Assessment of Antioxidation potential, Lipid peroxidation, and cytotoxicity of microbial pigment

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Abstract:

Bacterial pigments have found many applications in everyday life. Nontoxic nature of pigment produced by number of microorganisms makes them environmental friendly for utilization in dye, food, pharmacy, cosmetic and other industries. The current study involves isolation of Yellow (Zeaxanthin), Orange (β - carotene) and Pink (Canthaxanthin) pigment produced by *Staphylococcus aureus, Dunaliella salina and Monascus roseus* respectively and assessment of antioxidant potential by DPPH assay, Lipid peroxidation bb TBARS, cytotoxicity by MTT assay and DNA damage by Comet assay. The results of DPPH assay exhibited 81, 77 and 75% radical scavenging for Yellow, Orange and Pink by respectively. Lipid peroxidation by TBARS indicated that pigments efficiently lowered the MDA production in goat lever cells, MTT assay proved the non-cytotoxic nature of pigments to the tune of 92, 95 and 91% .

Introduction:

Ores, insects, plants and animals have been the source of natural pigments used as colorants since prehistoric period. In the mid -19th century synthetic dyes substituted

the natural pigments and are still in demand in spite of their toxicity, environment, humans, animals hazards (1). Toxicity problems of synthetic dyes have led to intense research in microbial pigment (2).Microbial pigments both algal and bacterial have promising applications in food, pharmaceutical, dyeing industry because of their bright colors, biodegradability and environmental compatibility (3).

The current stud y involved isolation of two bacterial and a microalgal species from soil and water samples. These bacteria were identified as *Staphylococcus aureus, Monascus roseus and Dunaliella salina* on the bases of their morphology, colony characters biochemical tests and the color of their pigment produced. Yellow (Zeaxanthin), Pink (Canthaxanthin) and Orange (β - carotene) pigment produced by *Staphylococcus aureus, Monascus roseus and* respectively.

These pigments not only add a bright coloration to the food and make it palatable but also act as radical scavengers and lower the oxidative stress. The sedentary lifestyle and improper eating habits generate free radicals in our body (4) these radicals in turn increase the chances of occurrence of diseases such as cancer, cardiovascular, diabetes and autoimmune diseases (5) . Antioxidants are used to overcome the oxidative stress by neutralizing free radicals (6).

The antioxidant potential of the pigments is evaluated by DPPH assay followed by Lipid peroxidation assay by TBARS (Thiobarbituric acid reactive substances) utilizing goat liver cells. Since the study suggests ingestion of pigments as antioxidants, the cytotoxicity and genotoxicity is also assessed.

Methodology:

Screening of pigment producing microorganisms:

The soil and water samples were collected from the college campus 18^0.150663 and

74.576782 with a GPS coordinates of 18^0 9′ 2.3868″ N and 74^0 34′ 36.4152″ E. The soil sample was serially diluted in 0.87% NaCl. 10^{-5} to 10^{-7} dilutions were plated on Luria - Bertani (LB) agar. The plated were incubated at 37^0C for 24 hours. After 24 hours of incubation, to induce pigmentation the plates were incubated at 42 ^{0}C till the pigmentation developed. Colony characters, morphology, motility Gram staining and biochemical test were performed.

Alternatively the 2ml water sample was inoculated in 1 liter beaker containing growth medium (500ml tap water , 0.01N KNO 3, 0.1N K2HPO4, 0.1N KH 2PO4, 5N NaCl) the beaker was exposed to 8 hour sunlight and incubated for 3 weeks with weekly microscopic observation.

Extraction of Pigment:

The pigment producing microorganisms were mass cultivated in 1 liter flask containing 500ml LB Broth incubated at 40^0C on shaker incubator with 90rpm for 3 days and/ or till pigmentation. Cells were pelleted using centrifugation at 300g, 15 minutes at 4^OC.

