

# Antioxidant, antibacterial, and ultraviolet-protective properties of carotenoids isolated from *Micrococcus* spp.

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## ABSTRACT

Carotenoids are the most common naturally occurring bioactive terpenoid pigments, which are commonly produced by a wide variety of plants and microbes. The present study was aimed to evaluate the antioxidant, antimicrobial and radio-protective properties of carotenoid pigments isolated from ultraviolet (UV)-C resistant *Micrococcus* spp. The UV-C resistant *Micrococcus roseus* and *Micrococcus luteus* were isolated from the soil samples of Savandurga hills region, Karnataka (India), and their pigments were identified as carotenoids based on spectral analysis. The UV-protective efficacies were determined by cling-film assay. Further, the antioxidant activities of pigments were evaluated by 2,2-diphenyl-1-picrylhydrazyl assay, and antibacterial activities by disc diffusion and broth microdilution assays. The optimum growth and pigment production by *M. roseus* and *M. luteus* were observed at temperature ranged between 35°C and 37°C, pH 7.0-8.0, NaCl 5.0-7.0%, and sucrose as major carbon and KNO<sub>3</sub> as major nitrogen sources. In the present investigation, the isolated carotenoid pigments of *M. roseus* and *M. luteus* showed significant UV protective activity along with antioxidant (IC<sub>50</sub> 3.5-4.5 mg/mL) and antibacterial (minimal inhibitory concentration 0.25-2.0 mg/mL) properties.

**KEYWORDS:** Antibacterial, antioxidant, carotenoids, *Micrococcus luteus*, *Micrococcus roseus*, ultraviolet-protection

## INTRODUCTION

Ultraviolet (UV) radiation is one of the energetic electromagnetic radiation, which causes both indirect and direct damage to living organisms.<sup>[1]</sup> Many synthetic UV-protective agents and pigments have been used in cosmetics, pharmaceutical and radiation industries, are known to have health hazards and safety problems. Hence, there is an increased interest for searching alternative UV-protective, as well as bioactive carotenoid pigments from natural origin due to their

less or no toxicity, as they are easily decomposable, not environmental pollutants and possess no residues.<sup>[2]</sup> Several researchers have reported that some pigmented bacteria, which are rich in carotenoids have been resistant to radiation when subjected to sub-lethal and lethal doses of ionizing radiations, due to the accumulation of the radio-protective pigments in the outer membrane.<sup>[3-6]</sup>

Carotenoids are the terpenoid pigments produced by a wide variety of plants and microbes, which are reported to have radio-protective property.<sup>[7]</sup> Carotenoid pigments of bacterial origin have been reported to have radio-protective and antioxidant properties, and as natural coloring agents.<sup>[8,9]</sup> Several epidemiological studies demonstrated that an increased consumption of a diet rich in carotenoids reduces oxidative damage of cells by scavenging free radicals and reactive oxygen species.<sup>[7-10]</sup> Carotenoid pigments extracted from bacteria are more acceptable, because of their safety, capability

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to use a wide range of carbon and nitrogen sources, predictable yield and pigments can be easily separated from the cell mass.<sup>[11]</sup>

The genus *Micrococcus* are Gram-positive, non-spore forming and aerobic coccoid, they are rich in carotenoid pigments, which are known to have radio-protective and bioactive properties.<sup>[12,13]</sup> Even though the carotenoid pigments produced by the genus *Micrococcus* are useful for the industry, particularly in food, pharmaceutical, cosmetic and dye industries,<sup>[12]</sup> but a scientific and systematic investigation with regards to UV-protective pigments and their various biological activities is lacking. Considering these, we screened different soil samples for isolation of UV-resistant bacteria particularly *Micrococcus* spp. The objective of this study was (i) to analyze the growth and resistance of the pigmented bacteria to UV-C, (ii) to isolate the UV-protective pigments from radiation-resistant bacteria, and (iii) to investigate their biological activities.

