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ORIGINAL ARTICLE

Radio-protective and antioxidative activities of astaxanthin from newly isolated radio-resistant bacterium *Deinococcus* sp. strain WMA-LM9

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Abstract A radio-resistant bacterium, designated as strain WMA-LM9, was isolated from desert soil. 16S rRNA gene sequencing indicated that the bacterium belongs to genus *Deinococcus* with maximum similarity to *Deinococcus radiopugnans*. *Deinococcus* sp. strain WMA-LM9 was found to be resistant to a ultraviolet (UV) dose of 5×10^3 J/m^2, hydrogen peroxide (50 mM) and mitomycin C (10 μg/ml). A carotenoid pigment was extracted using chloroform/methanol/acetone (7:5:3) and purified by high-performance liquid chromatography on a C_{18} analytical column. The compound was characterised as mono-esterified astaxanthin by ^{1}H, ^{13}C nuclear magnetic resonance and mass spectrometry. It was tested for antioxidant activity, total flavonoids and phenolic content, radio-protective potential in correlation to the prevention of protein oxidation and DNA strand breaks in vitro. The carotenoid pigment showed a very potent antioxidant activity and significantly stronger scavenging ability against superoxides, with an IC_{50} (concentration causing 50% inhibition of the desired activity) of 41.6 μg/ml. The total phenolic and flavonoid contents were 12.1 and 7.4 μg in terms of gallic acid and quercetin equivalents per milligram of dried mass, respectively. astaxanthin also showed a higher inhibitory action against oxidative damage to collagen, elastin and bovine serum albumin than did β-carotene. The carotenoid also inhibited breaks to DNA strands, as indicated by the results of the DNA damage prevention assay. We conclude that astaxanthin from *Deinococcus* sp. strain WMA-LM9 has protective effects against radiation-mediated cell damage, and it also protects cellular protein and DNA against oxidative stress and other anti-oxidant activities.

Keywords *Deinococcus* sp. · Astaxanthin · HPLC · NMR · Antioxidant · Protein oxidation

The original version of this article was revised: correct data is presented.

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Introduction

Extremophiles are organisms that can survive and optimally grow under extreme conditions, such as in volcanic areas, extreme depths of the sea, hot springs and low oxygen environments (Kumar et al. 2010). Among these extremophiles, radio-resistant organisms are very important because they can survive at high radiation levels (both ionising and non-ionising) that cause, for example, oxidative damage to biomolecules such as nucleic acid and proteins. Extreme energy radiation tolerance has been detected in various members of the archaea and bacteria domains, with members of the genera *Rubrobacter* and *Deinococcus* exhibiting high levels of resistance to ionising radiation (Fredrickson et al. 2004). In addition, a number of radiation-resistant bacteria have recently been reported from the Atacama Desert, Chile, the Sonoran Desert, Arizona, and a manganese mine in northern Argentina, including *Hymenobacter* sp. and *Geodermatophilus* sp.; these bacteria were found to have a higher survival profile than *Deinococcus radiodurans*, a bacterium generally considered to exhibit the highest resistance to radiation (Paulino-Lima et al. 2016). Many radiation-resistant organisms displaying diverse metabolic properties as well as desiccation resistance have been reported from the Taklimakan Desert in Xinjiang (Yu et al. 2015). In total, 52 γ-radiation-resistant bacteria were isolated from the

Taklimakan desert samples that were clustered into five group based on 16S rRNA sequencing (Yu et al. 2015). These extremophiles use various mechanisms to protect themselves from ionising radiation, including the synthesis of certain antioxidants to capture oxygen radicals and well-developed DNA repair mechanisms (Betlem et al. 2012). With regard to penetrating light, these bacteria reduce excess light and quench what is not required, thus escaping undesirable photochemical impairment. *Deinococcus radiodurans* is one of the most well studied of the radio-resistant, red-pigmented and non-photosynthetic bacteria. The function of the red pigment is assumed to be that of providing resistance against highly energetic radiation (Cox and Battista 2005).

Carotenoids play no role in the normal cell growth, reproduction or development of organisms, but its absence does affect survivability as these organic pigments are effectual trackers of reactive oxygen species (ROS), predominantly that of singlet oxygen ($^1O_2$) and peroxyl radicals (Tatsuzawa et al. 2000; Stahl and Sies 2003). As such, carotenoids are metabolic assets to microbes, and they have widely been studied for various industrial uses (Ferrer et al. 2007; Gostincar et al. 2010). The assembly of carotenoids from natural sources has been a major focus of research (Scarpa and Ninfali 2015). *Deinococcus radiodurans* produces deinoxanthin, a unique keto carotenoid that contributes to its resistance to oxidative stress through its potent ROS quenching ability compared to β-carotene and lycopene (Saito et al. 1998; Tian et al. 2007; Peng et al. 2009). In fact, carotenoids protect DNA strands from oxidative damage, membranes from lipid peroxidation and proteins from carbonylation (Zhang and Omaye 2000).

All types of ROS, such as the hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), superoxide (O_2^-) and $^1O_2$, and reactive nitrogen species, such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), can be hunted down by the carotenoids from *D. radiodurans* in vitro (Tian et al. 2007; Zhang et al. 2007). These carotenoids also have the ability to protect plasmid DNA (20%) exposed to •OH and to facilitate recovery of the supercoiled plasmid form that is completely disarranged (Tian et al. 2009). Advancements in metabolomics, proteomics and genomics have increased interest in the study of the genes and their proteins that help regulate microbial metabolic assets in such an extreme environment (Ferrer et al. 2007; Hammon et al. 2009; Singh et al. 2010).

We have investigated the role of astaxanthin, a keto-carotenoid extracted from *Deinococcus* sp. strain WMA-LM9, in the resistance of the cells to short wave ultraviolet B (UV-B), H_2O_2 and mitomycin C. This carotenoid was purified and investigated for its antioxidant and cytotoxic activity. Oxidative damage to various proteins, namely, bovine serum albumin, collagen and elastin, in the presence or absence of carotenoids and the possible role of astaxanthin in *Deinococcus* sp. WMA-LM9 resistance to intracellular protein carbonylation were also studied. We confirmed that this carotenoid can neutralise the effect of different ROS resulting from oxidative stress that can damage microbial DNA. To the best of our knowledge, this is the first report of an astaxanthin-producing *Deinococcus* strain with highly anti-oxidant and protein oxidation-preventing activities.

