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Characterization of Methanolic Extract of Red Pigment from *Penicillium purpurogenum* and its Antioxidant Activity

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The fungal pigments are a good alternative to currently used synthetic colourants and / or natural colourants derived from plant materials. An extracellular red pigment producing fungus, *Penicillium purpurogenum* was isolated from soil collected from Parambikulam Tiger Reserve Forest, Kerala. In the present study antioxidant assays *viz.*, Lipid peroxidation, hydroxyl radical and Nitric oxide radical scavenging assays were carried out with fungal red pigment. The pigment extracted from this fungus showed maximum inhibition of lipid peroxidation (30.15 %), hydroxyl radical (74.92 %) and nitric oxide radical scavenging activity (43.23%) at 20 mg ml^{-1} of pigment concentration. The red pigmentfrom *P. purpurogenum* was separated by (TLC) yielding three major fractions, *viz.*, Pinkish red, orange and yellow fractions. These fractions were further identified through Gas chromatography and Mass Spectrometry (GC-MS). The identification of the structure of the red pigment was detected using FT-IR spectra , and indicated that the presence of phenolic and quinone compounds and has broad stretching OH,C=C and C-H groups of the aromatic ring.

Keywords: Red pigment, *Penicillium purpurogenum,* antioxidant activity, FT-IR, natural food colourants.

The use of natural dyes in food has increased recently due to the marketing advantages with the development of natural ingredients and the consumer concern about the harmful effects of synthetic pigments on health[1]. Pigments are derived from natural sources such as plants, insects, and microorganism. There has been much interest in the development of new natural colourants for use in the food industry owing to strong consumer demand for more natural products. The production of many existing natural colourants of plant origin has a disadvantage of dependence on the supply of raw materials, which are influenced by agro-climatic conditions – in addition, their chemical profile may vary from batch-to-batch. Moreover, many of the pigments derived from the contemporary sources are sensitive to heat, light, and oxygen, and some may even change their colour in response to pH changes as in case of anthocyanins[2]. Many ascomycetous fungi naturally synthesize and secrete pigments and may thus provide a more reliable source for natural, "organic" food colourants with improved functionalities[3]. The diversity of fungal pigments is not only found in their chemical structures but also in the colour range of these pigments that may add new or additional hues to the colour palette of the existing colourants derived from contemporary sources[4].

Reactive oxygen species (ROS) are formed as a result of normal metabolic activity (oxidative stress) and from exogenous sources. ROS formed often results in causing cellular and subcellular damage by preoxidation of membrane lipids, denaturation of DNA strands and cellular

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proteins. In multicellular organisms, cell protects itself from the damage caused by the ROS system by various enzymatic pathways which includes catalase, glutathione peroxidase, glutathione reductase, superoxide dismutase, lipid peroxidise and non enzymatically by beta carotene, vitamin A, C, E[5]. To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects[6]. High dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases such as heart diseases, cancers and cataracts. The fungal pigments 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[7]. Recently, there is growing urge to discover natural antioxidants from microorganisms and microbial pigments are generally safer than the chemical and synthetic formulations. The pigments produced by *Penicillium* and *Monascus* (monascorubrine and monascuscorubramine) are structurally similar. Lovastatins or monacolins produced by *Penicillium, Monascus*, *Aspergillus* and *Rihzopus* inhibits cholesterol biosynthesis by binding to catalytic site of HMG-CoA reductase a key enzyme in cholesterol biosynthesis and scavenged DPPH radicals[8,9,10,11]. In the present study we report the antioxidant potential and characterization of fungal red pigment extracted from *P.purpurogenum*.

MATERIALS AND METHODS

Microorganism and culture conditions

The microorganism used in this study was isolated from soil, collected from Tiger reserve Parambikulam, Kerala, India. Stock cultures were maintained on potato dextrose agar (PDA) slants at 4ºC after being incubated at 30ºC for 5-7 days. PP-O production medium was used as fermentation medium to assess the pigment production. PP-O production medium containing 20 g of soluble starch and 2 g of yeast extract per liter of 50 mM citric acid/Na_3 citrate buffer with pH 5.0.

