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Evaluation of Antioxidant Properties of A Pigment Molecule Isolated from Radioresistant Bacterial Strain Inm-2

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ABSTRACT

Moleculesoffers radioresistantto an organism can be exploited as radioprotective drug for the human uses. Bacterial pigment assumed to be one of these molecules as itpossesses antioxidant properties.Present investigation was carried out to isolate a radioresistant bacteria (INM-2) and evaluation of its antioxidant properties. We have found that isolated bacterium wasradioresistant as it survived under high doses of gamma radiation i.e. 7.5 -10 kGy. Antioxidant properties of pigmentextracted from INM-2 were analysed through reducing power assay, DPPH and lipid peroxidation assay.Results of the study showed that isolated pigment could inhibit free radicals 30 to 35%. It can be assumed from the results that pigment isolated from bacterium (INM-2) having good free radical scavenging properties and may be one of the molecules which contribute to its radioresistant property.

KEYWORDS : Radioresistant, Antioxidant, DPPH, Antihemolytic, Radioprotective

INTRODUCTION

Ionizing radiation induced damage to DNA, oxidize proteins, lipids and other biomolecules via generation of free radicals (ROS) leads to cell death (Burg *et al.,* 2006). Free radicals adversely affect biomolecules and contribute to several human diseases as well as ageing (Harman and Gerontol 1956, Halliwell and Gutteridge 1997). Because>80% proportion of water in living matter radiolytic product of the water, mainly hydroxyl radicalsare responsible for damages to biomolecules (Walden *et al*., 1990). Hydroxyl radicals cause damages to biomolecules by extracting an electron from the biomolecules or by formation of the double bonds of aromatic moieties (Spothem-Maurizot*et al*., 2008). Ionizing radiation known to create strand breaks in cellular DNA, as well as other types of lesions in the chromatin of cells (American Joint Committee on Cancer 2010). Similarly, radiation producesperoxy radicals by peroxidation of lipids resulted in functional and structural damage to the cellular membranes (Rayleigh *et al.,* 1987). In order to reduce free radical damage,endogenous antioxidants in the human body counteract the harmful effects of free radicals(Moulisha*et al*., 2010). Current interest is being focused on the potential role of antioxidants in the treatment and prevention of atherosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus, and several others diseases (Ajitha and Rajnarayana, 2001).

Although, mammalian system is very sensitive to ionizing radiation but some microorganism shows extremely high resistant against lethal doses of ionizing radiation and can survive againstmegarad doses of gamma radiation. Probably, these novalradioresistantmicrobial species may have better adaptability to harsh environment (Kumar *et al*., 2010a). Logically, these organisms may have inbuilt resistant against radiation. These radioresistant bacteria may have developed a mechanism to synthesize some specific biomolecules which protect them from lethal radiation(Kumar *et al*., 2010).

Some prominent examples of radioresistant bacteria are*Deinococcusradiodurans, Bacillus sp. INM-1, rubrobacterradiotolerens*.A semiquinoneglucoside derivative (SQGD) was isolated from a radioresistant bacterium *Bacillus* sp. INM-1 (Kumar *et al*. 2010a) , possessing strong antioxidant activities and can effectively neutralize radiation induced free radicals in biological system (Kumar *et al.,* 2010a).

Bacterial pigments play a major role in photosynthesis. Besides photosynthesis, pigments have some other functions ranging from antibiotic activities (Nakamura *et al.,* 2003), UV protection (Tong and Lighthart 1997) and virulence (Liu and Nizet 2009) and anti-freezing agents.Carotenoids can inhibit various types of cancer and it enhances the immune responses (Guerin *et al*., 2003). These pigments are capable of quenching photosensitizers; interacting with singlet oxygen (Krinsky*et al*., 1994) and scavenging proxy radicals (Conn *et al*., 1992). Due to their antioxidant activity and pro- vitamin A function,they also protect from cardiovascular disease and age related macular degeneration (Steven *et al.* 2000). During present investigation, we have tried to findoutantioxidant properties of the bacterial pigment isolated from a radioresistant bacterium INM-2 which can be a contributing factortowards its radiotolerance capabilities.