Material and methods

Isolation of radio-resistant bacteria

Soil samples were collected aseptically from the desert of District Lakki Marwat, Khyber Pukhtoonkhwa, Pakistan, in sterile zipper plastic bags and immediately transported to the laboratory where they were kept at 4 °C before investigation for the radio-resistant microbial community. In the laboratory, the soil samples were serially diluted in phosphate buffer saline (PBS) and inoculated on basic medium [trypton glucose yeast extract agar (TGY) medium containing g/L: trypton, 10; yeast extract, 5; glucose, 1] by the spread plate method. TGY plates were exposed to UV-B radiation (wavelength 280 nm) for 5 min prior to incubation. Each sample was UV irradiated in UV chamber (119 × 69 × 52 cm) that was equipped with a 20 W, 280-nm UV light at the top. The choice of UV-B was based on the property of this radiation to cause a serious damage to cellular DNA due to its shorter wave length. Moreover, UV-B is considered to be the only radiation to cause both direct and indirect damage. The UV fluence rate (energy per area per time) to the test sample was calculated by using following equation in joules per metre squared (J/m^2):

$$He = Ee \times t$$

where He is the radiant exposure that is measured by energy reaching a surface area (Ee) for a specific time (t). Total UV dose was determined by time of exposure to the UV fluence rate. All UV irradiation procedures were performed under red light to prevent photo-reactivation. After irradiation, the plates were incubated at 37 °C for 5–7 days. The isolates were subcultured from irradiated plates and again exposed to UV radiation for further confirmation of their radio-resistance. Several fractionated doses ranging from 300 to 3300 Jm^{-2} (30–300 s) were used to determine the survivability rate of all the UV-resistant isolates. Strain WMA-LM9 was selected on the basis of tolerance to maximum doses of UV radiation.

Identification of radio-resistant bacterium

Based on high tolerance to UV radiation, H_2O_2 and mitomycin C, strain WMA-LM9 was identified morphologically as well as biochemically by previously described methods (Shah et al. 2013). Molecular identification was carried out by sequencing the 16S rRNA gene. For this purpose, the DNA was extracted

using a DNA extraction kit (QIAGEN, Hilden, Germany), and the 16S rRNA gene sequence was amplified using 27F′ (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R′ (5′-CTAC GGCTACCTTGTTACGA-3′) bacterial primers. The amplified PCR product was sequenced by Macrogen Service Center (Geunchun-gu, Seoul, South Korea). The nearest relatives of the sequence were searched in the NCBI database using the BLAST tool, and homologues were analysed for phylogeny using Molecular Evolutionary Genetic Analysis (MEGA) version 6. The neighbour-joining phylogenetic (Tamura and Nei 1993) tree was constructed for the identification of the isolated bacterial strain, and diversity among UV-resistant extremophiles was studied (Tamura et al. 2013). Finally, the sequence was submitted to NCBI GeneBank for assignment of an accession number.

Bacterial survival curves and oxidative stress

Strain WMA-LM9 was tested for radiation resistance and a survival curve was plotted (Mattimore and Battista 1996). The cell culture of strain WMA-LM9 was serially diluted (1:1000) with PBS and spread on TYG agar plates, then exposed to different doses of UV radiation at 280 nm. The survival rate was determined by dividing the number of colonies appearing on UV-irradiated plates by the number of colonies on un-irradiated culture plates. For the determination of oxidative stress and mitomycin C tolerance, an overnight grown culture of strain WMA-LM9 in TGY broth was diluted in sterile normal saline up to an optical density at 60 nm (OD_{600}) of 0.5. The cell suspension was treated with different molar concentrations of H_2O_2 (5–40 mM) for 30 min and mitomycin C (2–10 μg/ml) for 20 min, and then the cells were cultured on TGY agar plates. The plates were incubated at 30 °C for 3 days at which time the number of persisting colonies were counted. The survival rate was expressed as the difference in the number of colonies between the treated and untreated cultures. All experiments for the survival curve were run in triplicate.

Measurement of intracellular protein carbonylation

Protein carbonylation was measured using the 2,4-dinitrophenyl hydrazine (DNPH) method (Cao and Cutler 1995; Misra et al. 2004). Cells were lysed by sonication and the cell-free extract then collected for the protein carbonylation assay. The total protein concentration was estimated by the method of Lowry et al. (1951). The cell-free extract (2 mg/ml of protein) in 50 mM PBS (pH 7.4) was incubated with 400 μl of 10 mM DNPH in 2 M HCl for 2 h. Proteins were precipitated and then resuspended in 6 M guanidine hydrochloride. The solution was centrifuged, and the supernatant was analysed spectrophotometrically at 370 nm. A protein control was run in parallel whereby DNPH was replaced with 2 M HCl. The protein carbonyl content was expressed in millimoles per milligram protein.

Preparation of the carotenoid extract

An aliquot (500 ml) of *Deinococcus* sp. WMA-LM9 culture grown under aerobic conditions with continuous shaking was harvested after 48 h by centrifugation at 5000 *g* for 10 min. After washing with sterilised water, the cell pellet was extracted with acetone:methanol:chloroform (7:5:3) by probe sonication (150 W at 40 kHz). The cell suspension was then once again centrifuged (10,000 *g*, 10 min), and a clear red colour supernatant was recovered. The supernatant was allowed to dry and then dissolved in methanol for further study.

Reversed-phase high-performance liquid chromatography

The crude extracts were analysed by flash chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC) on a Waters 2690 Alliance system (Waters Corp., Milford, MA) consisting of a Hypersil ODS-C18 column (pore size 5 μm, 4.6 × 250 mm) protected by a guard column. All solvents of HPLC grade were degassed (Merck & Co., Kenilworth, NJ; Millipore00 Corp. Billerica, MA; Merck KGaA, Darmstadt, Germany) and filtered through a 0.2-μm filter prior to the HPLC analysis. The column was maintained at a constant temperature of 25 °C using a standard column thermostat. An isocratic technique eluted with a mixture of acetonitrile, methanol and isopropanol (40:50:10, v / v) at a flow rate of 0.8 ml/min was used as the mobile phase (Saito et al. 1998). The fractions eluted were monitored with a Waters 996 photodiode array detector (Waters Corp.).

Liquid chromatography–tandem mass spectrometry analysis

The carotenoid extracts from *Deinococcus* sp. WMA-LM9 were analysed in an liquid chromatography–tandem mass spectrometry (ABX3200 Q-TRAP mass spectrometer equipped with a TurboIonSpray ESI source, and connected to a Shimadzu HPLC system with dual LC-20 pumps, a SPD-M20A UV/Vis photodiode array (PDA) detector and auto sampler). A 10-μl sample dissolved in LC–MS grade methanol was injected onto a column (C18; internal diameter 5 μm, 250 × 4 mm; Bischoff, Germany). The mobile phase comprised methanol (solvent A) and acetonitrile (solvent B) with 0.1% (v/v) formic acid, used in a gradient mode for B [0.0/30; 25/100; 35/100; 45/30 (min/%)] at a flow rate of 0.8 ml/min. The system was controlled and data were analysed on a computer equipped with Thermo Scientific Xcalibur 2.2 (Thermo

Fisher Scientific, Waltham, MA). The mass spectrometer was used in positive ion mode to detect *m/z* transitions $[M + H]^+$.

