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Carotenoid accumulation in *Dietzia maris* NITD protects from macromolecular damage

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Abstract

Over the past decades, nutraceuticals have attracted considerable interest because of their potential nutritional, safety and therapeutic effects. Carotenoid molecules are potent nutraceuticals with an attractive colour and have been shown to play crucial roles in the immune system and susceptibility of organisms to certain diseases. Reactive oxygen species (ROS) are considered to be a major threat to cellular components and lead to protein oxidation, lipid peroxidation and DNA damage. In the present study, canthaxanthin (CX), an orange-red keto-carotenoid, was extracted from *Dietzia maris* NITD. Carbonyl content measurement showed that the presence of CX can prevent protein oxidation in *D. maris* NITD. The inhibition of protein oxidation *in vitro* revealed a high correlation coefficient of the extracted CX with positive controls. Both the linoleic acid model (IC_{50} = 932.48 μg/ml) and the goat liver model experiments showed that CX can also inhibit lipid peroxidation *in vitro*. CX from *D. maris* NITD was also proved to be effective in maintaining the observable intactness of a model DNA (pBR322). The present study indicated that bacterial CX can be a useful bioactive compound to prevent oxidative damage incurred by cellular macromolecules in terms of pharmaceutical and nutraceutical aspects.

Key words: canthaxanthin, ROS, protein oxidation, nutraceutical, linoleic acid

Introduction

Cellular metabolism involves the formation of reactive oxygen species (ROS) in their routine metabolic pathways and defends their master cells from invading pathogens. ROS also play a key role in intracellular signalling pathways (Bera, 2019). However, the augmentation of ROS may sometimes lead to oxidative stress, and consequently, the protection provided by the antioxidant defence system may become ineffective (Simon et al., 2000). In such cases, different ROS, for example, hydrogen peroxide or hydrogen and superoxide radicals produced during respiration, might cause an oxidative injury to the building blocks of cells and tissues, i.e. proteins, lipids and nucleic acids (Simon et al., 2000). Cellular oxidation can interfere with the protein function as the unfolded proteins lead to an enhanced hydrophobicity and often result in the formation of toxic aggregates (Costa et al., 2007). Free radicals also cause oxidation of the cellular thiols, which eventually affects key enzymes and lipid peroxidation as well as leads to chromosomal aberrations (Kumar et al., 2013). Because of increased ROS levels, humans develop serious health conditions, including cancer, cardiovascular diseases, inflammatory conditions, diabetic complications, Alzheimer's disease, aging etc. (Sonar et al., 2016). For their survival, cells use enzymatic and non-enzymatic defence systems such as production of antioxidants (Simon et al., 2000). Synthetic antioxidants are presently becoming popular as nutritional supplements. However, possible side effects (e.g. cytotoxic and carcinogenic) of synthetic antioxidants have further increased the interest in searching for potential antioxidants of natural origin (Kumar et al., 2013). Hence, different bioactive molecules, e.g. polyphenols, unsaturated fatty acids, oligosaccharides etc., from natural sources have gained rapid popularity because of their strong radical scavenging activities (Mitra et al., 2016). In this regard, microorganisms are more suitable for the production of bioactive compounds be-

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cause they can give high yield independent of culture conditions as they can be grown in a laboratory on a variety of substrates (Mitra et al., 2017a).

Dietzia sp. (family Dietziaceae, suborder: Corynebacterineae, order: Actinomycetales) are gram-positive bacteria that have been isolated from various environments such as soil, sweet water ponds, and effluents from sugar industry (Venugopalan et al., 2013). Although the genus *Dietzia* appeared to be a promising hydrocarbon degrader in recent years (Gharibzahedi et al., 2014), some species such as *Dietzia natronolimnaea* HS-1 (Khodaiyan et al., 2007), *Dietzia maris* NITD (Goswami et al., 2012) and *Dietzia* spp. (Venugopalan et al., 2013) showed a significant ability to produce canthaxanthin (CX) as their secondary metabolite. CX is an orange-red ketocarotenoid with significant applications in food and feed industry owing to its strong antioxidant property and anticancer, anti-inflammatory and immunomodulatory activities (Mitra et al., 2018). *D. maris* NITD is a gram-positive, aerobic, non-motile bacterium that has been established as an efficient CX producer (Goswami et al., 2012). Carotenoids are receiving worldwide attention because of their strong antioxidant activity and attractive color. They have gained a strong place in biopharmaceutical and nutraceutical industries in addition to their use as food additives (Bera and Dutta, 2017; Mitra et al., 2017b).