Pellet was washed twice in minimal salt solution and suspended in 50ml saline. The cells were lysed in saline using homogenizer. The homogenate was mixed in equal volume buffered dichloromethane (Equilibrated in Tris Cl buffer, pH =7.4) and kept in separating funnel overnight. The lower organic phase containing pigments was collected and its λmax was determined using Jasco 630 double beam UV -Visible spectrophotometer.

The radical scavenging DPPH assay:

The scavenging reaction between (DPPH•) and an antioxidant (H -A) can be written as:

Antioxidants (Microbial pigments) react with DPPH•, which is a stable free radical and is reduced to the DPPH -H and as consequence the absorbance decreased from the DPPH• radical to the DPPH -H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. DPPH radical scavenging activit y from all plant extracts was measured by taking 100µl sample, 900µl of acetate buffer (pH 6.5) and 3ml freshly prepared 100µM DPPH solution in methanol.

Reagent blank was 1ml buffer and 3ml DPPH solution. The absorbance was measured after 90 min of incubation in dark at 517 nm. DPPH radical scavenging activity (%) was determined by following equation:

DPPH radical scavenging activity (%) = Ab-As/Ab X 100

Lipid Peroxidation [TBARS Assay]:

In mammalian tissues, malondialdehyde (MDA) originates from oxidative degradation of polyunsaturated fatty acids (PUFAs) with more than two unconjugated double bonds. The main precursors of MDA are arachidonic acid (20:4) and docosahexaenoic acid (22:6). Two moles of TBA reacts with one mole of MDA, to form pink reaction product (λ max = 532nm), which is readily extractable in organic solvent such as butanol , and can be measured by spectrophotometerically .

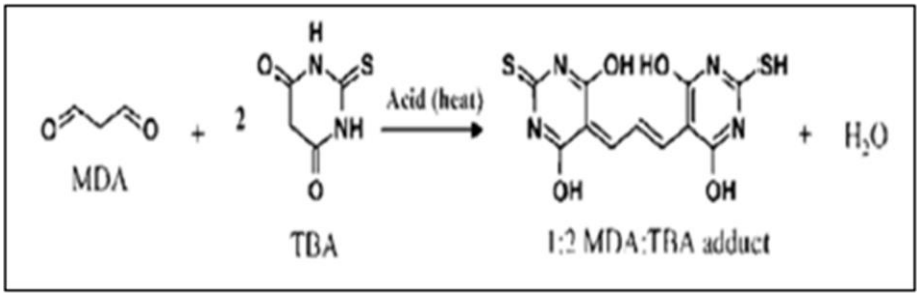


FIG 1: MDA reacts with TBA to form pink color

Erythrocyte ghosts were prepared by the standard procedure and diluted in PBS buffer (pH 7.2, 37ºC), so as to get a concentration of 106 ghosts/µl. The RBC ghosts were fractionated as depicted in Table 1

Test Parameters	RBC ghosts	FeCl3	Pigments
Positive control	100µl	--	--
Negative control	100µl	5mM	--
Test	100µl	5mM	40 µl[1mg/ml]

TABLE 1: TBARS ASSAY ADDITION OF REAGENTS

Samples were incubated at 37ºC for 30 minutes, so as to allow the formation of MDA. The so formed MDA was allowed to react with thiobarbituric acid which in turn produced 1:2 MDA: TBA adduct having pink color which can be measured by spectrophotometer at 532nm. In practice, TBARS are expressed in terms of malondialdehyde (MDA) equivalents. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted.