^1H and ^{13}C nuclear magnetic resonance studies

A nuclear magnetic resonance (NMR) spectrum provides prime evidence on the structure of a compound. NMR spectroscopic data were recorded at room temperature on a Bruker Avance 400 MHz NMR spectrometer (Bruker Corp., Billerica, MA) in dCDCl3 (CDCl3) referenced to residual protonated solvent (δ 7.24 ppm, δ 77.0 ppm, with tetramethylsilane as an internal standard. The purified sample was placed in an inert solvent (CDCl3), and the solution was placed between the poles of a powerful magnet. The different chemical shifts of the carbon and proton in response to their molecular environs within the molecule were measured in the NMR apparatus relative to a standard, usually TMS. The intensity of the signals was integrated to demonstrate the number of carbons and protons resonating at any one frequency. Each chemical shift value corresponds to a set of protons and carbons in a particular environment, and the intensity of each signal signifies the number of protons and carbon of each type.

Anti-oxidant activity and determination of total phenolic/flavonoid contents

The anti-oxidant activity of carotenoid was determined by the commonly used 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Xu et al. 2005). Various concentrations of carotenoid (5–20 μg) were placed in wells of a 96-well microtiter plate, and the volume was raised uniformly up to 200 μl using DPPH before the microtiter plate was incubated for 30 min at 37 °C. Ascorbic acid was used as a standard at same concentrations as the test samples. Absorbance was measured at 517 nm using methanol as the blank on the UV-visible spectrometer. The half maximal inhibitory concentration (IC_{50} value) for carotenoid as well as the standard preparation were calculated. The following formula was used to calculate the free radical scavenging activity:

$$\%\text{Scavenging} = (\text{Control}-\text{Sample}/\text{Control}) \times 100$$

Total phenolic and flavonoid phenolic contents were analysed in the extracted carotenoid from *Deinococcus* sp. strain WMA-LM9. The Singleton et al. (1999) method was used to determine the total phenolic content using gallic acid as the standard, and the aluminium chloride colorimetric assay was used to calculate total flavonoid contents (Zhishen et al. 1999) by plotting the quercetin standard curve. Phenolic and flavonoid contents were measured in microgrammes of gallic acid and quercetin equivalents per milligramme (GAE/mg and QE/mg) of dried extract.

Protein oxidation inhibition assay

The inhibitory effect of carotenoid on protein oxidation in vitro was quantified using bovine serum albumin, collagen and elastin as standard proteins. Samples of targeted proteins (200 μl; 1 mg/ml) were incubated with 100 μl of carotenoid dissolved in tetrahydrofuran and treated with 100 μl of (1 mmol/L) $FeSO_4$ and 100 μl of 80 mmol/L of H_2O_2 at 37 °C for 1 h, following which 15 U of catalase was added to stop the reaction. The mixture was then incubated with 600 μl of 10 mmol/L DNPH for 1 h, followed by the addition of 10% trichloroacetic acid to precipitate out the unbound protein. The mixture was dissolved in 6 M guanidine hydrochloride and quantified spectrometrically at 370 nm. Percentage inhibition of protein oxidation by carotenoids was calculated using H_2O to replace $FeSO_4$ and H_2O_2 as a blank:

$$(\%)\ \text{Inhibition of protein oxidation} = (\text{Control}-\text{sample}/\text{control}) \times 100$$

DNA damage prevention assay

The DNA damage-preventing capability of the carotenoid pigment was determined by incubating plasmid pUC18 in a reaction mixture that contained 2 μl plasmid, 3 μl (6 μg) and 6 μl (12 μg) carotenoid solution, 3 μl 2 mM $FeSO_4$, 4 μl 1 M sodium nitroprusside and 4 μl 30% H_2O_2, for 1 h at room temperature. H_2O_2 and sodium nitroprusside usually produces single-strand breaks in DNA. The pattern of bands of the treated samples, as well as of the positive and negative control, was examined using the gel electrophoresis technique.

Cytotoxic assay

The cytotoxicity of the carotenoid was determined by brine shrimp assay (Maridass 2008). Artificial seawater was prepared by dissolving 34 g of sea salt per litre of distilled water and placed in vials with 15–20 mg eggs of brine shrimp (*Artemia salina*). Carotenoid dissolved in DMSO was added to the vials at different volumes (15, 50, 100 μl) and incubated for 24–48 h at 30 °C. Cytotoxicity of carotenoid was determined by counting the number of live shrimps.

Statistical analysis

Student's *t* test was used to assess the significance between results, and $p < 0.05$ was considered to be significant. Bacterial sensitivities to UV, H_2O_2 and mitomycin C and percentage scavenging activity of carotenoid were studied by regression analysis between the percentage of survival/

inhibition and their respective concentrations. Single factor and two-way analysis of variance were applied for analysis of protein carbonylation and the in vitro percentage protein oxidation inhibition assay between groups and within a single group.

Results

UV-B selection and isolation of radio-resistant bacteria

The shorter wavelength UV-B (280 nm) radiation carries 3.94–4.43 eV of energy per photon and can damage cellular DNA and causes various types of damage to DNA. The most common damage products resulting from UV-B radiation are cyclobutane pyrimidine dimers and pyrimidine–pyrimidone (6–4) photoproducts, which and lead to CC-TT or C-T transitions.

In our study we isolated nine different radio-resistant bacterial strains from the desert soil samples. These were designated WMA-LM4, WMA-LM9, WMA-LM10, WMA-LM15, WMA-LM19, WMA-LM30, WMA-BD1, WMA-BD2 and WMA-BD4. Strain WMA-LM9 was selected on the basis of its high resistance to UV radiation, i.e. 50% survival rate following exposure to UV-B radiation dose (280 nm) of 5×10^3 Jm^{-2} (Table 1).

Identification of strain WMA-LM9

Strain WMA-LM9 was found to be a Gram-positive diplococcus, tetrad in arrangement, with round, red, raised, mucoid and opaque colonies. The 16S rRNA sequence of this strain was assembled using DNA baser software and subjected to a BLAST search in the National Centre for Biotechnology Information (NCBI) database. The results indicated that this strain belongs to genus *Deinococcus*, with 99% similarity with *Deinococcus radiopugnans*. The phylogenetic tree was constructed, and strain WMA-LM9 (KT008384) was clustered into *Deinococcus* group among the sequences obtained from NCBI (Fig. 1).

