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Radical scavengering of pigments from novel strains of *Dietzia schimae* and *Microbacterium esteraromaticum*

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ABSTRACT

Radiation resistant bacteria have adopted a variety of ingenious strategies for survival under the high dose of radiation, for example through their pigments. In the present study, two ultraviolet-C (UVC) radiation tolerant bacteria, named NM1 and NM3 strains, were isolated from the industrial waste and soil that identified by the molecular analysis. Survival assay of irradiated bacteria was performed by plate counting and flow cytometry (by a fluorescent dye, Rhodamin 123). Also, hydrogen peroxide tolerance of the isolated strains was analyzed by turbidimetric microplate technique. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and EC_{50} values in reducing power were measured to evaluate antioxidant activity and reductive power of their methanol extracted pigments. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that the NM1 and NM3 strains belonged to *Microbacterium esteraromaticum* and *Dietzia schimae* with 99% identity, respectively. Both of them showed much high resistance to 15 and 20 J/cm^2 UVC irradiation (254_{nm}). Visible spectra of their methanolic extracted pigments were considered identical with λ_{max} at 413, 439 and 468_{nm} for *Microbacterium* NM1 and λ_{max} at 451_{nm} for *Dietzia* NM3. EC_{50} values in reducing power were 35.26 and 36.13 µg/ml for pigments of NM1 and NM3 strains, respectively. Whereas scavenging abilities of DPPH radicals were 3.42 and 1.58 mg/ml for pigments of NM1 and NM3 strains, respectively. Based on the results, the pigments of isolated UVC tolerant bacteria displayed strong antioxidant activity. These bacteria may be a good source for antioxidative-related functional foods and the pharmaceutical industry.

Keywords: Ultraviolet radiation; Carotenoids; DPPH radicals; Phylogenetic analysis.

Introduction

Life is continuously threatened by abiotic stresses in the extreme environments, among them, wide temperature, high pressure, oxygen scarcity fluctuations, desiccation, lack of nutrients, and a variety of radiation (1-2). These extreme environments are excellent sources for isolation of bacteria with special features that these bacteria are specifically adapted to the harsh environmental conditions (3). The terms

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radioresistant organism applies to organisms including animals, plants and microbes, which are able to survive under ionizing and nonionizing radiation environments (1, 4). The radioresistant organisms induce genes that produce DNA repair enzymes, metabolites and pigment that are protective against the radiation. Some of these compounds will provide sunscreen, useful drugs, especially antibiotics and anticancer drugs (5).

Over 700 structurally distinct carotenoids have been identified in nature (6). The increasing utilization of natural pigments from two major source, plant and microorganism, in cosmetic, pharmaceutical, foodstuff and health care industries is due to concern about the harmful effects of synthetic pigments (7). Using of carotenoid-rich bacteria rather than the other boilogical resources provides the advantages of greater chemical extraction efficiency, lower production costs, and have not to be concerned with factors of weather or origin of the supply source (8).

The genus *Microbacterium* (phylum Actinobacteria, class Actinobacteria, and order Actinomycetales) belongs to the family *Microbacteriaceae* comprises 62 species with validly published names. The genus *Microbacterium* was described by Orla-Jensen in 1919 (9-10). *Microbacterium esteraromaticum* was isolated from accidental contamination in culture (11).

The genus *Dietzia* (phylum Actinobacteria, class Actinobacteria, order Actinomycetales and family *Dietziaceae*) was described by Rainey et al. (1995). At now, the genus *Dietzia* consists of 13 species (12). *Dietzia* strains are being increasingly isolated from widely different environments, as well as from the deepest sea mud, deep-sea sediments, plant tissues, soils and hospital environments (13). *Dietzia schimae* was isolated from a stem sample of plant genus *Schima* sp. (12).

In this paper, two new ultraviolet C (UVC) tolerant strains were isolated from the waste of Zob-e-Ahan factory in Isfahan and soil of Sirach town and were analyzed for UVC and peroxide hydrogen tolerances. Also, antioxidant activity and reducing power of their pigments were evaluated *in vitro*.

Materials and Methods

Chemicals

Potassium ferricyanide ($K_3(Fe(CN)_6)$), trichloroacetic acid (TCA), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), tryptic soy broth and agar (TSB and TSA) media were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Bacterial strains and culture conditions

In this study two isolated strains named NM1 and NM3 were used for all experiments. Also, *Escherichia coli* (ATCC: 25922) and *Deinococcus radiodurans* R1 (DR1) (ATCC: 13939) from the culture collection of the Iranian Biological Resource Center (IBRC) were used as standard sensitive and resistant strains to UVC irradiation, respectively. The strains were grown in tryptone glucose yeast extract (TGY) broth medium (containing% (w/v): tryptone 0.5; glucose 0.1; yeast extract 0.5; pH 7.2) under aerobic condition at 30°C, except for *E.coli* that was grown at 37°C. To evaluate the antioxidant activity, strains were grown in TSB medium (pH 7.3) under aerobic condition at 30°C in rotary shaker incubator up to 3 days.

