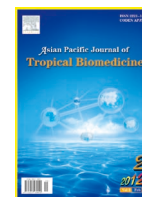




Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Document heading doi:10.1016/S2221-1691(11)60203-7 © 2012 by the Asian Pacific Journal of Tropical Biomedicine. All rights reserved.

## Evaluation of antigenotoxic effects of carotenoids from green algae *Chlorococcum humicola* using human lymphocytes

Bhagavathy S<sup>1</sup>, Sumathi P<sup>2\*</sup><sup>1</sup>Bharathiar University, Coimbatore, Tamilnadu, India<sup>2</sup>Department of Biochemistry, Government Women's College, Krishnagiri, Tamilnadu, India

## ARTICLE INFO

## Article history:

Received 17 July 2011

Received in revised form 2 August 2011

Accepted 20 August 2011

Available online 28 February 2012

## Keywords:

*Chlorococcum humicola*

Benzo(a)pyrene

Genotoxicity

Antigenotoxicity

Chromosomal aberration

Sister chromatid exchange

Micronucleus assay

Carotenoids

Green algae

## ABSTRACT

**Objective:** To identify the available phytochemicals and carotenoids in the selected green algae and evaluate the potential genotoxic/antigenotoxic effect using lymphocytes. **Methods:** Organic solvent extracts of *Chlorococcum humicola* (*C. humicola*) were used for the phytochemical analysis. The available carotenoids were assessed by HPLC, and LC-MS analysis. The genotoxicity was induced by the benzo(a)pyrene in the lymphocyte culture, the genotoxic and antigenotoxic effects of algal carotenoids with and without genotoxic inducer were evaluated by chromosomal aberration (CA), sister chromatid exchange (SCE) and micronucleus assay (MN). **Results:** The results of the analysis showed that the algae were rich in carotenoids and fatty acids. In the total carotenoids lutein,  $\beta$ -carotene and  $\alpha$ -carotene were found to be present in higher concentration. The frequency of CA and SCE increased by benzo(a)pyrene were significantly decreased by the carotenoids ( $P < 0.05$  for CA,  $P < 0.001$  for SCE). The MN frequencies of the cells were significantly decreased by the treatment with carotenoids when compared with the positive controls ( $P < 0.05$ ). **Conclusions:** The findings of the present study demonstrate that, the green algae *C. humicola* is a rich source of bioactive compounds especially carotenoids which effectively fight against environmental genotoxic agents, the carotenoids itself is not a genotoxic substance and should be further considered for its beneficial effects.

### 1. Introduction

The cellular macromolecules of humans, such as DNA, proteins and lipids, are continuously at risk for endogenous and environmentally induced structural alterations. Several man-made chemicals find their way into the environment and pose health risks to the human population. Acute and chronic exposure to these environmental chemicals, such as pesticides, metals, polycyclic aromatic hydrocarbons, solvents, and alkylating agents, has been shown to produce marked toxicity at their target sites. Some of these chemicals are used as therapeutic agents by virtue of their antitumor, antibiotic activity[1].

Reactive oxygen species (ROS) are another important class of damage agents for cellular macromolecules. ROS, such as  $O_2^-$ ,  $OH^-$  and  $H_2O_2$ , are highly genotoxic/ mutagenic

and harmful to cellular macromolecules such as DNA, proteins and lipids[2]. The adverse effect is represented by the oxidative stress that can arise from a lack of antioxidant defence or by an increase of oxidative processes in the body[3]. ROS produced in cells, largely as by-products of metabolic processes, constantly threaten the integrity and correct functioning of cellular DNA. Several oxidant species have the capacity to produce promutagenic lesions in DNA, which may play a significant role in the development of cancer[4].

It has been established that DNA damage induced by ROS is involved in aging and different human diseases such as atherosclerosis, cancer, neurodegenerative diseases and AIDS. The oxidative DNA damage arises from the attack of ROS on DNA bases or deoxyribose residues to produce damaged bases or strand breaks. In addition, ROS can attack protein or lipid molecules (process of lipid peroxidation) and generate intermediates that can react with DNA to form adducts[5].