Table. 1 Radio-resistant bacteria isolated from the extreme desert environment and their tolerance to UV radiation doses

Isolates	UV radiation resistance ($J\ m^{-2}$)	Survival rate (%)
WMA-BD1	3.3×10^3	45.45
WMA-BD2	2.0×10^3	43.18
WMA-BD4	2.0×10^3	48.27
WMA-LM4	2.60×10^3	45.28
WMA-LM9	3.30×10^3	79.47
WMA-LM10	1.30×10^3	46.15
WMA-LM15	3.30×10^3	38.72
WMA-LM19	1.30×10^3	51.69
WMA-LM30	3.30×10^3	68.03

Resistance to UV radiation, oxidative stress and mitomycin C

Strain WMA-LM9 was exposed to different energy doses of UV radiation to determine its radiation-resistant potential. This strain was observed to maintain nearly 50% viability at a UV radiation dose of 5 K Jm^{-2}, whereas the *Escherichia coli* strain (ATCC 10536) did not survive at this high level of UV radiation (Fig. 2a). A gradual decrease in the survival of strain WMA-LM9 was observed with increasing H_2O_2 concentration, but a viability of up to 49% was maintained at 50 mM H_2O_2 for 60 min (Fig. 2b). The bacterium was quite resistant to mitomycin C, with a >50% of survival rate observed at up to 8 μg/ml, whereas the *E. coli* cells were unable to survive at this concentration (Fig. 2c). The percentage values shown in Fig. 2 had an exponential distribution.

Measurement of intracellular protein carbonylation level

Total protein oxidation was significantly different ($p < 0.05$) in *Deinococcus* sp. WMA-LM9 and *E. coli* (ATCC 10536) (0.128091 ± 0.00585 vs. 0.197378 ± 0.0191 μM/mg, respectively) indicating lower protein oxidation in the radio-resistant WMA-LM9 strain as compared to *E. coli* (ATCC 10536). The results shown in Fig. 3 indicate that lack of ability to produce carotenoids in *E. coli* (ATCC 10536) became more sensitive to oxidative damage caused by UV stress and H_2O_2.

Carotenoid extraction and purification

The carotenoids were extracted from the cells of *Deinococcus* sp. WM-LM9 and analysed by HPLC. Fraction 7 of the flash chromatography showed the highest scavenging activity and was subsequently subjected to RP-HPLC. Two distinct peaks were observed with a retention time of 2.3 and 4.9, respectively; these were collected separately and labelled LM9F1 and LM9F2, as shown in Fig. 4. An aliquot of 15 mg of pure astaxanthin was extracted from *Deinococcus* sp. WM-LM9 with a purity of >90%.

Liquid chromatography–MS/MS

The LM9F1 fraction, which exhibited the highest antioxidant activity, was subsequently subjected to LC–MS/MS for mass identification of $C_{40}H_{52}O_3$ by elemental analysis and electron ionisation–liquid chromatography (EIMS). The positive electrospray ionisation mass spectrometry (ESI-MS) spectrum of the carotenoid extract exhibits signals at *m/z* 597 $[M + H]^+$ (LM9F1) and 610 $[M + H]^+$

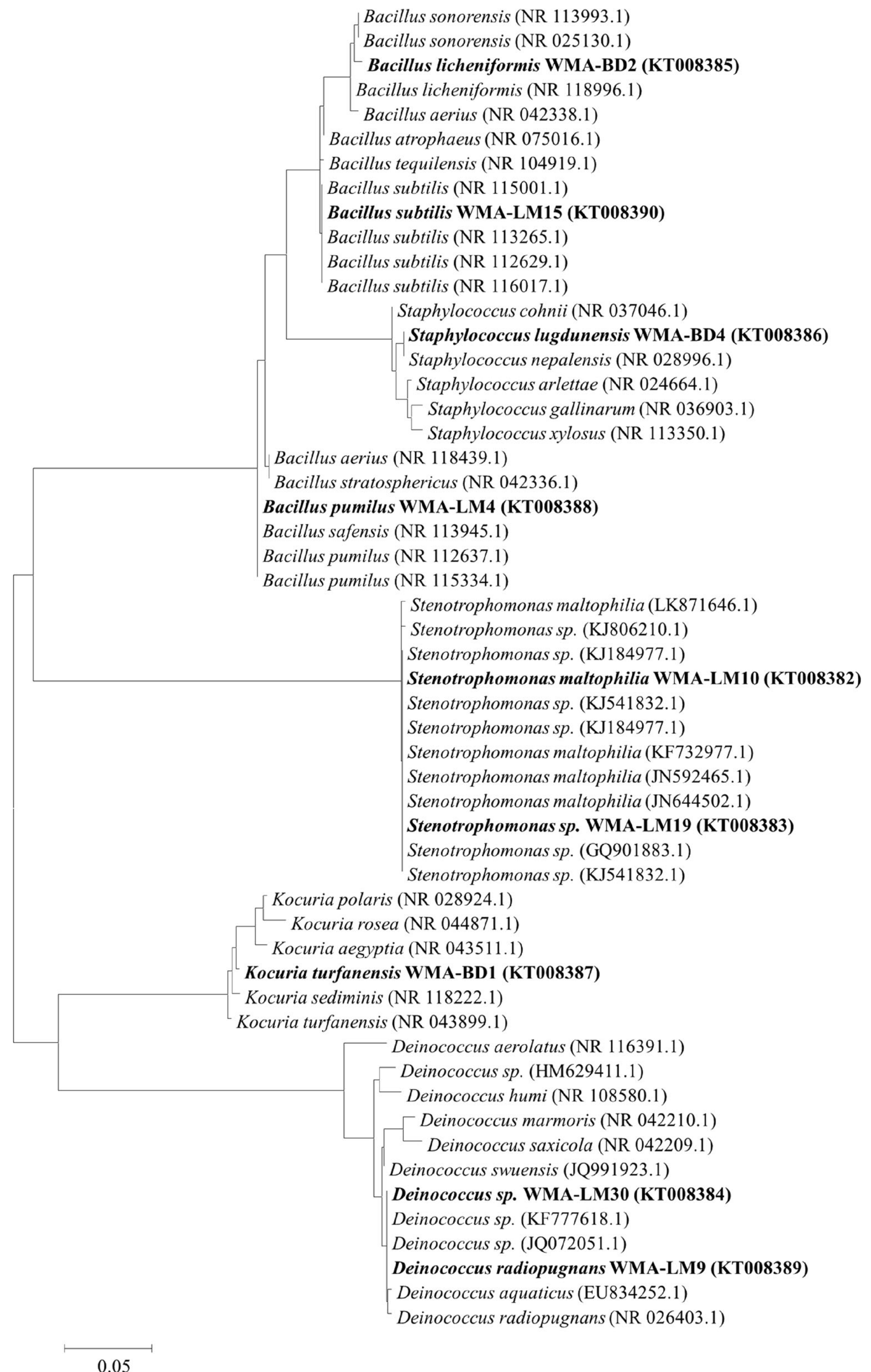


Fig. 1 Neighbour joining phylogenetic tree based on 16S rRNA gene sequence analysis, showing the position of isolate WMA-LM9 to other strains of *Deinococcus* identified following a search of the NCBI database. Accession numbers of the sequences used in this study are shown in *parentheses* after the strain designation. *Numbers at nodes* Percentage bootstrap values based on 1000 replications. *Bar*: 0.05 sequence variation. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

(LM9F2) (Fig. 4). The major component, m/z 596, accounted for more than 70% of the total carotenoids. The maximum wavelength (λ_{max}) of the UV spectrum of the compound was at 475 nm.