Sample Collection and Primary Screening of UVC Tolerant Strains

Waste samples of Zob-e-Ahan factory and Mobarakeh Steel Company near Isfahan city and soils (about 1–5 cm deep) from deserts of Khuzestan, near Kerman and Isfahan cities in Iran were collected during early spring 2014 in sterile containers. Samples were stored at 4ºC until further processing in the laboratory. One ml of waste sample and 1 g of soil sample were aseptically transferred into Erlenmeyer flasks containing 99 ml TGY broth medium followed by incubation at 30°C, 160 rpm for 72 h. After the incubation period, 100 µl of bacterial cultures was transferred onto TGY agar plate and pre-incubated for 2 to 4 h at 30°C for vegetation of spore forms. Then, the plate was exposed to UVC irradiation (10 J/cm^2) with artificial lamps (CROSSLINKER CL-E508.G) with a 254_{nm} UV source and incubated at 30°C for 3-10 days in the dark condition. The colored radiation resistant colonies in TGY agar, sub-cultured on TGY agar slants and restored at 4°C for further studies (14-15).

Secondary screening

The secondary screening was used to confirm resistance to the high dose of UVC irradiation. Isolated colonies were grown in TGY broth medium for 24 h at 30°C in a rotary shaker incubator. After incubation, when the culture reached 0.13 OD_{600}, 1 ml of each sample was inoculated into new fresh TGY broth and incubated at 30°C in a rotary shaker incubator until early log phase of growth. The culture grown in TGY broth was harvested and suspended in phosphate buffer saline (PBS) to a 0.13 OD_{600}. The number of 10^7-10^8 cells of early log phase in PBS were exposed to UVC irradiation at doses of 15 and 20 J/cm^2 with a 254_{nm} UVC source. After irradiation, 100 µl of each sample was transferred onto TGY agar plate and incubated for 3 days at 30°C for later counting of UV tolerant colonies (CFU/ml) (14, 16).

In vitro UVC irradiation experiments

Respiratory activity in the cell was measured with dyes sensitive to change in the proton electrochemical potential, such as the green fluorescent dye rhodamin. Flow cytometry and fluorescent dyes are important approaches to determining the cell viability (17).

A stock solution of rhodamin 123 was made up in ethanol and maintained at -20°C. To determine the effect of UVC irradiation, the strains were aerobically grown in 50 mL of TGY broth (160 rpm) at 30°C, whereas *E.coli* was grown at 37°C. The cultures were harvested by centrifugation (at 12000 g for 10 min) in the late log phase. Then, cells suspended in normal saline (0.9% NaCl) to a 0.5 OD_{600} and 2 ml of the each sample was transferred to sterile Petri dishes. The samples were exposed to UVC irradiation to a dose of 20 and 15 J/m^2 in a UV chamber (CROSSLINKER CL-E508.G) with a 254_{nm} UV source and analyzed by plate counting and flow cytometry. Before flow cytometry analysis, 100 µl of cell suspension was added to 400 µl of the ethanolic rhodamin 123 that was freshly prepared in PBS (14). The mixture was incubated at room temperature for 10 min in dark and analyzed on a Partec PAS flow cytometer. Controls were obtained by incubation of these bacteria without irradiation. Optical filters were set up such that green fluorescent was measured at 525_{nm} (FL1).

Tolerance to hydrogen peroxide (H_2O_2)

In order to quantify the effect of H_2O_2 concentration on the growth of isolates, 150 µl of TSB medium were dumped in the wells of standard 96-well microtiter plates (crystal polystyrene, smooth bottomed plates) at 0.2-4% (v/w) concentrations of H_2O_2. All wells were inoculated with a 50 µl of overnight grown culture in early log phase with a 0.13 OD_{600}. Then, the microtiter plate was covered and incubated at 30°C for 24 hours. Finally, bacterial growth was observed at OD_{600} by using microtiter plate reader (AWARENESS, Technology INC, stat fax 2100).

Pigment extraction

Bacterial isolates were grown in TSB medium at 30°C, 160 rpm shaking for 4 days and bacterial cells from stationary phase were harvested by centrifugation at 3100 g using the Eppendorf 5810R refrigerated centrifuge for 15 min. The harvested cells were washed with distilled water (DW). The pigments of the isolates were extracted from the harvested cells by pure methanol 95% after the pellet incubated for 1h at room temperature on rotary shaker 160 rpm. Then, the methanolic extracts of pigments were centrifuged at 3100 g for 15 min. The colored supernatant analyzed by scanning the absorbance in the wavelength spectrum of 200-800_{nm} using the Biochrom WPA Biowave II UV/Vis Spectrophotometer. Methanolic extracts were filtered through Whatman no.1 filter paper then dried at 40°C in the oven and stored at 4°C for further analysis (18-19).