Cell Viability test [MTT assay]:

Goat liver was procured from the local slaughter house aseptically in 250ml sterile chilled phosphate buffered saline. It was chopped to fi ne pieces with the help of sterile scissors in vertical laminar air flow. The chopped pieces were incubated with equal amount of TPVG for 45min in Orbitech rotary shaker at 37oC at 105 rpm. 10% Serum was added to inhibit the action of trypsin after the completion of incubation. The cells were centrifuged at 3250 rpm for 10min; the supernatant was subjected to Neubaur's Chamber for cell counting. Fifteen to twenty thousand cells were seeded in 96 well plates in 40µl of PBS, incubated on rotary shaker for 2 hours at 37oC at 95 rpm. 10µl, 20µl and 40µl of pigment were added in each well followed by addition of 10µl MTT reagent [Yellow tetrzolium salt]. The plates were incubated in Orbitech rotary shaker at 37^{o}C at 100 rpm for 4 hours. After the incubation 10µl of lysis solution [20% SDS in 50% DMSO] was introduced in each well. The plates were further incubated at 37^{o}C at 100 rpm to solubilize the purple coloration developed by conversion of Tetrzolium salt. The O.D was recorded using Eliza reader at 492nm and % cell viability determined by:

$$\%\text{viability} = \frac{\text{Mean absorbance of sample}}{\text{Mean absorbance of control}} \times 100$$

Results:

FIGURE 1: BACTERIAL COLONIES SHOWING PIGMENTATION

Colony characters:

Bacteria name	size	shape	color	texture	opacity	elevation	margin	motility
Staphylococcus aureus	2mm	circular	yellow	mucoid	opaque	convex	Entire	Non motile
Monascus roseus	2mm	circular	pink	smooth	Opaque	convex	Entire	motile

Table no. 1: Colony character of bacteria

Biochemical tests:

1. ***Staphylococcus aureus:***

TESTS ORGANISMS	*Staphylococcus aureus*	*Monascus roseus*
1.Catalase	Positive(+ve)	Positive(+ve)
2.Oxidase	Negative(-ve)	Negative(-ve)
3.Coagulase	Positive(+ve)	--
4.Glucose fermentation	Positive(+ve)	Positive(+ve)
5.Arabinose	Negative(-ve)	--
6.Mannose	positive(+ve)	--
7.gelatin hydrolysis	--	negative(-ve)
8.nitrate reduction nitrite	--	Positive(+ve)
9. Arginine Dihydrolase	--	Negative(-ve)

DPPH assay:

The radical scavenging assay revealed that the pigment have good antioxidant potential as seen in graph Zeaxanthin, B – carotene and canthaxanthin have antioxidant potential of 82%, 77% and 75% respectively

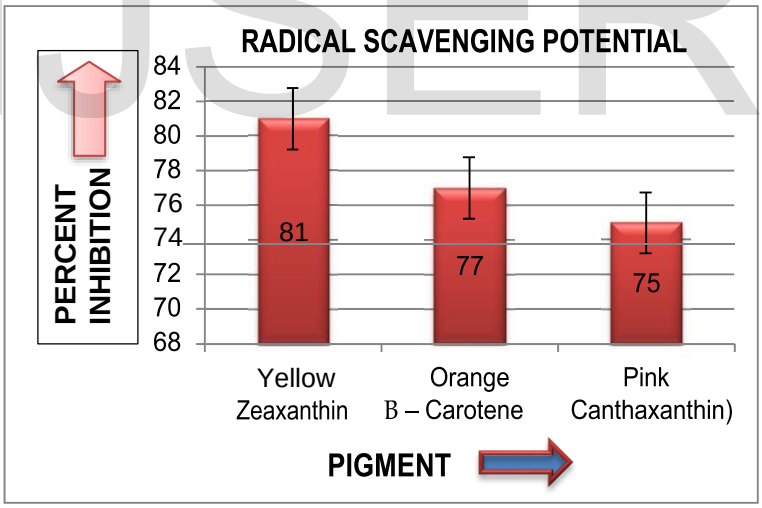
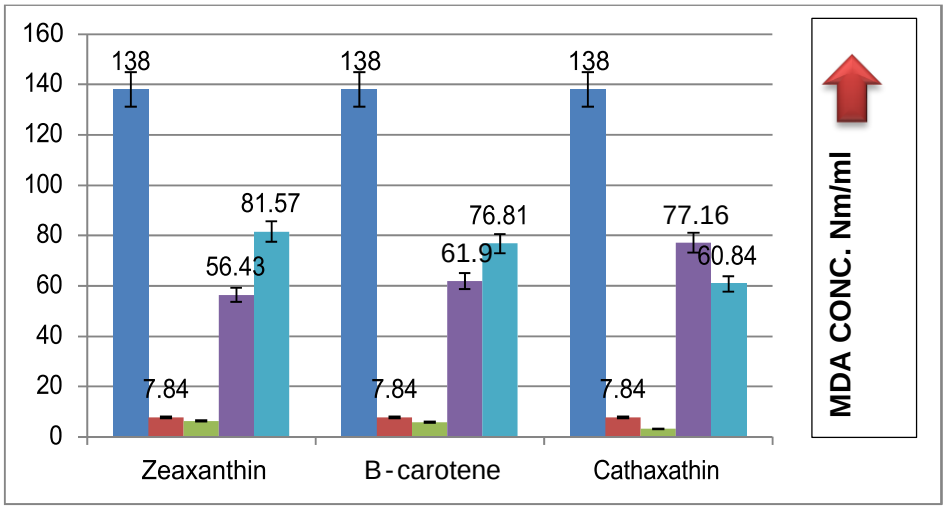