Cancer chemoprevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse

\*Corresponding author: Sumathi P, Assistant Professor, Department of Biochemistry, Government Women's College, Krishnagiri, Tamilnadu, India.

Tel: 9445283050

E-mail: sumathisiva12@gmail.com

Foundation Project: This work was financially supported by Bharathiar University, Tamilnadu, India.

the development of cancer in normal or preneoplastic tissue. A large number of potential chemopreventive agents have been identified, and they function by mechanisms directed at all major stages of carcinogenesis[6]. Because it is widely held that damage to DNA by reactive species is a significant contributor to carcinogenesis, it has been postulated that antioxidants in supplements or in foods containing them, which decrease oxidative DNA damage, should have a protective effect against cancer. It has been shown in many studies that DNA strand breaks can be reduced by antioxidant supplements or by various combinations of fruits and vegetables that are high in antioxidant nutrient content[7].

Therefore, as a strategy to prevent genotoxic event, antioxidant and radical scavenging properties of carotenoids, especially of  $\beta$ -carotene, are widely used. Carotenoids have been reported to have multiple biological activities such as anticarcinogen, antimutagen, antioxidant, anti-inflammatory, antiproliferative and antiatherogenic properties and chemopreventive agent against cancer diseases in various organs like lung, stomach, colon, breast and prostate. Also carotenoids play an important role in immune response, neoplastic transformation and control of growth and intracellular communication[8].

For the last couple of decades, natural products derived from plants, fruits, spices, herbs, *etc* have been the main focus of research to ameliorate the threat posed by harmful chemicals, toxins, *etc* from endogenous and exogenous sources. Overwhelming evidence from epidemiological studies indicates that diets rich in fruits and vegetables can be associated with a lower risk of numerous diseases. Natural products like fruits, vegetables, herbs, *etc* contain several promising chemopreventive compounds such as vitamins, minerals, carotenoids and an array of other phytochemicals[9].

Antioxidant activity is therefore considered to play an important role in the protective effects of fruits and vegetables against cancer.  $\beta$ -Cryptoxanthin, one of the six major carotenoids ( $\beta$ -carotene, lycopene, lutein,  $\beta$ -cryptoxanthin, zeaxanthin and  $\alpha$ -carotene) routinely measured in human serum, is obtained primarily from citrus fruits, like other carotenoids.  $\beta$ -Cryptoxanthin is an antioxidant and may help prevent free radical damage to biomolecules including lipids, proteins and nucleic acids. Retinoids, the cleavage products of carotenoids, as well as being antioxidants, have in some cases (including  $\beta$ -cryptoxanthin) vitamin A activity and may play an important role in the prevention and treatment of certain cancers[10].

There are over 600 known carotenoids, including compounds such as lutein,  $\alpha$ -carotene,  $\beta$ -carotene and lycopene, and they are commonly found in many red, yellow and orange fruits and vegetables. Carotenoids are only synthesized in microorganisms and plants, where they are involved in photosynthesis. They are important dietary sources of vitamin A[11].

Various studies, including those using short-term assays, have helped to identify a great number of antimutagenic

properties found in some foods such as:  $\beta$ -carotene, ascorbic acid, linoleic acid,  $\gamma$ -tocopherol, vanillin, chlorophyllin, polyphenols and components found in black and green teas and mushrooms. For this reason, these substances in natural foods are extremely important not only due to their nutritional value but also as prophylactic agents against diseases such as cancer[12].

Various reports confirm the existence of bioactive carotenoids in green algae such as  $\alpha$ -carotene,  $\beta$ -carotene, astaxanthin, lutein, zeaxanthin, cryptoxanthin, violaxanthin, *etc*. Also, there are some reports on the biological activities of several seaweed species, specifically the antiproliferative activity of ethanolic and aqueous extracts from green algae[13] and *Turbinaria orrata*[14] against human cancer cells. Two previous *in vitro* studies, noted the anti-genotoxic activity of *Ulva rigida* and the DNA damage protecting activity and antioxidant potential of crude ethanolic extract of *Codium tomentosum* Stackhouse in human lymphocytes[15].