In our previous work, CX from *D. maris* NITD (BCX) was shown to have high free radical scavenging activities in a purified form as measured by different antioxidant assays (Bera et al., 2015). In a follow-up study with different protective model systems, BCX was shown to have a high antioxidant activity after encapsulation in hydrogels (Bera and Dutta, 2017) or in a natural aqueous-based model system such as *aloe vera* (Bera et al., 2017). In addition to CX production ability, the bioremediation potential of *D. maris* NITD in terms of synthetic dye removal has also been reported recently (Bera et al., 2016). However, the effect of BCX on important cellular biomolecules (such as proteins, lipids or nucleic acids) in *D. maris* NITD has not yet been studied. In the present study, a non-carotenoid-producing strain *D. maris* 7011 was obtained to compare the protective mechanism of carotenoids in *D. maris* NITD (a carotenoid producer). Assays were performed to measure the levels of protein carbonylation and oxidation inhibition, and the results were compared with those of other standard carotenoids such as β-carotene, β-cryptoxanthin and zeaxanthin. The effect on lipid peroxidation was also analysed using the linoleic acid model (thiobarbituric acid reactive substances, TBARS) and the goat liver model. DNA damage prevention was determined by the DNA nicking assay by using the pBR322 plasmid DNA.

Materials and methods

Chemicals, bacterial strains and growth conditions

Media ingredients for bacterial cultivation were purchased from HiMedia, India. Chemicals for the protein oxidation assay, lipid peroxidation assay and DNA damage assay were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used in this study were HPLC grade (Sigma-Aldrich). The pBR322 plasmid DNA was purchased from Thermo Scientific, USA. Standard carotenoids were supplied by Sigma-Aldrich. *D. maris* NITD (accession number: HM151403) was previously isolated in our laboratory and was established as an effective CX producer (Bera and Dutta, 2017). Optimized medium (D-glucose: 15 g, yeast extract: 5 g, peptone: 10 g, sodium chloride: 5 g, distilled water: 1000 ml) supplemented with coconut water was used for a large-scale pigment production at the initial broth pH 6.7, 120 rpm and 25°C (Bera et al., 2017). The pigment was extracted according to the procedure of Goswami et al. (2012). The extracted pigment was concentrated using a rotary evaporator (Yamato, model RE301) and was freeze-dried (Digitech, India). Dried BCX was stored at 4°C for further experiments. *D. maris* 7011 was purchased from MTCC, Chandigarh, India, and maintained in a medium containing 30 g trypticase soy broth, 15 g agar and 1000 ml distilled water.

Measurement of protein carbonylation level

Cells of *D. maris* NITD and *D. maris* 7011 in a suspension with an optical density (OD) of approximately 0.3–0.5 at 600 nm were treated with H_2O_2 (30 mmol/l). The cells were harvested and re-suspended in phosphate-buffered saline (PBS) containing 1% v/v β-mercaptoethanol and 1 mmol/l PMSF (phenylmethylsulfonyl fluoride). To obtain a cell-free extract, the cells were sonicated and centrifuged (1000 *g* for 10 min). The protein concentration of the cell-free extract was determined (Bradford, 1976), and the proteins were diluted to 1 mg/ml of protein. The control protein was incubated

with 200 µl of 5 mmol/l DNPH (2,4-dinitrophenylhydrazine) and 1 mol/l HCl for 1 h in the dark and vortexed at regular intervals. TCA (10%, ice-cold) was used to precipitate the proteins which were washed 3 times with 50% ethyl acetate in ethanol to remove the excess DNPH. The decoloured precipitate was evaporated and dissolved in 1 ml of 3 M guanidine hydrochloride (Tian et al., 2009). The absorbance of the supernatant was measured at 370 nm. The protein control used was processed in a similar manner, but DNPH was replaced with 1M HCl. The protein carbonyl content was expressed as mmol/mg of protein.