## MATERIALS AND METHODS

### Chemicals and culture media

All culture media and ingredients were purchased from Hi-Media, Mumbai (India). All solvents, reagents, butylated hydroxytoluene (BHT) and iodo-nitro-tetrazolium were purchased from SRL, Mumbai. Microtiter-plates (96-well) were purchased from Axiva, New Delhi (India). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma, Germany. Silica gel 60 F<sub>254</sub>-coated preparative thin layer chromatography (TLC) plates were obtained from Merck, Germany.

### Isolation of ultraviolet-C resistant *Micrococcus* species

The UV-C resistant bacteria were isolated and identified following the standard procedures.<sup>[14-16]</sup> Briefly, soil samples were collected from the different regions of Savandurga hills area (Karnataka, India), where the soils were directly exposed to sunlight. The samples were subjected to UV-C irradiation (UV-C [30 W], Philips, Holland) for 30 min, serially diluted and plated onto the nutrient agar (NA) medium by agar pour plate method<sup>[16]</sup> and the plates were incubated at 37 ± 2°C for 3d. The pigmented bacteria appeared on NA were isolated, and pure-cultures were made. UV-resistance ability of the pure cultures were determined following the procedures of Jacobs and Sundin<sup>[15]</sup> with some slight modifications. Briefly, the bacterial cultures in the exponential phase (10<sup>8</sup> colony-forming unit [CFU]/mL) were transferred to a sterile plastic tissue culture dish (100 mm × 20 mm, depth of the liquid <2 mm) and exposed to UV-C at different intervals viz., 0, 30, 60, 90, and 120 min. The resistance potencies of the pigmented bacteria to UV-C

was assessed by determining the percent cell viability by recording the numbers of CFU after 3d of incubation on NA at 37 ± 2°C.<sup>[14,17]</sup> The standard *Streptococcus faecalis* served as an indicator organism. The percent cell viability was determined using the formula:

$$\text{Percent cell viability (\% CV)} = \frac{\text{Number of CFU in UV - C treated sample}}{\text{Number of CFU in UV - C untreated sample}} \times 100$$

### Identification of ultraviolet-C resistant *Micrococcus roseus* and *Micrococcus luteus* and their optimal growth conditions

The pure cultures of *M. roseus* (isolate 11R) and *M. luteus* (isolate 5Y) showed promising UV-resistant activities up to 120 min, were identified following the standard procedures.<sup>[18-21]</sup> Briefly, the cultures in the exponential phase of growth were observed microscopically (Olympus, CKX-41) for their cell morphology, and motility was determined by hanging drop method.<sup>[19]</sup> The biochemical characteristics of *M. roseus* and *M. luteus* were determined.<sup>[20,21]</sup> The % mol of G + C content was determined by thermal denaturation ( $T_m$ ) method.<sup>[18]</sup>

The optimum physiological growth parameters of *M. roseus* and *M. luteus* were determined following the procedure of Aneja.<sup>[21]</sup> Briefly, the cultures were grown at different temperature (25°C, 30°C, 35°C, 37°C, 40°C, 45°C, and 50°C), pH (6, 7, 8, 9, and 10) and NaCl concentrations (5.0%, 7.0%, 9.0%, 10.0%, and 12.0%). Different carbon and nitrogen sources were tested to determine best carbon and nitrogen sources for optimum growth and pigment production at 0.2% (w/v) concentration. After 3d incubation, the bacterial turbidity in different physiological parameters was measured spectrophotometrically by recording optical density at 550 nm (ELICO SL-210, India).

### Extraction, isolation and identification of pigments from ultraviolet-C resistant *Micrococcus roseus* and *Micrococcus luteus*

The extraction and isolation of pigments from *M. roseus* and *M. luteus* were carried out following the procedure of Lu *et al.*<sup>[2]</sup> Briefly, 10 mL of inoculum was transferred into Erlenmeyer flask containing 300 mL of nutrient broth, then incubated in rotary shaker incubator up to 5d. After incubation, the cultures were centrifuged at 10,000 rpm for 15 min at 4°C, and the cell pellets were collected. The collected pellets were extracted with cold methanol, then separated from the cells by centrifugation at 10,000 rpm for 15 min at 4°C. The methanolic extract of pigments was concentrated *in vacuo* and purified by column chromatography followed by preparative TLC, and the purified pigments were identified by λ-max

spectroscopic analysis and comparison with reported literatures.