^{1}H and ^{13}C NMR

The purified extract once dissolved in an inert solvent (CDCl3) was analysed for carbon and proton spectra to

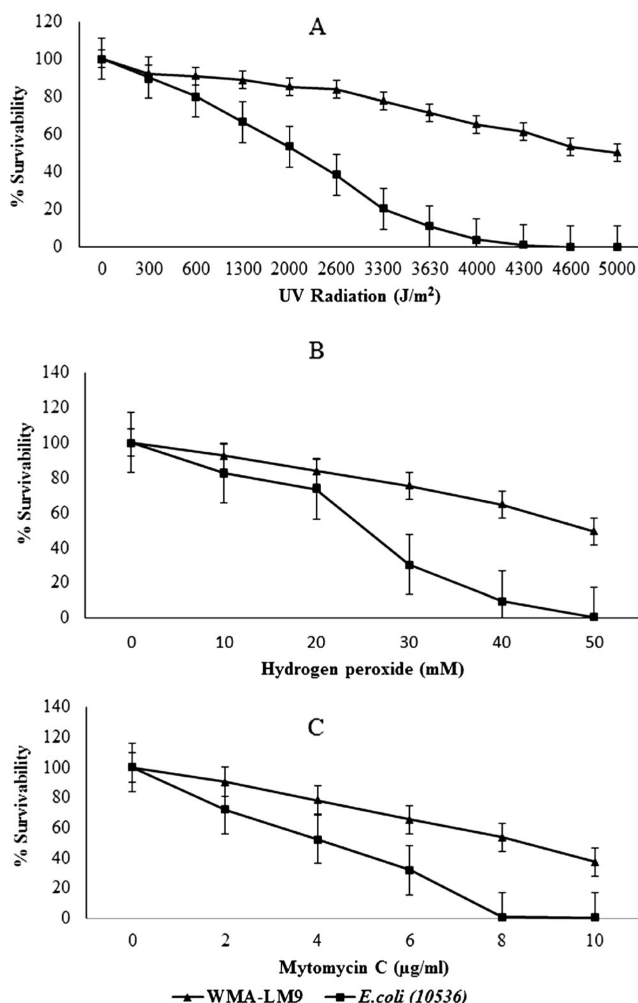


Fig. 2 Survivability of strain WMA-LM9 isolated from desert soil following exposure to different energy levels of ultraviolet B (UV-B) radiation. **a** UV radiation-resistant potential of WMA-LM9, **b** resistance to different concentrations of hydrogen peroxide (H_2O_2; mM), **c** resistance to different concentrations of mitomycin C (μg/ml). Percentage survivability value is calculated as $N_1/N_0 \times 100$ where Ni is the value after exposure to irradiation, H_2O_2 or mitomycin C, and N_0 is the value at time 0, for each condition tested. Results are highly significant among different groups at $p < 0.05$ (Student's unpaired *t* test). Values are given as the mean ± standard deviation (SD; *error bars*) of triplicate experiments

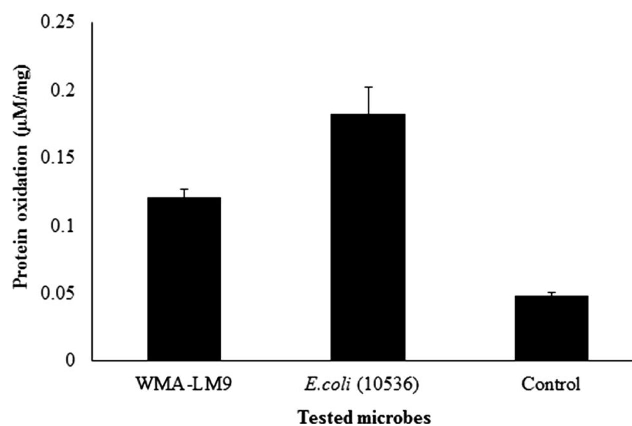


Fig. 3 Comparison of intracellular protein carbonylation level between the radio-resistant *Deinococcus* sp. strain WMA-LM9 and *E. coli* (10536) following exposure to UV radiation and H_2O_2. The results between the three groups are highly significantly different at $p < 0.05$. Values are given as the mean ± SD

investigate the properties of the chemical structure. The peaks obtained by NMR spectroscopy were found to contain astaxanthin. All of the peaks were compared with available spectra of astaxanthin and the identity confirmed.

^{1}H NMR spectroscopy of the pure extract showed a diastereomeric arrangement in the olefinic region that provides significant evidence for the presence of a carbohydrate back bone in the carotenoids. NMR spectra showed that the pure compound had a hydrophobic nature and was arranged in isoprene residues with a long conjugated chain of double bonds. Therefore, our attention was directed toward the chemical shift area for double bonds. The formation of a *cis* bond results in characteristic shift differences compared to all-*trans* compounds. The proton NMR data revealed that signals between 6.0 and 7.8 ppm represent the 14 (−CH) methine protons on the astaxanthin backbone and a near bilateral symmetry around the central double bond. The conjugation system described imparts carotenoids with excellent light-absorbing properties in the blue–green (450–550 nm) range of the visible spectrum. The signals in the range of 0.99 to 2.1158 ppm represent 30 protons from the methyl group, and two •OH protons show a peak at 3.627 ppm. In addition, the presence of unsaturated fatty acids was detectable by the appearance of multiplets between 5.2–5.4 ppm attributed to methine protons (Fig. 5a).

In the ^{13}C NMR spectra, the signals attributed to the carbon atoms found in the carbonyl moieties are good indicators of astaxanthin structure. The C-1 is a quaternary carbon, and it's peak was seen at 29.69 ppm. In the spectrum, the C-4 (C = O) signal is downfield at 200.14 ppm. The ^{13}C NMR spectra of the compound reveals the presence of C = C carbon-3 position, with 162.27 ppm on both sides of the ring showing the presence of the ester carbonyl. The spectra also confirm the monoester linkage in the compound. C-5, an alpha carbon to carbonyl, and an olefinic region shows a peak at 126.76–136 ppm. The eight methyl groups at these olefinic carbons show peaks at 14.00 and 26.13 ppm (Fig. 5b). The signals corresponding to the carbon atoms of the carotenoid were found to be the same as those of astaxanthin (Fig. 5c) reported in the literature.