Table no.2: DPPH assay

Lipid peroxidation assay:

The RBC ghost alone have had the malondialdehyde concentration of 7.84nM/ml and when these were incubated with pigments exhibited the 6.33, 5.87, 3.22 nM/ml as depicted in graph. When incubated in presence of $FeCl_3$ 56.43, 61.9 and 77.16 respectively RBC ghosts when incubated in presence of $FeCl_3$ gave a 138nM/ml concentration of malondialdehyde. Thus one can conclude that the pigments lower the malondialdehyde

concentration by 81.57, 76.81 and 60.84 nM/ml for Zeaxanthin, B-carotene and canthaxanthin respectively



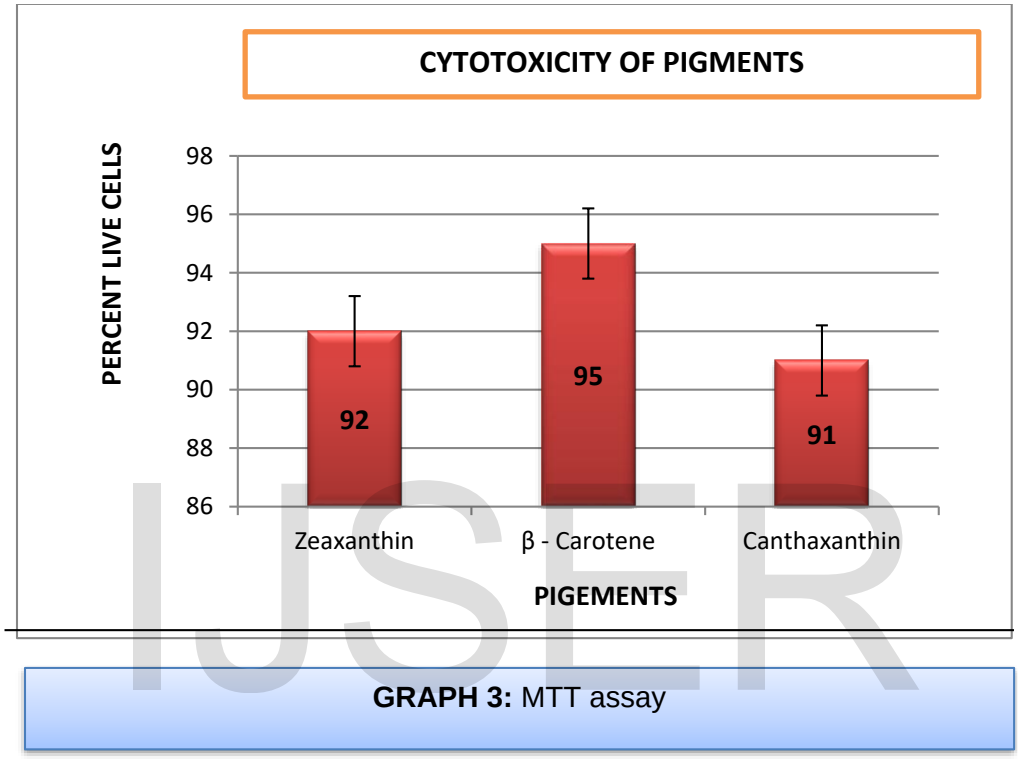
GRAPH 2: EFFFECT OF PIGMENTS ON RBC GHOSTS