DPPH radical scavenging assay

The percentage of antioxidant activity of bacterial pigment was investigated by DPPH free radical assay. Briefly, 1ml of the pigment extract, at different concentrations (0.05-10 mg/ml) was added to 1ml of 0.1 mM methanolic solution of DPPH. The samples were shaken and kept in the dark for 30 min. Then, the change in color was measured at 517_{nm} in a Milton Roy Spectronic 21D Spectrophotometer (18, 20). EC_{50} value (pigment mg/ml) is the effective concentration at which DPPH radical was scavenged by 50% and was obtained by interpolation from linear regression analysis. The blank solution was prepared by methan-

olic DPPH radical solution and DW. In this study, ascorbic acid was taken as a standard. The DPPH scavenging effect was determined according to equation (1):

(1) DPPH scavenging effect (%) = $\frac{A\ blank - A\ sample}{A\ blank} \times 100$

Reducing power assay

The reducing powers of extracted pigments were analyzed by the modified method of Oyaizu (1986). The different concentrations of pigments (0.5-10 µg/ml) were mixed with 2.5 ml of PBS (200 mM, pH 6.6) and 2.5 ml potassium ferricyanide 1% (w/v). The mixture was incubated at 50°C for 20 min. After cooling, 2.5 ml of trichloroacetic acid 10% (w/v) were added to the mixtures and centrifuged at 1740 g for 10 min. In the next step, 2.5 ml of the supernatant was mixed with 2.5 ml of DW and 1 ml of ferric chloride 0.1% (w/v) (21). EC_{50} value (pigment µg/ml) for reducing power is the effective concentration at which the absorbance was 0.5 and obtained by interpolation from linear regression analysis. The absorbance of resulting solution was measured at 700_{nm} against a blank in a Milton Roy Spectronic 21D Spectrophotometer. In this study, ascorbic acid was taken as a standard sample.

16S rRNA gene sequencing and phylogenetic analysis

Bacterial genomic DNA were extracted from pure cultures of the isolates by DNA extraction kit (Fermentas, Diagnostics). The partial sequences of 16S rRNA genes of the isolates were amplified using the universal primer 27F (5'-AGAGTTTGATCM TGGCTCAG-3') and 1492R (5'-TACGGYTACCT TGTTACGACTT-3') (22). The amplification reaction was performed by the CinnaGen PCR Master mix 2× in volume 25 µl using a thermal cycler (EPPENDORF GRADIENT MASTERCYCLER 5331) programmed for 30 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 90 s, after initial denaturation at 95°C for 5 min. PCR product (4 µl, 1500 bp) was electrophoresed on a 1.5% agarose gel. DNA sequencing was performed by Korean Macrogen Company. The gene sequence was compared to the other similar bacteria available in the GeneBank using the BLAST network service (www.ncbi.nlm.nih.gov/Blast.cgi, NCBI). Phylogenetic analysis was performed by Neighbor-joining and bootstrap analysis (1000 replicates) using CLC Sequence Viewer 6 software (https://www.qiagenbioinformatics.com/products/clc-sequence-viewer).

Statistical analysis

All data were expressed as the means ± SD (standard deviation) and were carried out in triplicates. The one-way ANOVA test was used to assess the significance of results. *P*-values < 0.05 were considered as significant. All statistical analyzes were done by using IBM SPSS Statistic ver. 20.0 for Windows (23).

Results

Characterization and molecular identification of the isolates

Among the 30 bacterial strains isolated in the primary screening from various habitats, the two bacterial isolates were selected with higher colony counts (2.01×10^3 CFU/ml and 1.35×10^3 CFU/ml) after UVC irradiation at a dose of 20 and 15 J/cm^2 and designated as NM1 and NM3 strains. For DR1 (positive control) and *E. coli* (negative control), 2.24×10^3 and 0 CFU/ml were obtained after UVC irradiation at doses of 20 and 15 J/cm^2, respectively. NM1 and NM3 strains were separated from the waste of Zob-e-Ahan factory in Isfahan province and soil of Sirach town in Kerman province, Iran, respectively. The isolates were identified based on microscopic, biochemical and molecular methods. The NM1 strain was short rod-shaped, Gram-positive, aerobic, motile, non-spore-forming, catalase positive, oxidase positive (weak) bacterium with yellow color colonies. The NM3 isolate was aerobic, Gram-positive cocci, non-spore-forming, non-motile, catalase positive and oxidase negative with deep pink pigmented colonies. Both of them showed no fluorescence emission under the UV light (UV) lamp.

16S rRNA gene sequences of NM1 and NM3 strains showed 99% similarity with *M. esteraromaticum* (NCBI GenBank accession No. KP064029) and *D. schimae* (NCBI GenBank accession No. KP207 685), respectively. The Phylogenetic tree constructed using the CLC Sequencer Viewer (6.1 version) and Neighbor-Joining for *M. esteraromaticum* NM1 and *D. schimae* NM3 confirmed the results of the 16S rRNA gene BLAST (Figure 1).