Anti-oxidant activity of carotenoid

The DPPH• radical scavenging activity of carotenoid extract dissolved in methanol was measured as shown in Fig. 6. The scavenging activity was found to be concentration dependent and almost 50% (IC_{50}) of the DPPH• radicals were scavenged at 41.6 μg/ml carotenoid, which was greater that the activity of

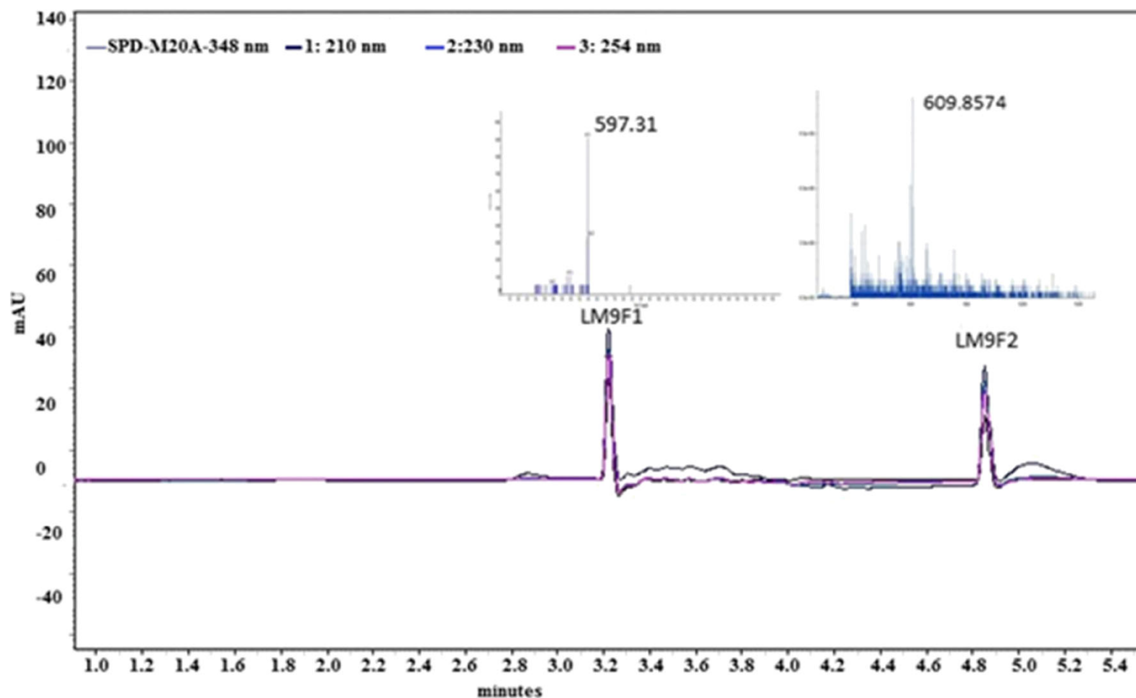


Fig. 4 High-performance liquid chromatography chromatogram/positive electrospray ionisation mass spectrometry (ESI-MS) spectrum of carotenoid extract exhibits signals at *m/z* 597 $[M + H]^+$, and 609 $[M + H]^+$ LM9F1 and F2, respectively

β-carotene (20%). However, the carotenoid extract scavenged less DPPH radical (80 ± 1.7%) than the ascorbic acid-positive control. The IC_{50} was obtained by linear regression analysis of the dose–response curve plotting percentage inhibition against carotenoid concentration, which led to 50% inhibition of free radical activity of DPPH.

The phenolic and flavonoid contents in the carotenoid extract (astaxanthin) were quantified by using the standard calibration curve equation. Various concentrations (5–20 μg) of gallic acid and quercetin were used as standards to plot the calibration curve, and the results were expressed as microgrammes of standard equivalents. Total phenolic and flavonoid contents were 12.1 ± 1.3 and 7.4 ± 1.0 μg/mg of the respective standards equivalence (μg of GAE/mg and μg of QE/mg), as determined from the calibration curve equation.

Protein oxidation inhibition assay

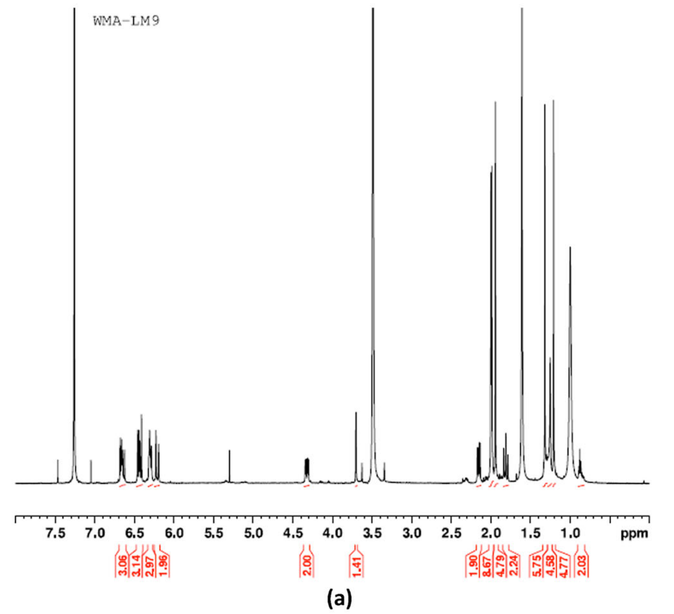
The inhibitory effect of astaxanthin from strain WMA-LM9 against oxidative damage of three standard proteins, i.e. bovine serum albumin, collagen and elastin were studied using β-carotene as the standard. astaxanthin was able to inhibit protein oxidation by 40–45 ± 10.3%, which is a greater inhibition than that achieved with β-carotene (15–20 ± 3.3%). Further, 10 μg of this carotenoid inhibited protein oxidation better than β-carotene, with the difference being significant at $p < 0.05$ using *t*-test (Fig. 7).