Assay of the inhibitory effect of carotenoids on protein oxidation in vitro

To investigate the inhibitory effect of carotenoids in protein oxidative damage, bovine serum albumin (BSA) was used as the targeted protein. One millilitre of 1 mg/ml BSA was mixed with 500 µl of carotenoid samples (dissolved in tetrahydrofuran or THF) and then treated with 500 µl of 1 mmol/l $FeSO_4$ and 500 µl of 80 mmol/l H_2O_2 at 37°C for 1 h. To stop the reaction process, catalase (30 U) was added. The obtained solution was incubated with 1 ml of 10 mmol/l DNPH for 1 h and then treated as mentioned earlier in the "Measurement of protein carbonylation level" section. The absorbance of the supernatant was determined at the wavelength of 370 nm. The inhibition of protein oxidation (%) by carotenoids was calculated using the following equation:

$$\text{Inhibition of protein oxidation [\%]} = = \frac{[(A_{control} - A_{blank}) - (A_{sample} - A_{blank})]}{(A_{control} - A_{blank})} \times 100$$

where $A_{control}$ is the absorbance of the control, A_{blank} is the absorbance of the background (using H_2O replace $FeSO_4$ and H_2O_2 and A_{sample} is the absorbance of the sample (Tian et al., 2009). Ascorbic acid and butylated hydroxyanisole (BHA) were used as positive controls. The data obtained were compared with the inhibitory efficiency of other standard carotenoids such as β-carotene, β-cryptoxanthin and zeaxanthin.

Effect of carotenoids from D. maris NITD on lipid oxidation

Lipid oxidation study in the linoleic acid model

The inhibition of lipid peroxidation by the carotenoid extract was studied in a linoleic acid emulsion model according to the method described by Lingnert et al. (1979) with some modifications. Briefly, an emulsion of linoleic acid was prepared with Tween 20 in 0.2 M sodium phosphate buffer (pH 7.2). Next, 20 mM of the linoleic acid emulsion was prepared by dissolving linoleic acid in 5.0 ml absolute methanol and adjusting the volume to 25 ml with the addition of 0.2 M sodium phosphate buffer (pH 7.2). Five hundred microliters of each carotenoid extract (concentration range of 100–500 µg/ml) in methanol was mixed with 2.0 ml of linoleic acid emulsion and tested for antioxidant activity. $IC_{(50)}$ values were determined. Ascorbic acid and BHA were used as positive controls.

Lipid oxidation study in the goat liver model

Lipid peroxidation inhibition by the carotenoid extract was studied in the goat liver model following the method of Kumar et al. (2013) with some modifications. In brief, a freshly excised goat liver (experiments were repeated 3 times) was collected in a sterile container and kept in an icebox till further use. Ten grams of the liver was homogenized in cold PBS of pH 7.4. The colloidal solution containing unbroken liver cells were subjected to sonication, and 10% homogenate was prepared. The obtained solutions were filtered and centrifuged to get clear supernatants. The entire process was performed ice. Varying concentrations (100-500 µg/ml) of the extract were added to 5 ml liver homogenate, and lipid peroxidation was initiated by adding 250 µl ferrous sulphate (25 mM). After 30 min of incubation, 1 ml aliquots were placed in tubes, and the proteins were precipitated using ice-cold 10% TCA. The solutions were centrifuged, and the supernatants were mixed with 0.67% TBA in 50% acetic acid. The obtained mixtures were heated for 30 min in a boiling water bath, and the intensity of the pink complex was measured at 535 nm. The degree of lipid peroxidation was assayed by estimating the TBARS content, and the results were expressed as a percentage of inhibition (Tan et al., 2014). Ascorbic acid and BHA were used as positive controls.