Extraction and estimation of red pigment yield

Pure culture of *P. purpurogenum* from PDA slants was transferred into 250 ml Erlenmeyer flasks containing 100 ml of the growth medium. After cultivation of 5-7 days, about 1 ml of the culture broth was dissolved with 5 ml of 90 % (v/v) methanol. The solvent and sample were kept on a rotary shaker at 200 rpm for 1 h, allowed to stand for 15 min and filtered through Whatman filter paper (47 mm). The clear supernatant was collected and after dilution, absorbance of red pigment was measured at 500 nm using Hitachi U-2000 spectrophotometer (M/s. Hitachi Ltd., Tokyo, Japan). The absorbance values were converted into pigment units using by the following formula[12] :

$$\text{Pigment yield (colour value units ml}^{-1}\text{)} = \frac{\text{OD} \times \text{Dilution} \times \text{Total volume of solvent}}{\text{Amount of sample (ml)}}$$

Antioxidant activity

Lipid peroxidation, Hydroxy radical scavenging and nitric oxide scavenging antioxidant power assays were used to determine antioxidant activity of red pigment by *P. purpurogenum* that was carried out by following standard method [13-15].

Characteristics of red pigment

Thin layer chromatography (TLC)

Pigments were detected by thin-layer chromatography using silica gel plates and developed in n-butanol:aceticacid:water (12:30:50 v/v)) as a mobile phase and determined their Rf values. The Rf values is a mathematical representation of the ratio of the distance travelled by the solvent[16]. After TLC separation, the plates were kept in room temperature for drying. The spots in the TLC plates were detected using UV light.

Detection of chemical groups by Gas chromatography and mass spectrometry (GC-MS)

GC-MS analysis was carried out to identify the pigment compounds present in the TLC separated bands by using DB-5 column with a length of 30m (M/s. Perkin Elmer - Clarus 500). Helium used as a carrier gas with flow rate at 1 ml min^{-1}.

Structure determination of pigment by Fourier Transformer Infra Red (FT-IR) Spectroscopy

To confirm the structure of fungal red pigment, FT-IR spectrometer (M/s. Impact 400D, Nicolet, Madison,WI) was used to measure the infrared spectra of extract solution in the wave number of 400-4000 cm^{-1} at room temperature. For each IR spectrometer samples 32 scans at 4 cm^{-1} resolution was collected in the transmittance mode.

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 might oxidize nucleic acids, proteins; lipid or DNA to initiate regenerative diseases. Estimation of Lipid peroxidation is the most commonly used method for determining antioxidant activity to measure the inhibition of linoleic acid. Scavenging of free radicals has been known as an established phenomenon in inhibition of lipid peroxidation which otherwise can be deleterious to cellular function. The antioxidant activities of the fungal pigment increased with increasing the concentration of the pigment extract of *P. purpurogenum* (Fig. 1). At 5 mg ml^{-1}, *P. purpurogenum* exhibited 14.40 per cent of antioxidant activity. However, antioxidant activity of standard BHA was 41.25, 43.37, 65.42 and 70.05 per cent at 5, 10, 15 and 20 mg ml^{-1} respectively. These results are in accordance with the literature data of Tseng *et al.* (2006)[17].

The hydroxyl radical is one of the representative reactive oxygen species generated in the body system. Hydroxyl radical produced may cause lipid peroxidation, sugar fragmentation, base loss and leakage of DNA strand[18,19]. The results of this assay revealed that the red pigment extract at the concentration of 20 mg ml^{-1} were considerably more active. The pigment extract was found to possess the hydroxyl radical scavenging activity in a dose dependent manner. It exhibited a maximum of 74.92 per cent activity at 20 mg ml^{-1} (Fig. 2). It is apparent from the present study that the *P. purpurogenum* and pigment not only scavenges off the free radical but also inhibits the generation of free radicals.