MATERIAL AND METHODS

Soil samples collection

A 100 gsurface (upper 5 cm) soilsamples were collected using sterile scoop in a sterile container from various locations of Chavera beach Kerala India. Storage and transportation of the soil samples was carried outat 4°C.

Isolation and screening ofradioresistantbacteria

5% w/v soil suspension was prepared in 10 ml of sterile deionisedwater. Soil suspension wasvortexes thoroughly and allowed to settle. Serial dilutions of samples were made with sterile water and 100 µl soil suspensionswerespread on solidified NAM (Nutrient Agar Media) and incubated at 37±2° C for 24 h. Single bacterial colonies were pickedup and streaked on NAM plates repeatedly to achieve pure culture. Plates were than incubated at 37±2° C for a period of 24 h and then subjected for their radioresistant properties.

Evaluation of radioresistant potential of isolated bacterium

To evaluate the radioresistant potential, isolated bacterium INM-2 was exposed to various doses (2.5, 5.0, 7.5 and 10 kGy of gamma radiation. Just after irradiation, broth was centrifuged at 4500 rpm for 10 minutes at 20°C and the pellet was re-inoculated in a fresh nutrient broth and incubated for 24h at 37°C. After incubation, broth (100ml) wasspread on NAM plates and incubated for 24h at 37°C. The colonies appeared on the NAM plates was considered to be radioresistant and maintained for further testing.

Isolation of yellow-orange coloured pigment from radioresistant bacterium INM-2

Fresh culture (24h old) of the INM-2 was centrifuged at 4500 rpm for 15 minute at 20°C. Supernatant discarded and pellet was washed in PBS two times and then mixed with 1ml PBS and sonicated (40hrz; 10 cycles) for 2.0 minutes. Supernatant containing crude extract was separated and pellet containing coloured cells debris were used to extract pigment. Methanol (100%) was used to extract pigment from the pellet until it get colourless i.e., complete pigment extraction has been achieved (Sasidharan*et al.,* 2013). The solvent containing pigment was centrifuged at 10000 rpm for 15 minutes at 20°C. The bacterial cell pellet was then discarded while the coloured supernatant (concentrated pigment) was transferred onto glass petri-dishes and vacuum dried.

Estimation of reducing power of bacterial pigment

The activity of reducing power was assayed using method described by Oyaizu (1986). A mixture of 0.02M phosphate buffer (pH-6.5), 1% potassium ferricyanide in equal volumes and varying pigment concentrations in the range of 10-2000 µg/ml was used to estimate the reducing power of the pigment. The monitoring bioassay was performed in two steps. Initially 200 µl of 10% TCA was added to each sample and centrifuged at 300xg for 10 minutes. 0.1% ferric chloride (100 µl)was added into collected supernatant. The resulted mixture (final volume 5 times of pre-aqueous phase) was re-incubated at 37°C for 10 minutes and the absorbance was recorded at 700 nm. An increase in absorbance was indicative of increased reductive power.

Estimation of antioxidant activity (electron donation potential) of bacterial pigment using DPPH Assay

To estimate the electron donation potential of isolated pigment DPPH assay was performed(Chen *et al.* 2013). A stock solution of extract was prepared in water and stirred for 5.0 minutes with the help of stirrer at room temperature. 1.0 ml of DPPH (100µM) was added to the different concentration ranging from 10-2000 µl of extract which made up to 1 ml by methanol. The mixture was then incubated at RT in the dark. The absorbance of the reaction mixture was recorded at 517nm and percent inhibition of free radical scavenging activity was calculated as follows:

$$\% \text{ Inhibition} = 1 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Estimation of lipid peroxidation inhibition by isolated bacterial pigment

The lipid peroxidation assay was performed as suggested by Devasagayam *et al.* (2003).

C57bl/6 mice, 4-8 week old (weighing 25-30 g)fed on chow diet and was sacrificed through cervicaldislocation method. Brain tissue excised, weighed and 10% homogenate in 0.1 M PBS (pH-6.6) was prepared using homogenizer and used as a source of lipid.