DNA damage protection activity

To test the effects of carotenoids from *D. maris* NITD on DNA (pBR322) damage, the reaction was conducted in a sterile tube. DNA damage was induced by free hydroxyl radicals generated by Fenton's reagent (Tian et al., 2007). Briefly, 1 µl of DNA was suspended in 2.5 µl PBS (50 mmol, pH 7.4) and 3.5 µl $FeSO_4$

(2 mM). Next, 2.5 µl of the carotenoid extract was mixed in varied concentrations with the solution, and 4.5 µl H_2O_2 was added to start radical generation. The mixture was incubated at 37 °C for 30 min. The mixture was then subjected to 0.8% agarose gel electrophoresis using EtBr staining. Quercetin was used as a positive control.

Statistical analysis

All experiments were performed in triplicates. The results were expressed as mean ± standard deviation (SD) of their values. An analysis of variance (ANOVA) was performed followed by Tukey's test using SPSS 16 software (SPSS Inc., Chicago, USA). A value of $P < 0.05$ was considered significant.

Results and discussion

Effect of carotenoids from D. maris NITD on protein oxidation

Oxidative stress may lead to cell injury, which is evident from the measurement of biochemical markers of protein oxidation. Protein carbonyls (PCOs) are the common by-products of protein oxidation, and their quantification can be used to measure the extent of oxidative modification (Rajendran et al., 2012). Carotenoids react with free radicals through an additional reaction at their conjugated double bonds, resulting in the formation of relatively stable products (Tian et al., 2009). Proteins are the major components of the cells and are highly susceptible to post-translational modifications due to radiation and free radicals; thus, the degree of cell resistance may be determined by the level of oxidative protein damage (Daly et al., 2007).

The carbonyl content may have a role as a biomarker for detecting oxidative DNA damage (Daly et al., 2007). Therefore, we determined the carbonyl content of the tested bacterial species by inducing oxidative damage with H_2O_2 and comparing the content with that of controls (without H_2O_2 treatment).

After H_2O_2treatment, the carbonyl content of *D. maris* 7011 was 0.0441 mmol/mg protein. It was significantly higher ($P < 0.001$) than the control value (0.0272 mmol/mg protein; without H_2O_2 treatment). Hence, it may be concluded that when *D. maris* 7011 is affected by oxidative molecules, the strain does not produce any carotenoids.

After H_2O_2 treatment, the carbonyl content of *D. maris* NITD (which produces CX) was 0.0276 mmol/mg protein. The value obtained (0.0258 mmol/mg protein) was not significantly different ($P = 0.017$) from the control value. The results for the protein carbonylation level assay for both normal and H_2O_2-treated cells agreed with those of a similar study conducted by Tian et al. (2009). The authors showed that the carbonyl content in a wild-type strain (carotenoid producer) and a mutant strain (non-carotenoid producer) of *Deinococcus radiodurans* after H_2O_2treatment was 0.0169 and 0.0212 mmol/mg, respectively. This result shows that the intracellular proteins in *D. maris* 7011 were more sensitive to oxidative damage than those of *D. maris* NITD because of a higher CX production by the latter strain than the former one. Apart from the primary defense proteins such as superoxide dismutases, catalases and glutaredoxin 2 (Tian et al., 2009), CX has recently been shown to be required in protection against protein oxidation, particularly in the stationary phase of growth in *Escherichia coli* (Nystrom, 2002). In our case, the functional group polarity and orientation of the terminal ring CX probably played a key role as an ROS scavenger (Bera et al., 2017).

Many important proteins, including DNA repair proteins and enzymes involved in cell recovery, might be protected by carotenoids in the cells (Tian et al., 2007). Iron ions released from oxidative-damaged proteins containing iron-sulfur clusters can react with H_2O_2 to form the most harmful hydroxyl radical in the Fenton reaction (Tian et al., 2007). In *D. maris* NITD, the inhibition of protein oxidation by the BCX *in vitro* was 13.28% and 30.18% at 50 and 300 µg/ml concentrations, respectively. Figure 1 shows the inhibition of protein oxidation by different standard carotenoids with respect to BCX. The results showed 12.91, 15.74 and 15.56% inhibition by standard zeaxanthin, β-cryptoxanthin and β-carotene, respectively, at 50 µg/ml concentration. At 100 µg/ml, the inhibition of protein oxidation by these three compounds was 27.55, 38.41 and 35.22%, respectively. Hence, *D. maris* NITD might be a good alternative for producing synthetic antioxidants for scavenging ROS, thus preventing protein oxidation. β-Cryptoxanthin and β-carotene showed a higher inhibition of protein oxidation because of their multiple functional groups in the terminal ring, which facilitate their high radical scavenging property. Figure 2 shows a correlation between BCX and a pure standard CX (PCX) and other synthetic antioxidants (i.e. ascorbic acid and BHA) when their concentration varied from 50 to 300 µg/ml. The correla-