### Ultraviolet-C protective effects of carotenoid pigments

The protective efficacies of carotenoid pigments of *M. roseus* and *M. luteus* were tested against UV-C following the procedure of Delpech.<sup>[22]</sup> Briefly, 1 mL of UV susceptible *S. faecalis* was taken in a sterile watch glass, and then covered by cling film at the top and 2 mL of pigment solution (dissolved in aqueous methanol [9:1, v/v]) was placed in a depression, and treated with UV-C at different intervals. Aqueous methanol (9:1, v/v) was used as control. After exposure, 2 µL of sample from watch glass was transferred to NA by pour plate method and the percent surviving cells were determined by counting CFU.

### Antioxidant activities of carotenoid pigments

The antioxidant activities of the isolated carotenoid pigments of *M. roseus* and *M. luteus* were determined using DPPH radical scavenging assay.<sup>[23]</sup> Briefly, desired different concentrations of the pigments were made using methanol (0.25–10 mg/mL). A volume of 1 mL of each dilution was mixed with 3 mL of freshly prepared methanol solution of DPPH (40 µg/mL) and incubated for 30 min in the dark at room temperature. The carotenoid pigments in 3 mL of methanol without DPPH were served as blank, methanol solution of DPPH (devoid of pigment) served as control and BHT was standard. The absorbance of the solutions was recorded using UV-VIS spectrophotometer (ELICO SL-210, India) at 517 nm. Percent inhibition of DPPH radicals was calculated by following formula:

$$I\% = \frac{(A_{\text{Control}} - A_{\text{Sample}})}{A_{\text{Control}}} \times 100$$

where  $A_{\text{Control}}$  is the absorbance of the control and  $A_{\text{Sample}}$  is the absorbance of the test samples.

### Antibacterial activities of carotenoid pigments

#### Bacterial strains

The human pathogenic *Escherichia coli* (NCIM 2065), *Staphylococcus aureus* (NCIM 2079), and *S. faecalis* (NCIM 5025) were obtained from the National Chemical Laboratory, Pune (India). All the tested bacteria were maintained on Mueller-Hinton agar (MHA) and 24 h old cultures were used for the assay.

#### Disc diffusion method

The disc diffusion method was employed for the determination of the zone of inhibition (ZOI) according to the method of Ebrahimabadi *et al.*<sup>[23]</sup> Briefly, sterile filter paper discs (6 mm in diameter) were

individually impregnated with 20 µL of carotenoid pigments (0.25–10 mg/disc) dissolved in dimethyl sulfoxide (DMSO), placed onto the preinoculated MHA plates (inoculum size: 100 µL of suspension containing 10<sup>8</sup> CFU/mL of bacteria) and incubated at 37°C. DMSO served as a negative control and neomycin (0.25 mg/disc) as standard. The ZOI diameters were measured in millimeters (mm).

### Determination of minimal inhibitory concentration/ minimal bactericidal concentration

The broth microdilution method was employed to determine the minimal inhibitory concentration/minimal bactericidal concentration (MIC/MBC) of carotenoid pigments of *M. roseus* and *M. luteus*, following the procedure of Hajji *et al.*<sup>[24]</sup> Briefly, 200 µL of desired different concentrations of carotenoid pigments in MHB (0.25–10 mg/mL) was added separately to the wells of a sterile 96-well microtiter plate and inoculated with 15 µL of a microbial suspension containing 10<sup>8</sup> CFU/mL of test bacteria, and incubated at 37°C for 24 h. DMSO served as a negative control, and neomycin was used as a standard. After incubation, 5 µL of the cultured broth was transferred onto the NA and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the pigment required for inhibiting the growth of bacteria, whereas MBC was defined as the lowest concentration of the pigment required to prevent the bacterial growth completely on pigment-free agar medium.