	MDA concentration nMol / ml	Difference in MDA nMol/ ml
RBC ghosts	7.84	---
RBC ghosts + FeCl3	138	---
RBC ghosts + *Zeaxanthin*	6.33	---
RBC ghosts + *Beta - carotene*	5.87	---
RBC ghosts + *Canthazathin*	3.22	---
RBC ghosts + FeCl3 + *Zeaxanthin*	56.43	81.57
RBC ghosts + FeCl3 + Beta - carotene	61.90	76.81
RBC ghosts + FeCl3 + Canthazathin	77.16	60.84

Table no.3: Lipid peroxidation assay

MTT assay:

Cells that are live imbibe tetrazolium yellow and their mitochondria reduced this dye to purple color, whereas cells that die cannot do so. The liver cells exhibited 92, 95 and 91% survival indicating that the dyes are not toxic to cells



GRAPH 3: MTT assay

Reference:

1. Chidambaram Kulandaisamy Venil, Zainul Akmar zakaria, Wan Azlina Ahamad: (2013); *Bacterial pigments and their applications:* Process biochemistry 48 (1) 1065-1079

2. Hardeep S.Tuli; Prachi Chaudhary; Vikas Beniwal; Anil K. Sharma: (2015); *Microbial pigments as a natural color sources: current trends and future perspectives* Journal of food science technology Aug; 52(8):4669-4678

3. Tanuka Sen; Colin J. Barrow; Sunil Kumar Deshmukh; (2019); *Microbial pigments in the food industry-challenges and the way forward:* Frontiers in Nutrition *10.3389/fnut.2019.00007*

4. Alugoju Phanianendra; Dinesh Babu Jestadi; Latha Periyasamy: (2015) *Free radicals: properties, sources, Target and their implication in various disease;* Indian Journal of Clinical Biochemistry; 30(1) :11-26

5. Gabriele Pizzino; Natasha Irrera; Mariapaola Cucinotta; Giobanni Pallio; Federica Mannino; Vinocenzo Arcoraci; Francesco Squadrito; Domenica Altaviila; Alessandra Bitto; (2017) *Oxidative stress: Harms and benefits for human health* Oxidative Medicine Cell Longevity. 10.1155/2017/8416763

6. V.Lobo; A.Patil; A.Phatak; M. Chandra; (2010) *Free radical, Antioxidant and Functional foods: Impact on human health*: Pharmacognasy Review. 4(8): 118-126

7. Aruoma O I (2003) *Methodological consideration for characterization for potential antioxidant actions of bioactive component inplant foods mutate*: Research; 532; 9-20.

8. Young I S, Woodsid J V (2001) *Antioxidant in health and diseases*. Journal of Clinical pathology. 54 - 176-86.

9. Mccord J N (2000).*The evolution of free radical and oxidative stress* American Journal of Medicine; 108:652-9

10. Rao A L; Bharani M; Pallavi V. (2006) *Roll of antioxidant and free radical in health and disease.* Advanced Pharmacological toxicology - 29-38