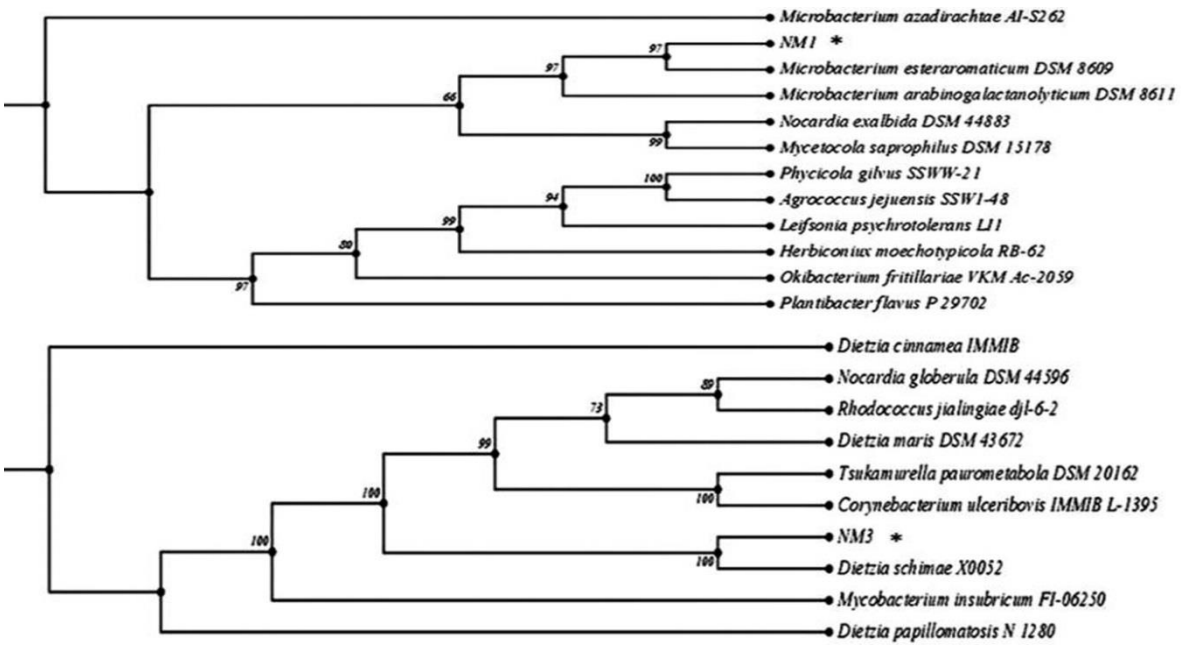

Figure 1. Phylogenetic trees generated using the Neighbor-joining algorithm for partial 16S rRNA gene sequences of NM1 strain (*Microbacterium esteraromaticum*, upper tree) and NM3 strain (*Dietzia schimae*, lower tree) including reference sequences from GenBank that showing the phylogenetic relationships among strains (NM1 and NM3) and related species. Bootstrap values are shown at the branching points (percentage of 1,000 replicates).

Strains viability assessment by flow cytometry after UVC irradiation

The histograms showing strain viability for NM1, NM3, DR1 and E. coli are presented in Figure 2 where the live and dead cells could be detected by using rhodamine 123. Figure 2a shows the histogram for unstained bacteria (negative control). The viability of NM1, NM3, DR1 and E. coli without UVC irradiation (positive control) were determined as 96%, 99%, 98% and 94% (Figure 2b, 2d, 2f, and 2h), respectively. Strains viability after 15 and 20 J/cm2 irradiation, according to Ds index, were D95, D90, D91 and D49 for NM1, NM3, DR1 and E. coli (Figure 2c, 2e, 2g, and 2i), respectively. Based on the results shown in the Figure 2c, UV resistance in NM1 strain was similar to DR1 (Figure 2g) in response to a dose of 20 J/cm2 of UVC irradiation. DR1 and NM1 isolate have high metabolic activity after UVC irradiation than the other strains (that are seen by height pick in their histograms). However, plate count of DR1 after irradiation was higher than the plate count of NM1 strain.

Evaluation of the bacterial reduction was calculated according to the equation (2):

(2) Bacterial reduction (%) = $\frac{A-B}{A} \times 100$, in which A is the percentage of viable cells before irradiation and B stands for percentage of viable cells after irradiation.

H2O2 tolerance by the isolates

The tolerance of the UVC resistant isolates to H2O2 as an oxidant agent are shown in table 1. wherein the cultures showed a varying level of sensitivity to H2O2. Escerichia coli exhibited extreme sensitivity to all concentration of H2O2. The bacterial isolates showed high resistance at 0.2% to 3% concentration of H2O2, but these strains did not show any resistance at 4% and 5% concentration. The MIC and MBC of H2O2 were determined at 3%, 3%, 5% and 5%, 4% and 8% concentrations for NM1, NM3 isolates and DR1, respectively. Also, according to ANOVA analysis, significant differences were observed at different concentrations ($p < 0.05$).