## RESULTS AND DISCUSSION

The damage caused by UV irradiation, and serious environmental and safety problems caused by many synthetic pigments have led to search for UV-protective and safe pigments from natural sources.<sup>[2]</sup> Hence, the isolation of UV-protective pigments from microbes is one of the promising alternative strategy. In the present investigation, we have isolated UV-resistant different pigmented bacteria from soil samples collected from the different regions of Savandurga hills area (Karnataka, India), where the soils were directly exposed to sunlight.

Among the bacteria isolated, red-pigmented *M. roseus* and yellow-pigmented *M. luteus* were found to be dominant in the UV-C irradiated soil samples and these bacterial counts ranged from 1.5 × 10<sup>3</sup> to 2.5 × 10<sup>3</sup> cells/g of soil. A total of 22 red-pigmented and 19 yellow-pigmented colonies were isolated and sub-cultured based on the colony characteristics, and then exposed to UV-C. Out of these, 12 red-pigmented and 9 yellow-pigmented bacteria showed strong resistance to UV-C with percentage coefficient of variation (%CV) ranged 70-95% at 120 min exposure. Among which the red-pigmented (11R) and yellow-pigmented (5Y)

bacteria showed strong UV-resistance with %CV value ranged 90-95% and more pigmentation, were selected for identification and pigment extraction. On the NA, colonies of the selected isolates (11R and 5Y) were circular, convex with a smooth margin and their diameter varied from 0.5 to 2.0 mm [Figure 1]. Under microscopic observation, both isolates appeared as Gram-positive with tetrads of cocci in shape [Figure 2]. Morphological and biochemical properties of the isolated bacteria are shown in Table 1. The % mol of G + C content was ranged 71.8–73%. Based on the physicochemical and biochemical characteristics, the red-pigmented isolate (11R) was identified as *M. roseus* and yellow-pigmented isolate (5Y) was *M. luteus*.

As shown in Figure 3a-e temperature, pH, NaCl concentration, carbon and nitrogen sources have affected the growth and pigment production significantly. The *M. roseus* exhibited optimum growth at 37°C and pH 8.0, whereas *M. luteus* at 35°C and pH 7.0. The optimum NaCl required for growth was 7.0%, but the growth was not completely inhibited even at 12% concentration. The cultures could grow best when sucrose and KNO<sub>3</sub> supplemented as major carbon and nitrogen sources, respectively. The present investigation confirms that the bacterial growth and pigment production was dependent upon physiological parameters.

After 5d incubation, the yield of pigment and biomass of *M. roseus* was ranged between 18.9-19.1 g/L and 28.4-28.9 mg/g, whereas *M. luteus* was 15.33-15.76 g/L and 26.1-26.9 mg/g, respectively. The purified pigments of *M. roseus* and *M. luteus* were analyzed using a spectrophotometer, and the maximum absorptions ( $\lambda$ -max) were observed at 476.31 and 437.16 nm, respectively [Figure 4]. Based on the spectral analysis ( $\lambda$ -max) and comparison with reported literature, the pigments isolated from *M. roseus* and *M. luteus* were identified as carotenoids. The UV-C protective efficacies of carotenoid pigments of *M. roseus* and *M. luteus* were tested against UV-C sensitive *S. faecalis*. The %CV of test organism in control was 0.0%, while the test organism covered with red-carotenoid of *M. roseus* and

yellow-carotenoid of *M. luteus* was resistant to UV-C even up to 120 min exposure [Table 2]. The results clearly confirm that both the red and yellow carotenoid pigments isolated from *M. roseus* and *M. luteus* showed UV protective properties.