The assay was performed with varying concentrations of drug ranging from 25 to 2000 µg/ml concentration. 250 µl tissue (brain) homogenate was added to each tube and volume made up to 1 ml with PBS. Samples were incubated for 30 minutes at 37 °C and then exposed to 250 Gyradiation. Irradiated samples were incubated for 1h at 37 °C. 100 µl volume of TCA (10%) and 100 µl TBA (0.675% w/v) was added to each tube and incubated at 100 °C for 1 h. Samples were centrifuged at 300 rpm for 5 minutes and absorbance was recorded at 534 nm. Percent Inhibition was calculated using following formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Antihemolytic Assay

The membrane stabilizing activity of the pigment was assessed using erythrocyte suspension (5 % RBC solution in PBS) (Srour *et al.*, 2000). To prepare erythrocyte suspension whole blood was obtained using syringes (containing anticoagulant EDTA) from a healthy human after taking prior consent. The blood was centrifuged and the blood cells were washed three times with PBS through centrifugation at for 10 minutes at 3000xg. Varying concentration of drug/pigment were mixed with 5% RBC suspension and incubated for 30 minutes at room temperature. Samples were exposed to radiation at 100Gy dose and then incubated 2-3 at RT.After that samples were centrifuged at 3000 rpm for 2-3 minutes and absorbance of supernatant was recorded at 415 nm.

The percent inhibition of either hemolysis or membrane stabilization was calculated using the following Formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

RESULTS AND DISCUSSION

Isolation and screening of bacteria

Bacteria were isolated from the soil samples collected from the extreme radiation environment of Chavera beach Kerala, India. Four colonies were isolated and irradiated to find out the radioresistant potential. A yellowish orange bacteriumnamed INM-2with smooth appearance was found radioresistantupto 10 kGy of gamma irradiation.

Evaluation of radio resistantpotential of isolated strain INM-2

Bacterial broth was radiated at different doses (5-10 kGy) of gamma radiation and then checked for its radioresistance potential by spreading on Nutrient Agar Plates. Theappearance ofbacterialcolonies on culture plates reflected radioresistantof the bacterium upto 10 kGy irradiation. Higher number of colonies 552 at 7.5 kGy(Fig. 1, a) appeared on lower dose as compared to that with higher dose of 10 kGy where 72 colonies were appeared on plate (Fig. 1, b).It can be inferred that selectedstrainINM-2was capable of surviving at high radiation and carried significant resistance against gamma radiation. Our results were in conformity with*Deinococcusradiodurans,* a well-knownradioresistant bacterium, whichcould grow continuously in presence of chronic radiation (60 Gy/h) and survivedin gamma radiation environment exceeding 15 KGy (Anderson *et al.,* 1956, Makarova*et al.,* 2001).

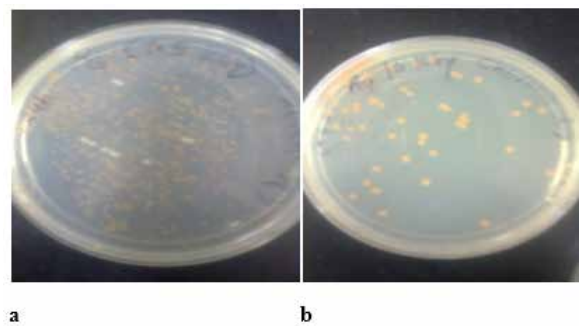

Fig.1Growth of INM-2 bacterium upon irradiation at 7.5 kGy (a) and 10kGy (b) radiation doses

Antioxidant activity of bacterial pigment in terms of its reducing power capability

Reducing power of pigment was observed as a function of its concentration. Reducing power was found to increase with increasing concentration of pigment. Higher absorbance of the reaction mixture indicates higher reductive potential. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*., 1995; Mishra et al. 2013, 2014).

In the present investigation, a concentration dependent reducing ability of isolated pigment was observed. Pigment reflected a substantial increase in reducing power between concentration range 500-2000 µg/ml(Fig. 2).Reducing power found to be increase as the concentration of the isolated pigment was increases.These observations indicated that some compounds in the pigment acting as electron donor and could react with the free radicals to terminate radical chain reaction.