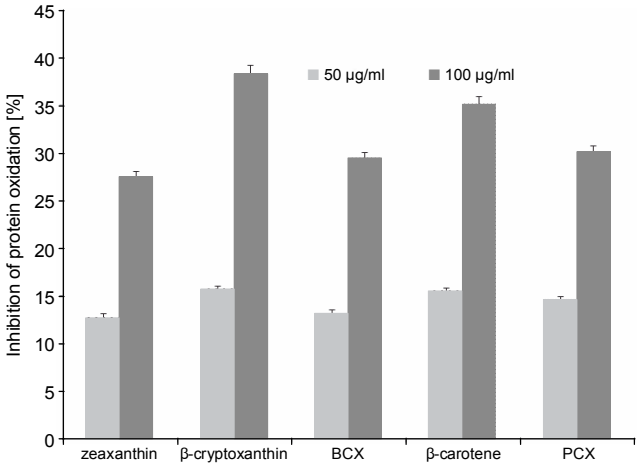

Fig. 1. Inhibition of protein oxidation by different carotenoids

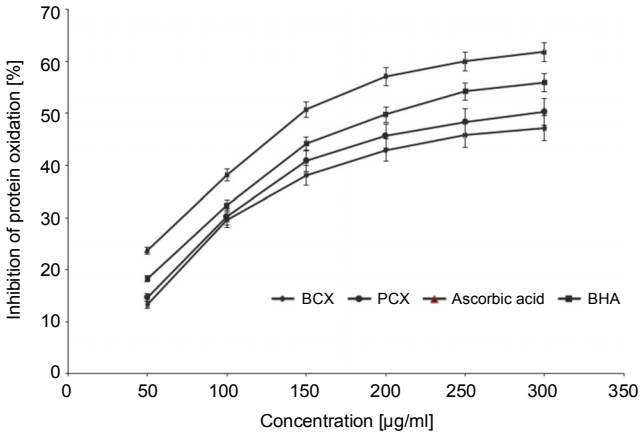

Fig. 2. Typical correlation graph between different antioxidants in terms of protein oxidation *in vitro*

tion coefficient between BCX-PCX, BCX-ascorbic acid and BCX-BHA was 0.9989, 0.9950 and 0.9943, respectively. These values indicate that CX from *D. maris* NITD has more functional similarity with the standard CX. On the other hand, the inhibition of protein oxidation by PCX was 14.65 and 50.28% at 50 and 300 µg/ml concentrations, respectively, which was higher than that extracted from BCX. Ascorbic acid showed highest inhibition of protein damage (23.67 and 61.81%) at 50 and 300 µg/ml concentrations, respectively.

Effect of carotenoids from D. maris NITD on lipid peroxidation

Lipid peroxidation by ROS is believed to be associated with the pathology of many diseases and health conditions (Van der Paal et al., 2016). Thus, the inhibition of lipid peroxidation is considered to be one of the most important indices of antioxidant potential (Van der