**Table 1: Morphological and biochemical characteristics of *M. roseus* and *M. luteus***

Parameters	<i>M. roseus</i>	<i>M. luteus</i>
Pigmentation	Red	Yellow
Motility	Positive	Negative
Morphology	Tetra head coccus	Tetra head coccus
Catalase test	Positive	Positive
Oxidase test	Negative	Positive
Glucose fermentation	Positive	Negative
Esculin hydrolysis	Negative	Negative
Gelatin hydrolysis	Negative	Positive
Nitrate reduction to nitrite	Positive	Negative
Arginine dihydrolase	Negative	Negative

*M. roseus*: *Micrococcus roseus*, *M. luteus*: *Micrococcus luteus*

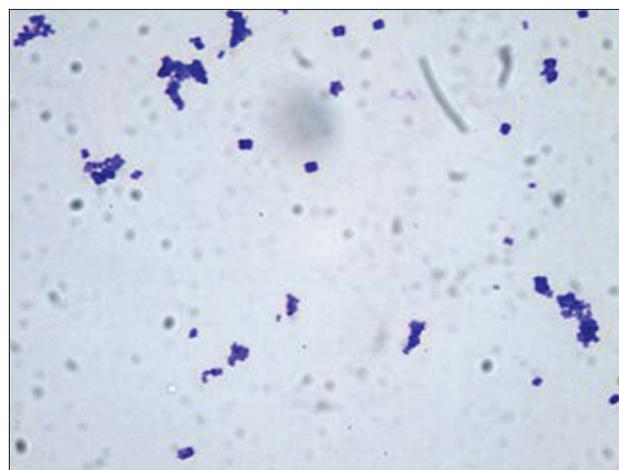
**Table 2: Efficacy of UV-C protective activity of carotenoid pigments isolated from *M. roseus* and *M. luteus* on growth of *S. faecalis***

Exposure time (min)	Carotenoid of <i>M. roseus</i>	Carotenoid of <i>M. luteus</i>	Control (without carotenoid)
0	+++	+++	+++
30	+++	+++	+
60	+++	+++	-
90	+++	+++	-
120	++	++	-

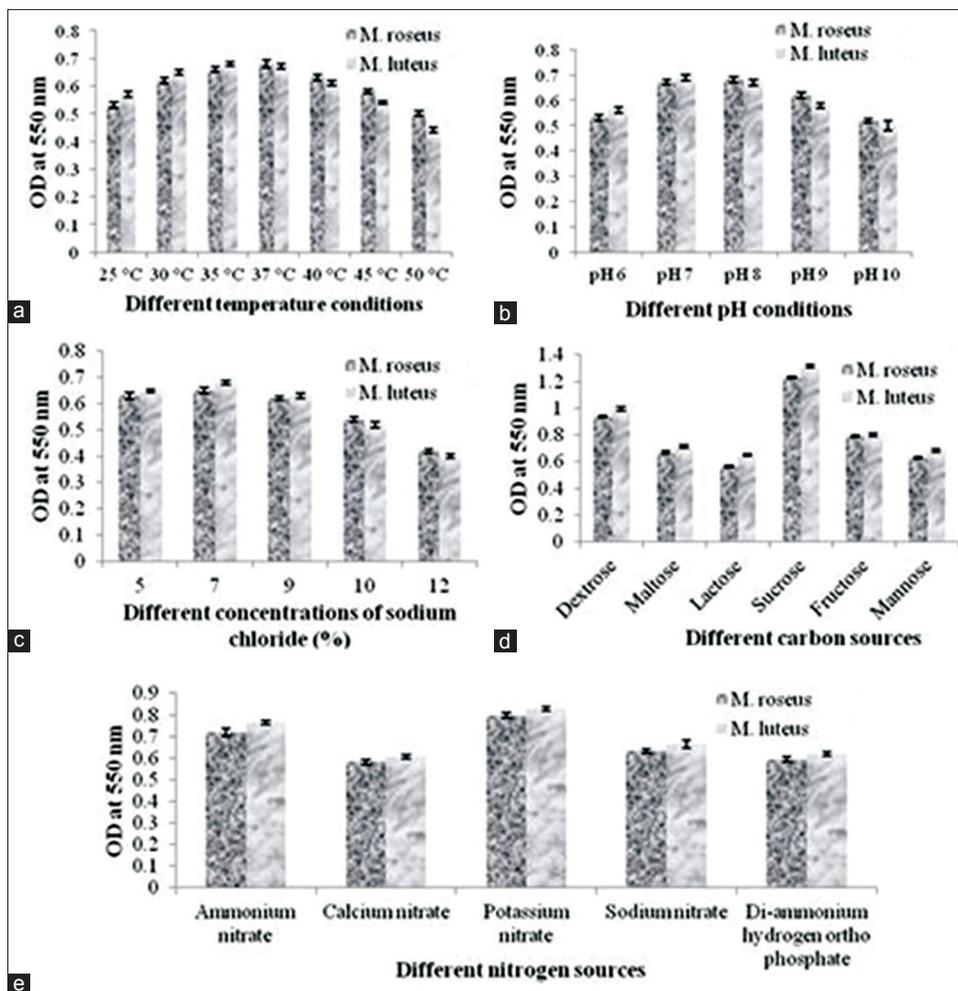
Data given are mean of four replicates. The notations were used to estimate UV-C protection ability of the pigments as follows – +: %CV is 5-25, ++: %CV is 26-50, +++: %CV is above 50, -: Complete inhibition of growth. UV: Ultraviolet, *M. roseus*: *Micrococcus roseus*, *M. luteus*: *Micrococcus luteus*, *S. faecalis*: *Streptococcus faecalis*, CV: Coefficient of variation