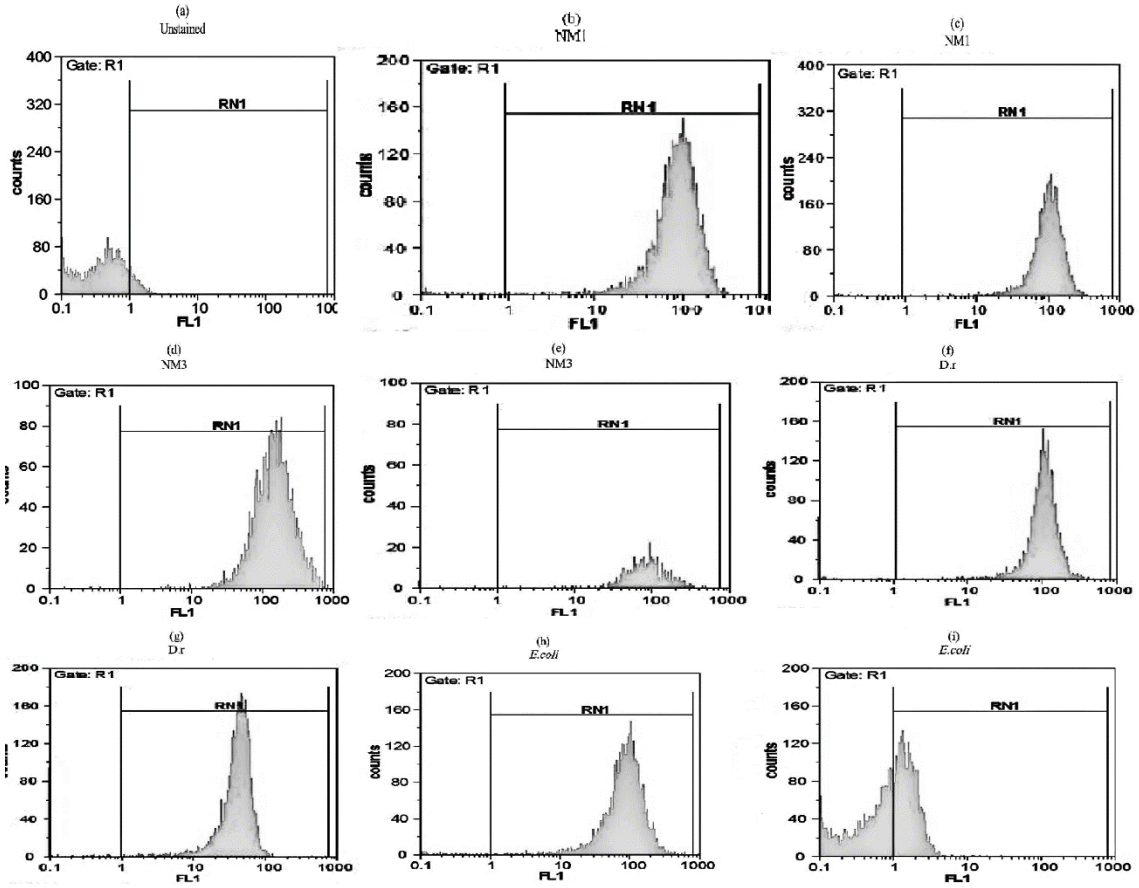

Figure 2. Effects of UVC irradiation on isolates NM1, NM3, *Deinococcus radiodurans* R1 and *Escherichia coli* using a Partec PAS flow cytometer after staining with rhodamine 123. Unstained bacteria (negative control) (a); non-exposure bacteria (positive control): NM1 isolate (b), NM3 isolate (d), *D. radiodurans* R1 (f) and *Escherichia coli* (h); ultraviolet radiation exposure bacteria 20 j/cm^2, NM1 isolate (c), *D. radiodurans* R1 (g) and *Escherichia coli* (i) and 15 j/cm^2, NM3 isolate (e). RN1 region: The percent of living bacteria.

Table 1. Statistical analysis of the optical densities of *M. esteraromaticum* NM1, *D. schimae* NM3, *D. radiodurans* R1 and *E.coli* in the medium containing hydrogen peroxide (H_2O_2)

H_2O_2 (%)	*M. esteraromaticum* NM1		*D. schimae* NM3		*D. radiodurans* R1		*E. coli*	
	Mean OD_{630nm}	SD*	Mean OD_{630nm}	SD	Mean OD_{630nm}	SD	Mean OD_{630nm}	SD
0	0.535	± 0.005 a	0.443	± 0.004 a	0.539	± 0.006 a	1.128	± 0.002 a
0.2	0.399	± 0.008 b	0.395	± 0.005 b	0.45	± 0.007 b	0.017	± 0.005 b
0.4	0.344	± 0.009 c	0.283	± 0.005 c	0.418	± 0.004 c	0.005	± 0.002 c
0.6	0.227	± 0.003 d	0.265	± 0.006 d	0.393	± 0.006 d	0.002	± 0 c
0.8	0.188	± 0.011 e	0.201	± 0.011 e	0.362	± 0.004 e	0	± 0 d
1	0.149	± 0.007 f	0.165	± 0.002 f	0.322	± 0.006 f	0	± 0 d
2	0.116	± 0.007 g	0.123	± 0.008 g	0.27	± 0.006 g	0	± 0 d
3	0.092	± 0.006 h	0.092	± 0.003 h	0.226	± 0.002 h	0	± 0 d
4	0.053	±0.005 i	0.064	± 0.004 i	0.124	± 0.005 i	0	± 0 d