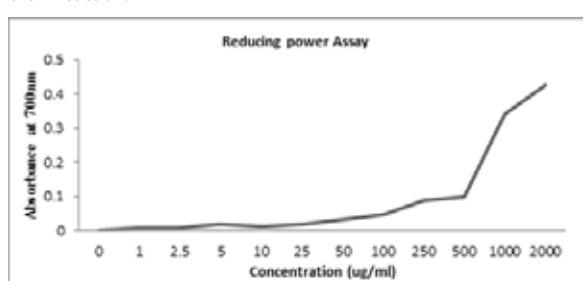

Fig. 2Reducing power of bacterial pigment isolated from radioresistant bacterial strain INM-2

Antioxidant activity of pigment in terms of its electron donation ability

DPPH is a stable free radical, when an antioxidant molecule react with DPPH, it extract an electron from it and becomes paired off. Therefore, bleaching of the DPPH colourstoichiometrically depends on number of electrons taken up from the antioxidant molecule (Soares *et al.*, 1997). Pigment showed 28%inhibiton in DPPH oxidation at maximum

concentration of i.e. 2000 µg/ml (Fig. 3).Analysis of DPPH assay exhibited that as the pigment concentration increases, optical density of the samples decreases which indicates dose dependent scavenging property of isolated bacterial pigment.

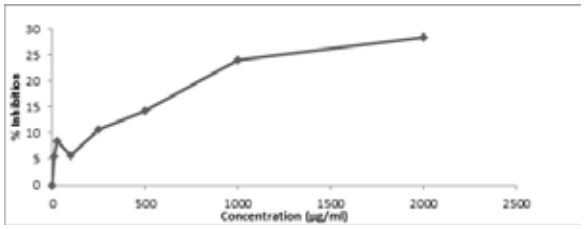

Fig. 3DPPH neuterilizing activity of isolated radioresistant bacterial pigment

Anti-lipid peroxidation potential of bacterial pigment

Several concentrations of pigment ranging from 25-250 µg/ml were tested for its antilipid peroxidation activity. An inhibition of 34.66 % has been observed (radical scavenging) at the concentration of 250µg/ml (Fig. 4). Inhibition was found to be more prominent at higher concentrations as compared to lower concentrations. The highly reactive hydroxyl radicals can damage biological membranes by oxidizing polyunsaturated fatty acid moieties of the cell membrane phospholipids resulted lipid hydro-peroxide generated(Valentao *et al.*, 2002). Lipid hydro-peroxide known to decomposed to produce alkoxy and peroxy radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, induction of cancer and aging related diseases (Riemersma *et al.*, 2000). Thus, inhibition of free radicals with the increasing concentration of the pigment justified its role in maintenance in redox homeostasis in bacterial itself during oxidative stress (Kumar et al, 2010b; Mishra et al. 2013, 2014).

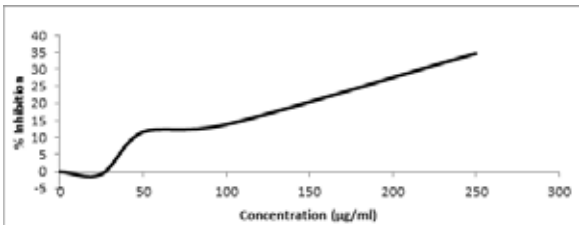

Fig. 4Anti-lipid peroxidation activity of isolated bacterial pigment

Antihemolytic activity of bacterial pigment

Bacterial pigment was analysed for its anti-hemolyticactivity. RBC treated with pigment showed no reduction in hemolysis, though a little reduction was observed at very low concentration which was almost negligible. So it is concluded that the radiation induced damage to RBC cannot be protected by pigment pre-treatment, however low concentrations provided some level of protection, but higher concentrations were found to be unsuccessful in providing desired shielding effect against gamma radiation.

CONCLUSION

The present study was undertaken to evaluate the survival capacity in presenceof radiation and inherent antioxidant activity of the bacteria. Antioxidants prevent damage to biological systems by neutralizing the free radicals interactively and synergistically. So, from various antioxidant activity assays, it was found that the bacterial pigment isolated from bacterial strain INM-2carried moderate level of free radical scavenging activities.Therefore, this pigment can be used to neutralize gamma radiation induced oxidative stress. Though, it is preliminary study, more detailed study pertaining to the chemical characterization of bacterial pigment and its probable biological activity determination will unravel its futuristic applications.

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