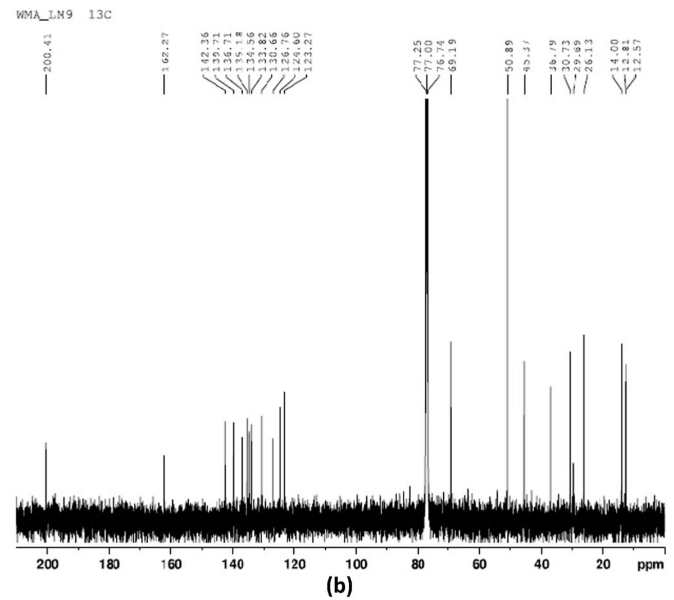
Inhibition of protein oxidation and DNA damage prevention

The effect of astaxanthin on DNA damage prevention was investigated using a hydroxyl radical-induced DNA breaks system in vitro. Specifically, plasmid pUC18 was incubated with H_2O_2 and sodium nitroprusside in the presence and absence of carotenoid. The plasmid DNA was broken down by the attack of the•OH generated from the Fenton reaction, as indicated by smear formation in the negative control in Fig. 8. The DNA was completely protected from oxidative damage by H_2O_2 and sodium nitroprusside by the presence of carotenoid in test samples T1 and T2 (6 and 12 μg) in the reaction mixture, showing promising results in DNA prevention (Fig. 8).

Cytotoxic activity of carotenoid

The cytotoxic activity of astaxanthin was determined by the brine shrimp assay, carried out at four different concentrations of carotenoid. We observed that there was no toxic effect at the





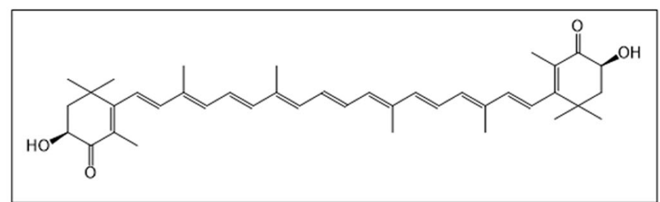


Fig. 5 ^{1}H (**a**) and ^{13}C (**b**) nuclear magnetic resonance (NMR)spectra of purified compound LM9F1 from the carotenoid extract. The purified compound was identified as "astaxanthin" with the chemical structure shown in **c** as determined from NMR peaks using ChemDraw software

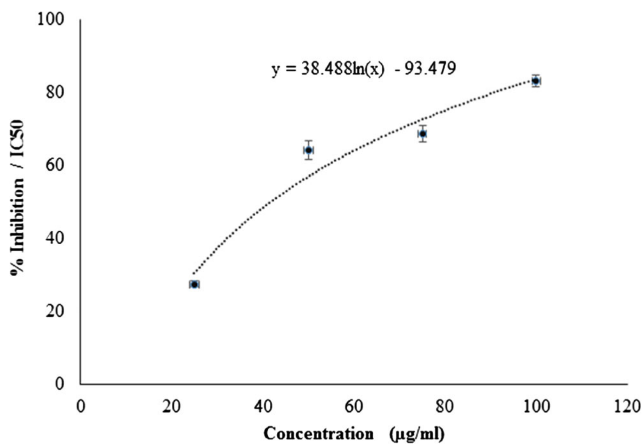


Fig. 6 Anti-oxidant activity of carotenoid extracted from strain WMA-LM9. The IC_{50} (drug concentration causing 50% inhibition of the desired activity) of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was obtained by linear regression analysis of the dose–response curve plotting between percentage inhibition (*% inhibition*) or percentage activity (*y-axis*) and carotenoid concentration (41.6 μg/ml; *x-axis*). Results are expressed as the mean ± SD or standard error and are compared using the Student's unpaired *t* test. Significance was set at $p < 0.05$

lowest concentration of carotenoid tested, i.e. 25–100 μg, and only 30% cytotoxicity at the highest concentration (200 μg/ml) with an IC_{50} of 1567.62 μg.

Discussion

A hot and dry desert can be considered as a paradigm of an extreme environment for all forms of life due to several life-limiting factors, such as nutrient availability, extreme dryness

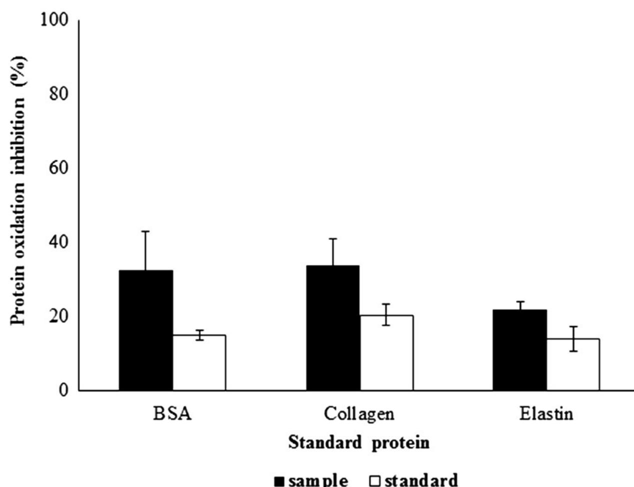


Fig. 7 Inhibitory effect of carotenoid from strain WMA-LM9 on the oxidation of different proteins in vitro. *Black bars* Carotenoid from strain WMA-LM9, *white bars* percentage inhibition of protein oxidation by commercially available standard β-carotene (Sigma–Aldrich, St. Louis, MO). Values are given as the mean ± SD. *BSA* Bovine serum albumin

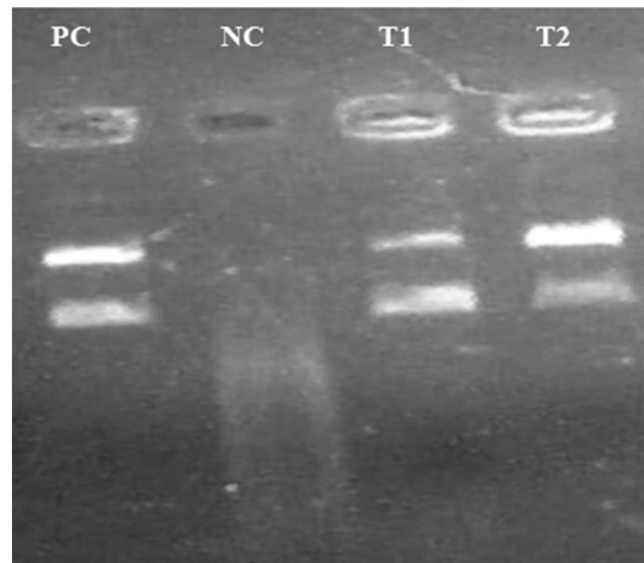


Fig. 8 Role of carotenoids in preventing oxidative damage to pUC18 plasmid DNA after exposure to oxidative agents. *Lanes*: *PC* Positive control (only plasmid DNA), *NC* negative control (plasmid DNA treated with hydrogen peroxide and sodium nitroprusside), *T1*, *T2* test samples (plasmid DNA, hydrogen peroxide, sodium nitroprusside and carotenoid at different concentrations)

and high temperature, as well as continuous exposure to high levels of UV radiation in sunlight. In recent years, improving our understanding of ROS-induced oxidative stress mechanisms and searching for suitable strategies to protect organisms from oxidative stress have been two major goals of medical research efforts (Vilchez and Manzanera 2011). In the present study surface soil collected from the desert of District Lakki Marwat was screened for the isolation of radio-resistant bacteria. The carotenoid extract from this bacterium was purified and evaluated for its ability to inhibit DPPH radical, protein and DNA oxidation activity.