The present study, was aimed to investigate the presence of phytochemicals and carotenoids in the selected unicellular green algae *Chlorococcum humicola* (*C. humicola*) and, to evaluate the oxidative DNA damage-protecting activity of carotenoid extracts using human lymphocytes *in vitro*.

## 2. Materials and methods

### 2.1. Collection and culturing of algae

Fresh water, unicellular, nonmotile green algae *C. humicola* was obtained from the culture collected from the Department of Plant Biology and Plant Biotechnology, RKM Vivekanantha College, Chennai, India. Algal culturing was carried out with 100 mL Bold's basal medium supplemented with sterile compressed air and kept under fluorescent light (20  $\mu$  mol/m/s) with 16 h light period and at (25 $\pm$ 2)  $^{\circ}$ C temperature. Algae samples were cleaned of epiphytes and necrotic parts were removed. Then the samples were rinsed with sterile water to remove any associated debris.

### 2.2. Preparation of algal crude extracts and phytochemical analysis

The algal samples were centrifuged at 2 500 rpm for 10 min to remove the water content. 25 g of fresh algae was extracted for 15 min with 50 mL of organic solvents, *i.e.* acetone, benzene, chloroform, diethyl ether, ethyl acetate, ethanol, hexane and methanol. All the crude extracts were used for the existence of available phytochemicals such as total carbohydrates, total amino acids, total proteins and total lipids. Saponins, tannins, glycosides, carotenoids, alkaloids, flavanoids and phenolic compounds were carried out by the standard methods[16].

### 2.3. Carotenoid extraction and estimation

Algal sample (1 g dry weight) was extracted with ethanol until all the pigments were removed, and filtered through a

sintered glass filter (porosity 3; pore size 20–30 Q). An equal volume of diethyl ether was added to the combined ethanol extracts, followed by the addition of water droplets until two layers were formed. The ethereal epiphase, containing all the pigments, were washed free from ethanol with water, and the solvent was removed. The residue was then saponified with equal volume of 10% methanolic KOH and kept in overnight in the room temperature at dark, after which the carotenoid solution was washed with water to remove the alkali (pH: 7.0) dried over  $\text{Na}_2\text{SO}_4$ . The unsaponifiable residue was dissolved in a little ether and then in 10 mL of petroleum ether (b.p. 40–60 °C). This extraction was used for further analysis. The total carotenoids were estimated spectrophotometrically at 450 nm.

#### 2.4. High performance liquid chromatography analysis (HPLC) of carotenoids

The carotenoid profile of *C. humicola* was analyzed by HPLC (LC-10A, Shimadzu) using a reversed phase (25 cm × 4.6 mm) column with an isocratic solvent system consisting of acetonitrile/methanol/dichloromethane (70:10:20) at a flow rate of 1.0 mL and detected at 467 nm<sup>[17]</sup>.

#### 2.5. Liquid chromatography–mass spectrometry (LC–MS) analysis of carotenoids

Identification of the different carotenoids was made using Hewlett Packard HB 5890 liquid chromatography (LC) coupled with 5989 B series mass spectrometer (MS). Mass spectra of carotenoids were acquired with an m/z detector and 400–600 scan range at 450 nm by a diode array detector and confirmed with respected standards. Identification of the individual components was performed by comparison of mass spectra with the profiles from the Wiley GC–MS 275 libraries<sup>[18]</sup>.

### 2.6. Genotoxicity/antigenotoxicity assays

#### 2.6.1. Leucocyte culture

Heparinised peripheral blood obtained from five healthy donors, who were non-smoking, non alcoholic, non users of drugs, antibiotics, analgesics were used in all experiments. Leucocyte cultures were set up for chromosomal aberration (CA) and sister chromatid exchange (SCE) analysis together along with control cultures for each. 5 mL of F-10 medium supplemented with 5 mL 10% AB serum and 0.3 mL of phytohemagglutinin were taken in sterile glass bottles. About 0.3 mL of blood was added to this and the bottles tightly corked and incubated at 37 °C for 24 h.