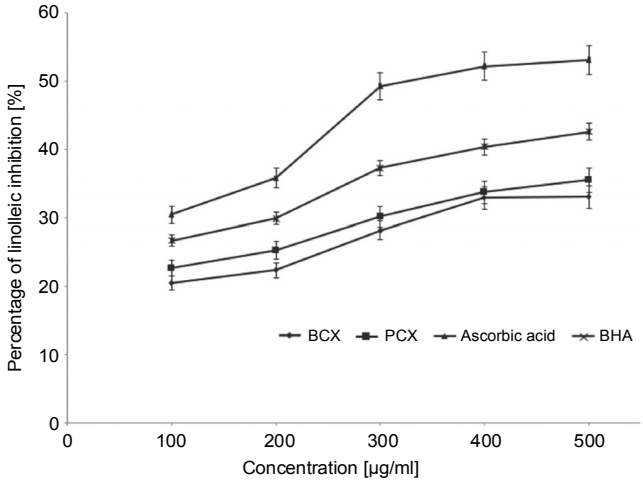

Fig. 3. Linoleic acid inhibition percentage for different antioxidants *in vitro*

Paal et al., 2016). During lipid peroxidation, different hydroperoxides lead to the transformation of a substrate into by-products such as malondialdehyde (MDA). An increase in the MDA content in the linoleic acid emulsion is an evidence of a continuing oxidation process of the lipids (Gramza-Michałowska and Stachowiak, 2010). Figure 3 shows typical patterns of the linoleic acid inhibition percentage for BCX and PCX with varying concentrations from 100 to 500 µg/ml. The results revealed that 100 µg/ml BCX and PCX showed 20.42 and 22.64% of linoleic acid inhibition, which increased to 33.02 and 35.45% at 500 µg/ml concentration, respectively. IC_{50} values were determined to be 932.48 and 905.64 µg/ml for BCX and PCX, respectively. Ascorbic acid and BHA showed low IC_{50} values at 395.09 and 647.99 µg/ml, respectively. Gramza-Michałowska and Stachowiak (2010) showed that carotenoids retard hydroperoxide formation on azo-initiated lipid peroxidation in homogeneous methyl linoleate/AMVN {2,2′-azobis (2,4-dimethylvaleronitrile)} systems. The authors found 0.45% antioxidant activity with 1% extract of astaxanthin from *Phaffia rhodozyma*, and they proposed that carotenoids with oxo-groups may retard hydroperoxide formation more efficiently than non-oxocarotenoids. The functional group present in the terminal CX may be responsible for the inhibition of linoleic acid peroxidation *in vitro*.

In this regard, it is worthy to mention that another effective approach to assess MDA formation is TBARS measurement. The reaction requires an acidic environment where two moles of TBA react with one mole of MDA to form a pink product, which is readily extractable

in organic solvents and gives absorbance at 530–535 nm (Britton et al., 2009). The reaction is presented below:

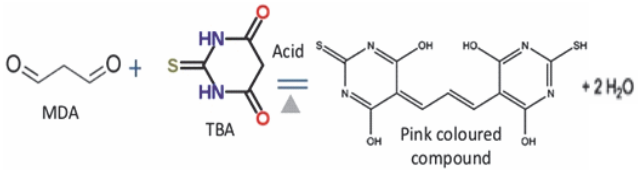

Figure 4 shows percentages of the inhibition of lipid peroxidation by BCX, PCX, ascorbic acid, and BHA at varying concentrations from 100 to 500 µg/ml. The result obtained in this model followed similar trends to that noted in the linoleic acid model. At 100 µg/ml, the percentage of inhibition of lipid peroxidation by BCX and PCX was 32.43 and 35.21%, whereas at 500 µg/ml, the percentage increased to 56.47 and 57.28%, respectively. Ascorbic acid and BHA showed 47.96 and 42.61% inhibition of lipid peroxidation at 100 µg/ml, respectively. A study conducted by Lavy et al. (2005) on *Deinococcus radiophilus* extract showed an approximately 90% inhibition in TBARS formation during the lipid peroxidation study. A recent study by Matumoto-Pintro et al. (2017) showed a high lipid peroxidation inhibition by egg powder enriched with lycopene (a red carotenoid). Our experimental results of TBARS in the goat liver model were slightly higher than those obtained in the linoleic acid model study for the same concentrations of carotenoids (Fig. 3 and Fig. 4). This may be due to the apparent presence of fresh and active enzymes such as aspartate transaminase and alanine transaminase in a freshly excised goat liver.