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[21]. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals[22]. The nitric oxide radical scavenging activity of the fungal pigment extract was less than that of BHA, which was used as standard for all the assays. At 20 mg ml^{-1} concentration the pigment extract of *P. purpurogenum* exhibited higher activity of 43.23

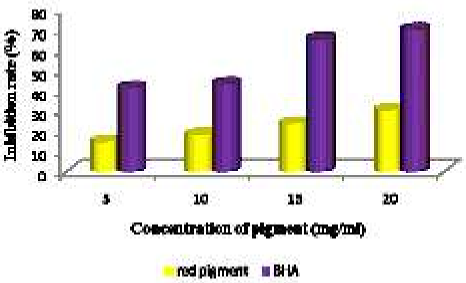

Fig. 1. Lipid peroxidation activity of red pigment

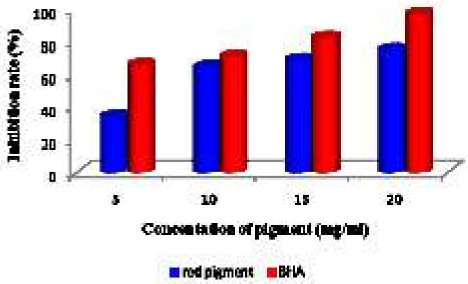

Fig. 2. Hydroxy radical scavenging ability of red pigment

Table 1. TLC of methanolic extracts of red pigment

Pigment fraction	Colour of the pigment fraction	Distance travelled (cm)	Rf value (cm)	Absorption maximum (λ max)
1	Pinkish red	10.3	0.79	500
2	Orange	11.5	0.88	420
3	Yellow	12.2	0.93	420

per cent of inhibition. Whereas BHA standard recorded 81.58 per cent of inhibition (Fig. 3).

Identification of fungal pigments by TLC, GC-MS and FT-IR

In the present study, best separation of the red pigment compounds was achieved with the mobile phase consisting n-butanol:aceticacid:water (12:30:50). The pigment fractions of *P. purpurogenum* were separated in to 3 bands (pinkish red, orange and yellow) whose Rf values and ë$_{max}$ were presented in Table 1. Close agreement was obtained between absorption maxima of these fractions and Rf values. The results are in accordance with the earlier reports[23, 24].

GC-MS studies were performed to identify the pigment compounds present in the TLC separated bands of methanolic extract of *P. purpurogenum* culture. The peak was obtained for red, orange and yellow of TLC separated bands from *P. purpurogenum* in GC-MS. The compound present in the red band have the retention time of 31.65 min. The given library match for this compound may be 3-(9a-methyl-3-octanyl-2,9,dioxo-2, 7,9,9a-dihydro-2H-furo[3,2-g]glisochromen-6yl)-acrylic acid. It has the formula $C_{23}H_{26}O_7$ established from GC-MS (Fig. 4). For the orange and yellow bands in GC-MS, the given library match for these compounds may be 6-ethyl-5 hydroxy-2,3,7-trimethoxy napthoquinone and 2-tert-butyl-4-isopropyl-5-methylphenol respectively (Fig. 5 and 6). The results are similar to who reported that *Penicillium* sp. AZ has been found to produce PP-O when cultured in a medium composed of soluble starch and yeast extract at pH 7. They reported that presence of an azaphilone skeleton in PP-O pigment[25]. *Penicillium purpurogenum* has been found to produce Monascus pigment homologs in culture with a specific medium [26]. This strain does not produce citrinin (mycotoxin),