#### 2.6.2. CA and SCE

At the end of 24 h, the total carotenoid extracts at a concentration of 100, 200 and 300  $\mu\text{g/mL}$  were added to the cultures used for scoring CA. The cultures used for SCE were supplemented with BrdU at the concentration of 5  $\mu\text{g/mL}$  and all the bottles were further incubated at 37 °C for 24 h in complete darkness. After 48 h of setting up the cultures used

for CA analysis were terminated. While the cultures used for SCE analysis were treated with carotenoid extracts at a concentration of 100, 200 and 300  $\mu\text{g/mL}$  and incubated at 37 °C for 24 h at the end they were terminated. For induction of oxidative and chromosomal damage, the lymphocytes were treated with benzo(a)pyrene [B(a)P] (25  $\mu\text{g/mL}$ ) as positive controls. The mutagenic solution was prepared immediately before use, to avoid degradation of the agents. As a negative control, distilled water was also used.

#### 2.6.3. Harvesting the cultures

Harvesting the cultures was carried out at the end of 48 h for CA and 72 h for SCE. Colchicine was added at a concentration of 0.2  $\mu\text{g/mL}$  to the cultures 90 min prior to harvesting, to arrest the cells at metaphase. 90 min after colchicines addition, the cultures were centrifuged for 10 min at 1 000 rpm. The supernatant was discarded and pellet was mixed gently. The cells were then exposed to hypotonic KCl solution (0.075 M; pH 7.0) for 20 min at 37 °C. After 20 min, the cells were spun down and KCl was discarded, then the cells were fixed by slow addition of freshly prepared, chilled fixative solution (methanol: acetic acid in 3:1 ratio). The pellet was aspirated gently and mixed with the fixative evenly using a Pasteur pipette. The tubes were incubated at 4 °C for at least 30 min, after which the fixative was removed by centrifugation. The pellet was washed well 3–4 changes of fixative till a clear supernatant was obtained. A few drops of cell suspension were dropped on a clean, grease free slide which was stored under chilled distilled water prior to the slide preparation. The slide was dried at 60 °C on a hot plate.

#### 2.6.4. Staining for CA

The slides were stained with BDH liquid Giemsa and were diluted 20 times with  $\text{KH}_2\text{PO}_4$  solution (0.3402%; pH 6.8) for 10 min. The slides were washed well with distilled water and air dried. Fifty to hundred complete well spread metaphases were scored for CA from each culture. The results were expressed as the number of CAs/metaphases.

#### 2.6.5. Staining for SCE

The slides were aged for 3 days for at least 3 days prior to staining. The slides were stained in the dark with Hoechst 33258 stain dissolved in PBS to give a concentration of 10  $\mu\text{g/mL}$  for 15 min. They were then rinsed with distilled water and arranged in a petridish. Distilled water was added onto the slides, so that a thin film of water was formed on the slides. The slides were then exposed to UV lamp (360 nm) at a height of 15–17 cm for 2 h. After UV exposure, the slides were treated with SSC solution maintained at 60 °C for 2 h. The slides were then washed thoroughly with distilled water, care being taken not to precipitate the salts. The slides were then counter stained with diluted Giemsa for 15 min. After staining, the slides were aged for 7 days. At least 20 well spread metaphases/cultures were scored for the occurrence of SCE. The results were expressed as the number of SCEs/metaphases<sup>[19]</sup>.

#### 2.6.6. Micronucleus assay (MN)

Two thousand binucleated cells for each experimental

point were examined, following the scoring criteria adopted by the Human Micronucleus Project. We evaluated the binucleated micro nucleated lymphocyte (BNMN) frequency as the number of binucleated lymphocytes containing one or more MN per 1 000 binucleated cells. In the anti-clastogenicity experiments, the present reduction by treatment with carotenoids in the number of cells with micronuclei that showed protective activity was calculated according to Bonassi *et al*[20] using the following formula:

$$\text{Reduction (\%)} = \frac{\text{Number of cells with MN in B} - \text{Number of cells with MN in D}}{\text{Number of cells with MN in B} - \text{Number of cells with MN in A}} \times 100$$

Where B was the group of cells treated with the corresponding positive control *i.e.* B(a)P, D was the group of cells treated with carotenoids plus the positive control and A was the negative control group.