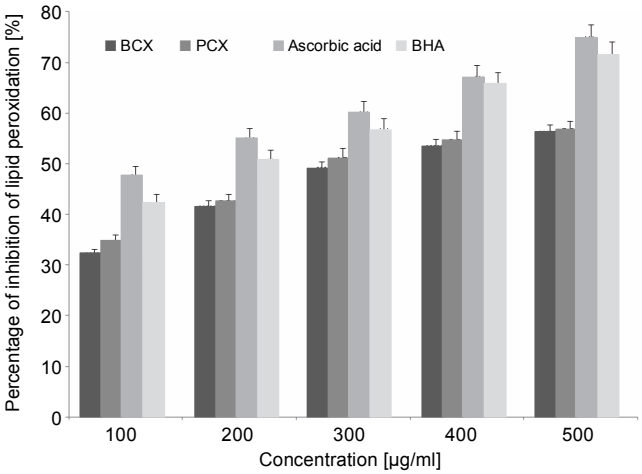

Fig. 4. Inhibition percentage of lipid peroxidation by different antioxidants *in vitro*

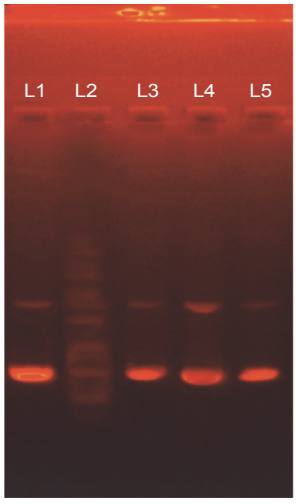

Fig. 5. DNA damage protection study by agarose gel electrophoresis: L1 – only pBR322 DNA, L2 – DNA + fenton's reagent, L3 – DNA + BCX, L4 – DNA + quercetin, L5 – DNA + PCX

Effect of carotenoids from D. maris NITD on DNA damage protection

Studies on simple prokaryotic models such as a bacterial system enable to identify risk factors in the form of accumulation of oxidative-damaged proteins. Elevated levels of oxidized proteins may be caused by an increased production of misfolded or malformed polypeptides (Nystrom, 2002). Any damage to regulatory proteins participating in the cellular expression machinery by ROS may lead to a catastrophic degeneration of nucleic acids inside the cells, which eventually induce apoptosis, followed by death of the cells (Milani et al., 2017). Therefore, the protection of DNA by carotenoids may be partial because of the fact that carotenoids prevent protein oxidation. In this experiment, hydroxyl radicals generated from Fenton's reaction caused damage to DNA (pBR322). In Figure 5, lane 1 shows an intact DNA band, while shows a smear of DNA, indicating the presence of a degraded DNA due to ROS generated by Fenton's reaction. Lanes 3, 4, and 5showed less smeared DNA (observable intactness of pBR322) due to the presence of the antioxidant compounds quercetin, BCX and PCX, respectively. The results of our experiments are in accordance with those of Tian et al. (2007) where the authors investigated the effect of carotenoids (e.g. deinoxanthin) extracted from *Deinococcus radiodurans* on DNA damage protection. Kumar et al. (2013) also conducted similar experiments and proved that *Helicteres isora* L. fruit extract was a promising antioxidant to pro-

tect DNA intactness from ROS generated in Fenton's reaction. The high antioxidant activity of BCX may be attributed to its distinctive chemical nature with long extended conjugated double bonds and terminal functional group, which eventually provide protection from DNA degeneration by ROS *in vitro*.

Conclusions

In summary, the present study revealed that CX from *D. maris* NITD may have a significant effect on the inhibition of protein oxidation *in vitro*. Moreover, non-CX-producing *D. maris* 7011 is more prone to internal damage due to oxidative stress than CX-producing *D. maris* NITD. The study on inhibition of lipid peroxidation also showed protective effect of CX against lipid peroxidation in both linoleic acid and goat liver models. The DNA damage protection experiment suggested that CX is more likely to prevent pBR322 DNA damage. Further detailed experiments need to be conducted to investigate the molecular mechanism of protective action of CX in *D. maris* NITD for its future application as a therapeutic agent.

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