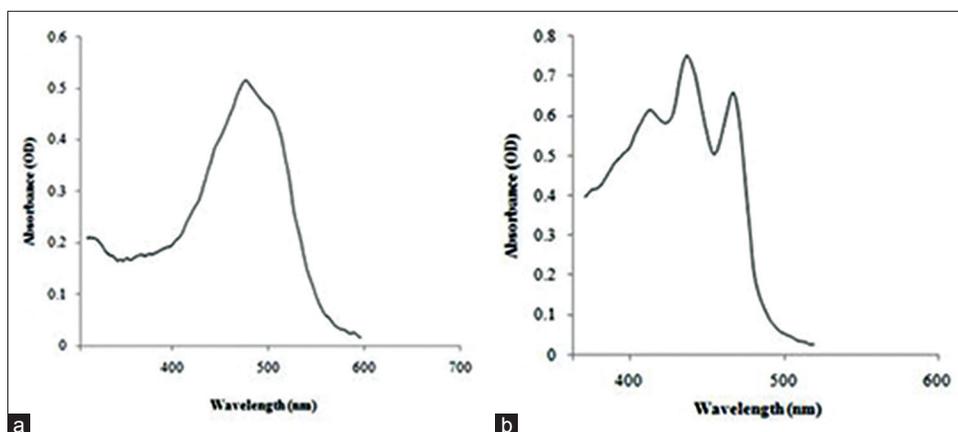
**Figure 1:** Ultraviolet resistant pigmented *Micrococcus roseus* (a) and *Micrococcus luteus* (b) grown on nutrient agar



**Figure 2:** Microscopic observation of Gram stained *Micrococcus luteus* ( $\times 100$ )



**Figure 3:** Effect of different physiological parameters on growth of *Micrococcus roseus* and *Micrococcus luteus* (a → Effect of temperature; b → Effect of pH; c → Effect of sodium chloride concentration; d → Effect of carbon sources; e → Effect of nitrogen sources)



**Figure 4:** Ultraviolet spectra of carotenoid pigments isolated from *Micrococcus roseus* (a) and *Micrococcus luteus* (b)

Both red- and yellow-carotenoids of *M. roseus* and *M. luteus* showed concentration-dependent antioxidant and antibacterial activities [Table 3]. In antioxidant activity assay, the IC<sub>50</sub> values of red- and yellow-carotenoids were found to be 3.5 mg/mL and 4.5 mg/mL, respectively, and

the results were compared with BHT. The order of antioxidant activity was BHT > red-carotenoid (*M. roseus*) > yellow-carotenoid (*M. luteus*). In antibacterial activity assay, both the carotenoid pigments did not show any inhibitory activity against Gram-negative *E. coli*, but they were active against Gram-positive

**Table 3: Antioxidant and antibacterial activities of carotenoid pigments isolated from UV-C resistant *M. roseus* and *M. luteus***