We isolated a radio-resistant bacterium, *Deinococcus* sp. strain WMA-LM9, from desert soil. Strain WMA-LM9 showed prolonged resistance (50% survivability) to different energy doses of UV-B radiation and also was found to be viable following culture with mitomycin C (10 μg/ml) for 20 min and 50 mM H_2O_2 for 60 min, suggesting that this strain has a strong catalase and superoxide dismutase antioxidant system that protects the cell from oxidative damage (Prazdnova et al. 2014). Several researchers have reported that resistance to ionising radiation is directly linked with resistance to H_2O_2, mitomycin C and desiccation (Daly et al. 2007; Fredrickson et al. 2008; Daly 2009). The resistance of *Deinococcus* sp. strain WMA-LM9 to a high concentration of mitomycin C for 10 min generates 100–200 cross-links per genome without a loss of viability (Kitayama 1982). However, the detailed antioxidant mechanisms of this bacterium are still unknown.

The carotenoid compound in LM9F1 was analysed for astaxanthin ($C_{40}H_{54}O_4$) by elemental analysis and EIMS,

giving a molecular ion at *m/z* 597. Compared to *E. coli* (10536), astaxanthin protected cellular proteins of strain WMA-LM9 from oxidative damage. The carotenoids are effective scavengers of different toxic oxides and as such block the formation of all superoxides and Fenton reaction pathways that contribute to protein oxidation (Imlay 2003; Zongtao et al. 2009). The ability of members of genus *Deinococcus* to survive under several extreme conditions has been suggested to be due to three combined mechanisms, namely, prevention, tolerance and repair (White et al. 1999).

The mono-esterified astaxanthin from WMA-LM9 showed a twofold stronger quenching ability of superoxides than has been reported for deinoxanthin and β-carotenes, which may be attributed to the extra keto-group substitution and length of their conjugated double-bond system as compared with the other carotenoids. The antioxidant potential of carotenoids from radio-resistant microbes has been recognised as a contributory factor to the radioprotection offered by different compounds (Albrecht et al. 2000). A carotenoid extract from *Deinococcus radiodurans* was clearly able to scavenge superoxide anions, as demonstrated by Zhang et al. (2007) using the DPPH assay. In that study, the antioxidant results against free radicals by the DPPH assay showed that astaxanthin was capable of donating electrons to neutralise free radicals and that it can scavenge free radicals; hence, this molecule has potential as a chemotherapeutic drug to eliminate pathological diseases related to free radicals from a system. The photoprotection against toxic superoxides provided by astaxanthin is based on electron exchange energy transfer quenching (Galano et al. 2010). These authors suggested that protein rather than DNA is a potential target for UV-B radiation and free radicals. In our study, the carotenoid isolated from *Deinococcus* sp. strain WMA-LM9 was more effective in preventing the oxidation of different standard proteins, such as BSA, collagen and elastin, than *E. coli* (10536) . The protective effect of purified astaxanthin on proteins in *Deinococcus* sp. is the result of its free radical quenching ability. DNA repair proteins and many other important cell proteins and enzymes involved in cell recovery are protected by these naturally occurring carotenoids in the cells. Carotenoids can react with free radicals and are involved with some additional reactions at their conjugation double bonds, resulting in the formation of a relatively stable product, which in turn contributes to the inhibition of protein and lipid damage from oxidative products formed during stress (Krinsky and Johnson 2005; Cheng et al. 2014). We also noted that this newly reported monoesterified astaxanthin from *Deinococcus* sp. strain WMA-LM9 neutralised the effect of superoxide, H_2O_2 and sodium nitroprusside and protected pUC18 plasmid DNA from oxidative damage. It also restricted protein oxidation by inhibiting protein carbonylation that led to the prevention of indirect damage to DNA. The carotenoid may block the formation of 8-oxo-2-deoxyguanosine during the oxidation of DNA under stress conditions. Many important proteins, including DNA repair proteins and other enzymatic antioxidants, are protected by carotenoids as a means to prolong cell survivability under extreme conditions. Antioxidant-rich metabolites from radio-resistant extremophiles significantly reduce the risk of DNA damage (Singh and Gabani 2011).

The carotenoid isolated from *Deinococcus* sp. strain WMA-LM9 was found to be either non-toxic or only slightly toxic at higher concentrations, which suggests that it can be effectively used in drugs and for other therapeutic applications. Little is known about the total phenolic and flavonoid contents of the carotenoid of *Deinococcus radiopugnans*, which may contribute to the radio-protective ability and anti-oxidant activity of the carotenoid. The presence of aromatic hydrocarbons, a double-bond system and different keto groups indicated that the extracted carotenoid from *Deinococcus* sp. WMA-LM9 has a high resonance structure. We therefore investigated the phenolic and flavonoid groups that can contribute to the antioxidant ability of such carenoids, as well as to their ability to prevent DNA damage. Flavonoids impart mostly a characteristic colour (orange, violet, crimson, scarlet, mauve, blue) with beneficial health effects (Žilić et al. 2012). The OCH_3 group shifts the colour toward red (Grotewold 2006). The presence of oxo-groups at position 4 and nine or more double bonds in the carotenoids enhances the quenching activitys for singlet oxygen and superoxides (Terao 1989). The contribution of carotenoids and phenolic contents to radical scavenging activity has been described by several researchers (Fernandez and Galvan 2007; Wang et al. 2010). Our results confirm that both carotenoids and phenolics contributed to the radical scavenging property of the extract. It would seem that a high phenolic and flavonoid spectrum determines the medicinal importance of a specific carotenoid. *Deinococcus* sp. strain WMA-LM9 has higher levels of catalase and, therefore, has a greater resistance to UV, H_2O_2 and mitomycin C than *E. coli*.

Based on these results, we conclude that astaxanthin from the newly isolated *Deinococcus* sp. WMA-LM9 plays a key role in protecting the microorganism against UV-photooxidation and protecting proteins and DNA from oxidative damage, as well as contributing to cell resistance. Further investigations are required to determine the biosynthetic pathway of this astaxanthin in order to produce a highly active carotenoid via metabolic engineering.

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