*Standard deviation

Pigment characterization and antioxidant activities of pigment by DPPH

Since the pigment production is carried out in the stationary phase of bacterial growth, pigments were extracted from isolates on the third day of their cultivation. Methanol extracted pigments from the NM1 isolate (yellow pigment) was exhibited three peaks by UV/visible spectroscopy at 413, 439 and 468_{nm}. NM3 isolate pigment (deep pink pigment) and DR1 pigment (red pigment) were exhibited only one peak and maximum absorptions (λ_{max}) were 451 and 479_{nm}, respectively.

The radical scavenging of the pigments, increased with their concentrations and were directly related to increasing in pigment quantity (Figure 3a, b). The EC_{50} values of pigments of *M. esteraromaticum* NM1, *D. schimae* NM3, and DR1 strain were obtained as 3.42, 1.58 and 3.28 mg/ml, respectively. If the EC_{50} concentration is lower, the antioxidant activity of the pigments will be stronger. Antioxidant activity of NM1 pigments was approximately equal with deinoxanthin (the pigment of DR1) (data not shown), but the antioxidant activity of NM3 pigment was higher compared to other pigments. The radical scavenging by the NM1 pigment was lower than NM3 pigment at concentration 0.05-1.00 mg/ml (17.93%-32.87%), but both pigments (NM1 and NM3) were scavenged 77% of free radicals in methanolic solution at 5 mg/ml concentration (Figure 3a, b). According to the results, antioxidant activities of pigments were lower than ascorbic acid (data not shown) (95.12%-96.13%) at low concentration (0.05-5 mg/ml); however, antioxidant activities of the pigments of isolates were similar to ascorbic acid (96.01%) at a concentration of 10 mg/ml that showed high scavenging ability above 90%. It seems that with regard to antioxidant activity, the deep pink pigment extracts from *D. schimae* NM3 strain were more effective than yellow and red pigments extracts from *M. esteraromaticum* NM1 and DR1 strains. Also, according to ANOVA analysis, significant differences were observed at different concentrations ($p < 0.05$).

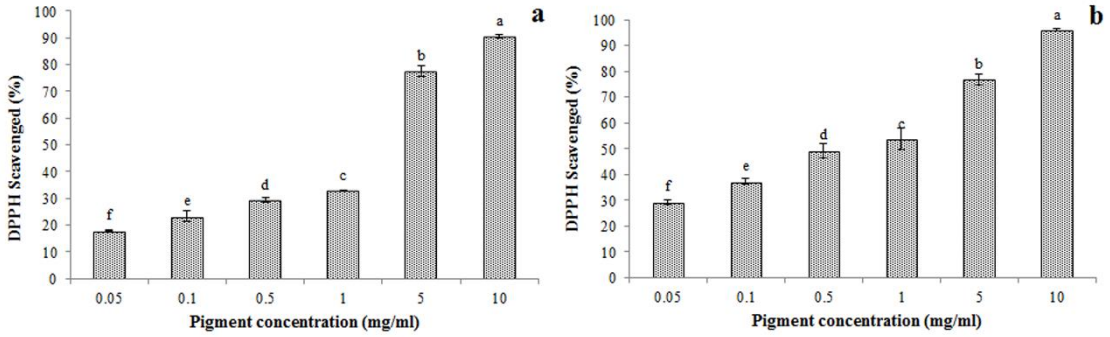

Figure 3. DPPH free radical scavenging activities of carotenoid extracted from *M. esteraromaticum* NM1 (a) and *D. schimae* NM3 (b). All values are mean of three replicates. Error bars represent ± standard deviation (SD). Bars with different letters show significant ($p < 0.05$) difference (Duncan's test).

Reduction power

The reductive abilities of the pigments were measured by the Oyaziu (1986) method. Like the antioxidant activity, the reducing power of the pigment extract from *M. esteraromaticum* NM1, *D. schimae* NM3 strains and DR1 increased with their concentrations (Figure 4a, b). The EC_{50} was obtained according to the standard curve and linear equations. The reducing power of the pigments from NM1, NM3 isolates and DR1 (data not shown) were 0.5 at 35.26 µg/ml, 36.13 µg/ml and 20.19 µg/ml concentrations, respectively (Figure 4a, b). Reducing abilities of the pigments were higher than the ascorbic acid compound (data not shown) (0.0016-0.062) at concentration investigated (0.5-10 µg/ml). Also, according to ANOVA analysis, significant differences were observed at different concentrations ($p < 0.05$) (Figure 4).

