comparing with the optical density of control.

(vii) Reduction Potential[10]

The reduction potential is determined on the basis of ability of antioxidant to form a coloured complex with potassium ferricycnide, TCA and ferric chloride.100µl of the different concentrations of the sample was mixed with

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2.5ml of 1% Potassium Ferrricyanide and 2.5ml of Phosphate Buffer (pH 6.6). The mixture was then made upto 6ml by using distilled water and incubated at 50ºC for 20min. After the incubation, 2.5ml of 10% Trichloro Acetic acid was added and mixed thoroughly. To 2.5ml of this reaction mixture equal amount of water was added and 0.5ml of 0.1% Ferric Chloride was added to it. The absorbance of the colour was measured spectrophotometrically at 700nm.

(viii) Phosphomolybdenum Method[15]

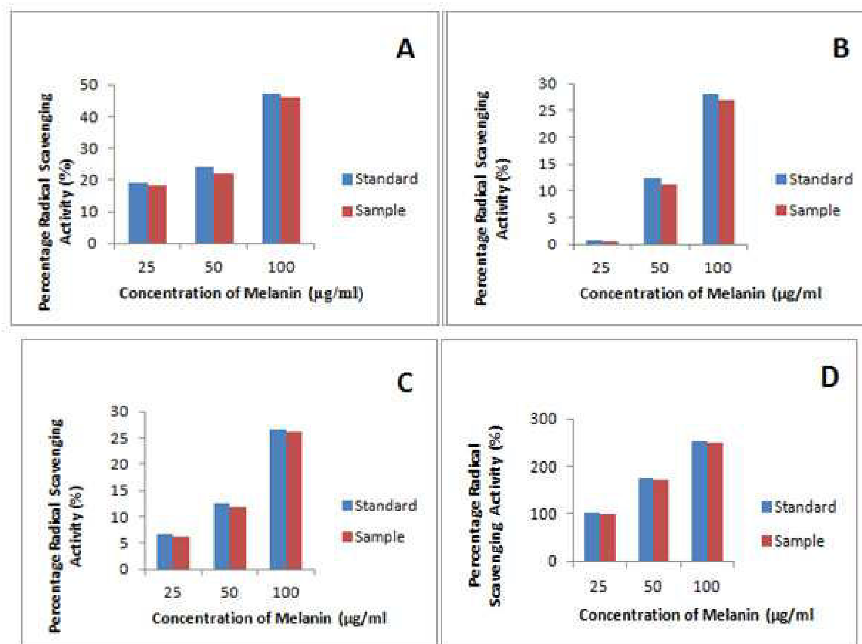
This assay is used to determine the antioxidant capacity based on the reduction of molybdenum ions by the antioxidant compound and the formation of green reduced product at acidic pH. The tubes containing 50µl of samples at different concentrations and 2ml each of reagent (0.6M H2SO4, 28mM Na2PO4 and 4mM Ammonium Molybdate) and distilled water were incubated at 95ºC for 90 min. After the mixture is cooled to room temperature, the absorbance of each solution was measured at 695nm against blank and the scavenging activity was determined.