Sample concentrations (mg/mL)	% DPPH radical scavenging activity		Antibacterial activity (ZOI in mm)			
	Carotenoid of <i>M. roseus</i>	Carotenoid of <i>M. luteus</i>	<i>S. aureus</i>		<i>S. faecalis</i>	
			Carotenoid of <i>M. roseus</i>	Carotenoid of <i>M. luteus</i>	Carotenoid of <i>M. roseus</i>	Carotenoid of <i>M. luteus</i>
Control	0.0	0.0	0.0	0.0	0.0	0.0
1	32.8±0.97	29.1±1.13	6.5±0.24	0.0	7.5±0.44	6.9±0.16
2	41.2±1.12	34.7±1.10	7.9±0.43	6.7±0.24	8.5±0.37	7.8±0.33
4	55.3±1.01	46.7±1.07	8.7±0.55	7.6±0.16	9.2±0.41	8.7±0.27
6	66.2±1.03	59.5±1.31	9.5±0.61	8.0±0.34	10.5±0.46	9.5±0.49
8	79.1±0.83	74.5±0.95	10.8±0.49	9.5±0.39	13.0±0.53	10.5±0.53
10	88.5±0.93	82.1±1.01	12.5±1.01	10.5±0.64	15.0±0.87	11.8±0.56
Standard <sup>#</sup>	98.5±0.65	98.5±0.65	18.5±0.35	18.5±0.35	22.2±0.34	22.2±0.34
IC50*/MIC**	3.5	4.5	0.5	1.0	0.25	0.5

<sup>#</sup>Standard: BHT for DPPH radical scavenging activity and neomycin for antibacterial activity, \*IC50 value (mg/mL) for DPPH radical scavenging activity, \*\*MIC value (mg/mL) for antibacterial activity. Values are expressed as mean±SE (n=5). SE: Standard error, *M. roseus*: *Micrococcus roseus*, *M. luteus*: *Micrococcus luteus*, *S. faecalis*: *Streptococcus faecalis*, *S. aureus*: *Staphylococcus aureus*, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ZOI: Zone of inhibition, MIC: Minimum inhibitory concentration, IC: Inhibitory concentration, BHT: Butylated hydroxytoluene

bacteria with ZOI, MIC, and MBC ranged 6.5-15.0 mm, 0.25-2.0 mg/mL, and 6.0-10.0 mg/mL, respectively, and the results were compared with standard antibiotic neomycin. The order of inhibitory activity against bacteria was neomycin > red-carotenoid (*M. roseus*) > yellow-carotenoid (*M. luteus*). These results are of great importance, particularly for *S. aureus*, which is well known for being resistant to a number of antibiotics.<sup>[25]</sup>

Synthetic antioxidants play an important role in preventing major degenerative diseases caused by free radicals and protecting foodstuffs from lipid oxidation.<sup>[26]</sup> However, with increasing documentation of possible adverse effects of some synthetic antioxidants on human health, there is an increasing interest in finding natural and biologically produced antioxidants. Further, the emergence of bacterial strains resistant to clinically used antibiotics and changing patterns of susceptibility to clinically available antimicrobial agents require continuous updating of knowledge concerning the treatment of diseases caused by such pathogens.<sup>[27,28]</sup> Considering these factors, both red and yellow carotenoids of *M. roseus* and *M. luteus* could be explored as potential alternatives for managing diseases caused by free radicals and microbes. Further, *in vivo* studies of these carotenoids are being investigated.

## CONCLUSION

In this investigation, the carotenoid pigments isolated from *M. roseus* and *M. luteus* showed promising UV-protective, antioxidant and antibacterial activities. The growth and pigment production was dependent on temperature, pH, NaCl, and carbon and nitrogen sources.

To the best of our knowledge, we are reporting here the UV-protective, antioxidant and antibacterial activities of carotenoids of *M. roseus* and *M. luteus* for the first time. The findings indicate the possible exploration of these pigments as natural coloring agents in food and pharmaceutical industries and UV-protective agents in cosmetics, after clinical evaluations.

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