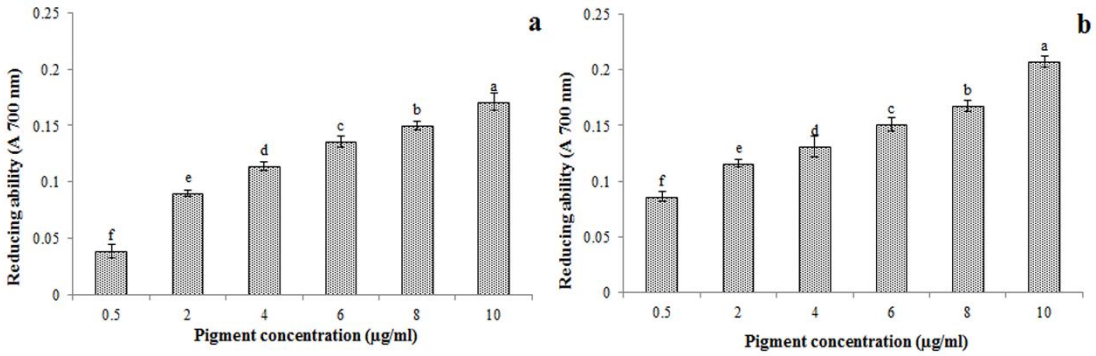

Figure 4. Reducing abilities of carotenoid extracted from *M. esteraromaticum* NM1 (a), and *D. schimae* NM3 (b). All values are mean of three replicates. Error bars represent ± standard deviation (SD). Bars with different letters show significant ($p < 0.05$) difference (Duncan's test).

Discussion

UV-resistant bacteria have been found in wide environments such as higher elevations, mountain ranges, radioactive waste, oceans, lakes and open fields where UVR levels are high (e.g. deserts) (1). In the current study, we studied the antioxidant activity and UVC tolerance of two environmental isolates including *M. esteraromaticum* and *D. schimae*. To confirm bacterial resistance to UVC irradiation and cell viability assay, the samples were analyzed by the flow cytometry method. According to our observations, *M. esteraromaticum* NM1 was highly resistant to UVC radiation after 20 J/cm^2 irradiation, but *D. schimae* NM3 showed moderately UVC resistance after 15 J/cm^2 irradiation. Also, survival percentage was determined about 91% and 89% for NM1 isolate and DR1 strain and 89% and 36% for NM3 isolate and *E. coli* after 20 and 15 J/cm^2, respectively. The NM3 strain showed higher resistance to UVC irradiation than *E. coli*, whereas, its radio-resistance is moderately comparing to DR1.

In many cases, UVC resistant strains can be found in bacterial communities from natural high UVC irradiated environments. *Dietzia schimae* NM3 strain was isolated from an area with high UV irradiation in Sirach town in Kerman province, Iran. An arsenite-resistant strain of *M. esteraromaticum* was detected in deep-sea sediments on the Southwest India (24), and another strain of this bacterium was isolated from a ginseng farm whose β-glucosidase gene (*bgp*1) was cloned and expressed in *E. coli* BL21 (DE3) (25). In other studies, *M. phyllosphere (*irradiation dose above 25 J/cm^2) and *M. radiodurans* were isolated which could tolerate a high dose of UV irradiation (26-27). *Dietzia schimae* was originally isolated from a stem sample of *Schima* sp. from Yunnan Province in south-west China (13). A new strain of *Dietzia* sp. was isolated from Sirch Hot Spring in Kerman, Iran that could tolerate an irradiation dose above 25 J/cm^2 (16). *Dietzia* sp. is able to survive in multiple extreme conditions. It was shown that at least some strains of *D. cinnamea* such as P4, is highly resistant to UV ABC (28). However, no previous studies of radiation resistance by *M. esteraromaticum* and *D. schimae* have been reported yet.

UVR leads to production of reactive oxygen species (ROS) and oxidative condition that cause the break in single and double-strand DNA (29). The antioxidant defense machinery (e.g catalase, superoxide dismutase, and peroxidases) in radiation resistant bacteria is active against all three primary reactive oxygen species (hydroxyl, superoxide radicals, and H_2O_2). *Microbacterium esteraromaticum* NM1 and *D. schimae* NM3 isolates, with the catalase activity, show higher tolerance not only to UVC but also to a high concentration of H_2O_2, similar to DR1. The concentration of 5% and 4% H_2O_2 have completely inhibited the growth of NM1 and NM3 strains, respectively. DR1 showed resistance to 5% H_2O_2. NM1, NM3, and DR1 strains are much more resistant to H_2O_2 compared with the UVC sensitive strain (*E.coli*). In fact, catalase and other antioxidants have

important roles in UV defense, but the resistance to H_2O_2 and UVC irradiation in bacteria indicate that there are nucleotide excision repair mechanisms (UVR ABC and UVDE) and a genetic recombination mechanism (30). Another line of defense against oxidative stress is carotenoid pigments.

In recent years, scientists have paid much attention to the pigments of microorganisms and their biosynthetic pathways. The production of artificial colors is economically affordable, but these colors are causing problems such as toxicity, non-renewable and health issues. Natural colors extracted from fruits, vegetables, roots and microorganisms are without foul odor, no toxicity and can be recycled. Bacteria can be a valuable resource for the production of bio-colors (18).