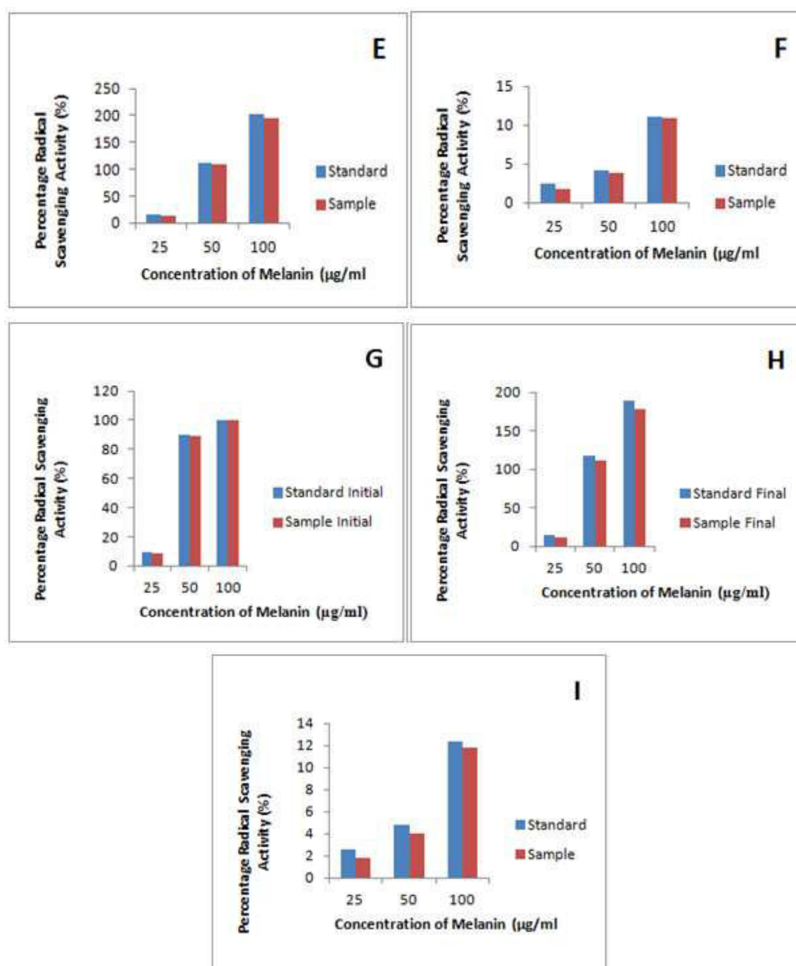
RESULTS AND DISCUSSION

In vitro free radical scavenging efficacy of extracted melanin from *A. fumigatus* was assessed by its ability to scavenge DPPH, ABTS, Reduction Potential, Nitric Oxide (NO), Phosphomolybdenum Method, H_2O_2, Superoxide radicals and Hydroxyl radicals (Figure 1) by illustrating an comparative analysis of percentage radical scavenging activity using synthetic melanin at each of the respective concentrations. These standard antioxidant assays conducted as a comparative analysis with respect to the standard synthetic melanin

Graph 1

Efficacy of (A) DPPH Radical Scavenging Assay; (B) ABTS Radical Scavenging Assay; (C) Reduction Potential; (D) Nitric Oxide Scavenging activity; (E) Phosphomolybdenum Method; (F) Hydrogen Peroxide Radical Scavenging Assay; (G)& (H) Superoxide Radical Scavenging Activity; (I) Hydroxyl Radical Scavenging Activity





DPPH radical[16] and ABTS radicals are commonly used substrate for fast evaluation of antioxidant activity because of its stability in the radical form and simplicity of the assay. This assay is known to give reliable information concerning the antioxidant ability of the tested compounds[17]. It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions directly competing with oxygen in the reaction with NO[18]. It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions.H_2O_2 is highly important because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells[19]. Hydroxyl radicals are most reactive species, initiating the peroxidation of the cell membrane[20]. The lipid radical, thus generated would initiate a chain reaction in the presence of oxygen, giving rise to lipid peroxide, which break down to aldehydes such as malondialdehyde.With respect to all the above discussed parameters, an initial study of DPPH and ABTS radical scavenging ability of all concentrations of extracted melanin compared to synthetic melanin was conducted. These results showed significant activity and the comparison showed that the activity of the extracted melanin from *Aspergillus fumigatus* was on par with that of synthetic melanin. In order to conduct a detailed study, the antioxidant capability of the extracted biopolymer was extended with respect to the remaining radicals that also revealed similar

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results. The presence of antioxidant activity in varying degrees in all the concentrations used, the extracted sample did show results of being slightly more than that of synthetic samples. This may be due to the extraction from a biological source (fungi), in turn confirming that the samples that were isolated may be protein bound.

CONCLUSION

The melanin isolated from *Aspergillus fumigatus* had a higher scavenging rate of ·OH, DPPH· and $O^{2-·}$, which indicated it has a strong antioxidant activity. Although melanins, as biopolymers, are said to play a crucial role in the absorption of free radicals generated and protecting the host from UV light, further studies are required to clarify the relationship between biological activities and structure of this melanin. On the basis of the results obtained in the present study, antioxidant scavenging efficacy of melanin extracted from *A. fumigatus* gives scientific basis for its use as potent antioxidant to the individuals under oxidative stress such as inflammation.

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