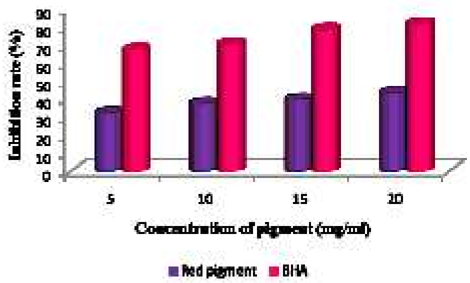

Fig. 3. Nitric oxide scavenging ability of red pigment

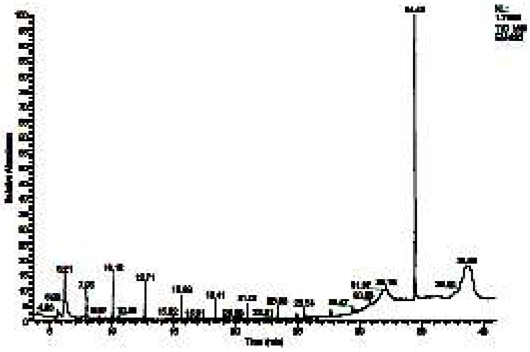

Fig. 4. GC-MS chromatogram of band 1 of crude extract of *P. purpurogenum*

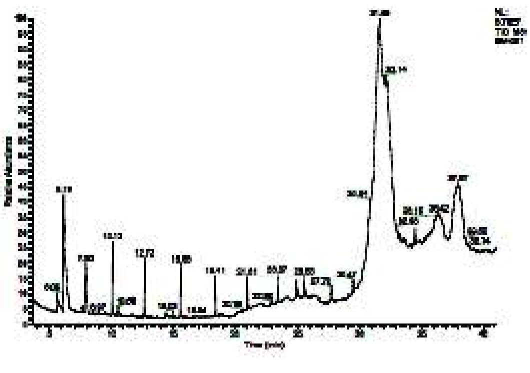

Fig. 5. GC-MS chromatogram of band 2 of crude extract of *P. purpurogenum*

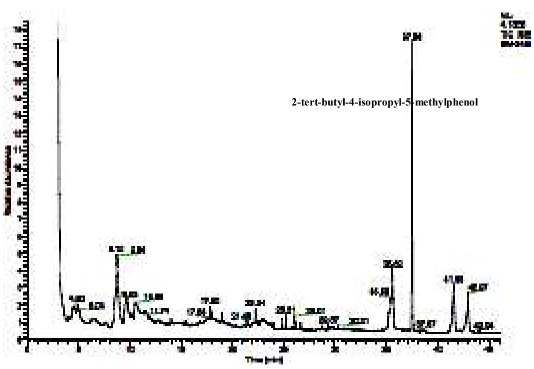

Fig. 6. GC-MS chromatogram of band 3 of crude extract of *P. purpurogenum*

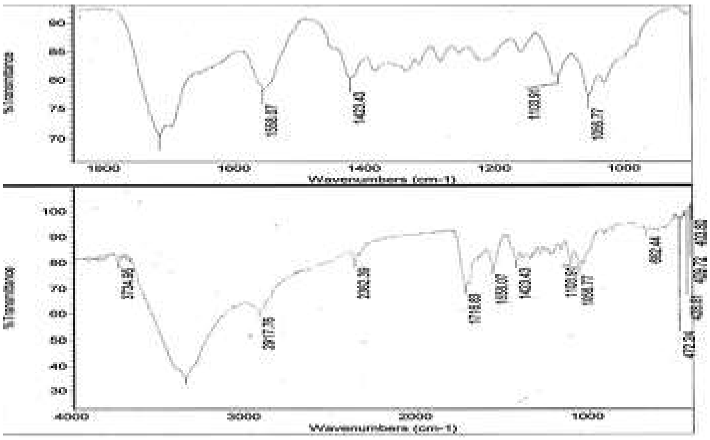

Fig. 7. FT-IR spectrum of *P. purpurogenum* pigment extract

making *P. purpurogenum* a potentially valuable commercial source of natural food colourant.

FT-IR spectroscopy has widely been used for the characterization and identification of fungi, bacteria and yeasts which are hydrophilic microorganisms and can easily be suspended in water for sample preparation[27]. In the present study, the infra red spectra of red pigment showed the presence of hydrogen bonded OH groups at 3247 cm^{-1}. The carbonyl stretching vibration frequency of the pigment is in the region of 1716 cm^{-1} for red pigment (Fig. 7). The observed stretching frequencies are however close to phenol and quinones. The aromatic C=C stretching observed at 1423 and 1558 cm^{-1} for red pigment, Similar results were also reported by Dhale and Vijay-Raj (2009) in *Penicillium* sp[28]. The IR spectrum showing broad stretching at 3379 cm-1 for hydroxyl group of phenolics. In the IR spectrum stretching frequency were also observed at 1643 and 1414 cm^{-1} assignable to C=C and C-H of the aromatic ring respectively.

CONCLUSION

In recent years, production of natural food colourants through microbial fermentation is an extensive area of investigation, since they overcome concerns of unfavorable side effects by synthetic colours. In this study, under *in vitro* condition red pigment efficiently inhibits lipid peroxidation, scavenged free radicals and hydroxyl radical scavenging activity exhibited 30.15 and 74.92 per cent inhibition at the concentration of 20 µg ml^{-1}. Identification of compounds in methanolic extract of red pigment from *P. purpurogenum* shown that the presence of phenols and quinones. This study reveals that fungal pigments are a better source of natural pigment and antioxidants than synthetic dyes, therefore, the investigations of the antioxidant activity of natural products have created new ways for drug development to reduce the usage of synthetic drugs.

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