UV-visible spectroscopy is one of the first criteria in detection and characterization of carotenoids. Carotenoids have strong optical absorption in the visible light region and in some cases are also in the UV region. The spectroscopy only provides information on the color functional groups and does not provide information about its functional groups. Visible light spectroscopy specifies maximum light absorption, absorption rate and absorption curve (31). It has been determined that neoxanthin have highest optical densities in the area of 415, 439 and 467 nm wavelengths (32). Probably, the yellow pigments extracted from *M. esteraromaticum* NM1 isolate is neoxanthin pigment. Neoxanthin pigment is an intermediate in the biosynthesis of the plant hormone abscisic acid (33) and potently reduced the viability of three prostate cancer cell lines, DU145, LNCaP, and PC-3 (34). Production of pigment in all strains of *Dietzia* have been reported that their color is ranging from yellow to coral-red, but production of canthaxanthin was not reported in *Dietzia* species except *Dietzia* sp. K44 strain and *D. natronolimnaea* HS-1. The canthaxanthin isolated from K44 showed two major peaks absorbing at 465.3 and 475_{nm} (20). The zeaxanthin pigment displayed UV-Vis λ_{max} at 428, 450 and 478_{nm} and ß-carotene showed λ_{max} at 450_{nm} and 478_{nm} (35). The pigment of *D. schimae* NM3 isolate may belong to zeaxanthin or ß-carotene pigments or their derivatives according to the maximum absorbance at 451_{nm}. But for the isolation and accurate identification of pigments thin layer chromatography (TLC), FT-IR, MS, NMR and HPLC methods should be used. In further studies on these pigments the mentioned methods should be conducted accurately.

The antioxidant activity of pigment could be one of the factors in UVR resistant bacteria against the damaging effects of UV radiation. Bacterial pigments have an important role in the protection from damages of UV and gamma radiation and are effective scavengers of all types of ROS which protect DNA, protein and cellular membranes from oxidative damage and have important roles in photosynthesis and nutrition (1, 36). Pigments of UVC resistant bacteria with high antioxidant ability can be used as anti-cancer drugs as well as antibiotics and agricultural products of commercial significance, anti-inflammatory, immune stimulatory activity, antitumor and food colorants (1).

The antioxidant activities of pigments were investigated by the DPPH model system. The measurement of free radical scavenging by using DPPH is one of the reliable, accurate, easy and affordable methods with high repeatability. DPPH radical scavenging activity of NM1, NM3, and DR1 pigments exhibited a dose-dependent increase in maximum activities. The pigment of NM3 isolate showed the highest radical scavenging activity. *Exiguobacterium* sp. can be used as a potential source of astaxanthin and scavenged 60-70% of DPPH radicals at different concentration (25-500 mg/ml) (18). Carotenoid extract from *M. esteraromaticum* NM1 and *D. schimae* NM3 isolates showed higher antioxidant activities than the astaxanthin pigment at less concentration. It was reported reported that deinoxanthin from DR1 scavenged 40.2% of DPPH radicals at a concentration of 0.5 mg/ml (36), which is similar to the results of the radical scavenging ability of deinoxanthin in our study.

The reducing power assay determines the electron donating capability of pigments using the potassium ferricyanide reduction method. The reductive agents can convert ferricyanide complexes to ferrous form ($Fe^{+3} \rightarrow Fe^{+2}$) (21). Various mechanisms related to anti-oxidant activities were suggested, including radical scavenging, binding of transition metal ion catalysts, chain initiation, decomposition of peroxides,

prevention of continued hydrogen abstraction and reductive capacity (37). The reducing powers of pigment extracts from both UVC tolerant *M. esteraromaticum* NM1 and *D. schimae* NM3 isolates were excellent, but it seems that the pigments extracts from *M. esteraromaticum* NM1 and *D. schimae* NM3 were less effective in reducing power than deinoxanthin. The reducing powers of pigment were higher than the ascorbic acid compound at a range of concentration (0.5-10 µg/ml). It was also formerly reported that the reducing ability of deinoxanthin was higher than those of ascorbic acid and *β*-carotene compounds which were similar to the results of our study. The pigment of DR1 demonstrated a strong antioxidant activity *in vitro*, and this effect was further verified by its ability to suppress hepatic damage resulting from CCl_4-induced oxidative stress in mice. These results confirm that pigments of UV-resistant bacteria possess high antioxidant activity. For application in the various industries, the fractionation of two pigments and further identification of active components merit more investigations.

In conclusion, most of the radiation resistant bacteria produce various antioxidant metabolites and enzymes that can use in biotechnology and therapeutics. We found high antioxidant activity in the pigments of UVC tolerant *M. esteraromaticum* NM1 and *D. schimae* NM3 strains. Natural pigments provide opportunities in pharmaceutical, nutraceuticals, cosmetic, food and feed industries. High quantities of carotenoid pigments from these strains could be produced by engineering of the enzymatic pathway in an industrial process.

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