#### 2.6.7. Mitotic index (MI), proliferative index (PRI) and nuclear division index (NDI)

The MI was calculated from the number of metaphases in 2 000 cells, analyzed per culture for each dose group and donor in CA and SCE assays. In the SCE assay, PRI was calculated using the following formula:

$PRI = (M1 + 2M2 + 3M3)/N$  where M1, M2 and M3 indicate those metaphases corresponding to first, second and third divisions, and N is the total number of metaphases scored. Moreover, in the micronuclei assay, 500 lymphocytes were scored to evaluate the percentage of binucleated cells, and NDI was calculated according to the following formula:

$NDI = [MONO + 2BN + 3TRI + 4TETRA]/500$  where MONO, BN, TRI and TETRA are mononucleated, binucleated,

trinucleated and tetranucleated lymphocytes, respectively.

## 2.7. Statistical analysis

The different frequencies of DNA damage on lymphocyte cultures were compared by using two-way analysis of variance (ANOVA) with 95% confidence intervals. These analysis were carried out with commercial software programs SPSS 12.

## 3. Results

### 3.1. Phytochemical analysis

The existence of phytochemicals of *C. humicola* was shown in Table 1. Fatty acids and chief colouring pigment carotenoids were present at higher concentrations. Carbohydrates, proteins and aminoacids are also found to be available. Saponins and alkaloids were found at the major concentration. Flavanoids and phenolic compounds were identified. Tannins and glycosides were absent.

### 3.2. HPLC/GC-MS analysis of total carotenoids

In *C. humicola*, six different carotenoid pigments were separated and detected. The carotenoid samples were eluted under isocratic conditions within 21 min. The carotenoid profile of *C. humicola* through  $C_{18}$  column was violaxanthin, astaxanthin, lutein, zeaxanthin,  $\alpha$ -carotene and  $\beta$ -carotene (Table 2 and Figure 1). The carotenoids were analyzed using authentic standards. The carotenoids were identified by mass spectra showed in Figure 2. Spectra

**Table 1**  
Phytochemical analysis of various organic extracts of *C. humicola*.

Identified compounds	Acetone	Benzene	Chloroform	Diethyl ether	Ethyl acetate	Ethanol	Hexane	Methanol
Carbohydrates	+	+	+	+	+	+	+	+
Fatty acids	+	+	+	+	+	+	+	+
Proteins	+	+	+	+	+	+	+	+
Aminoacids	+	+	+	+	+	+	+	+
Saponins	+	–	+	+	+	+	+	–
Tannins	–	–	–	–	–	–	–	–
Carotenoids	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+	+	+
Phenolic compounds	+	+	+	+	+	+	+	+
Glycosides	–	–	–	–	–	–	–	–

+: Present; -: Absent.

**Table 2**  
List of carotenoids, spectral data in the mobile phase and in different solvents.

No	Pigment	Acetone	Benzene	Ethanol	Hexane	Eluent
1	Violaxanthin	–	428 454 483	419 441 471	443 472	416 440 472
2	Astaxanthin	–	433 457 488	428 449 473	422 445 475	420 448 476
3	Lutein	–	–	420 445 475	–	424 448 472
4	Zeaxanthin	440 463 492	–	–	425 450 479	452 480
5	$\alpha$ -carotene	–	–	–	420 442 472	420 448 476
6	$\beta$ -carotene	–	–	–	425 449 477	428 452 476

**Table 3**

Chromosomal aberration by total carotenoid treatment on lymphocyte culture.

Treatment	Normal	Del	AC	DC	TC	No of aberrations	% Aberration	% Protection
Control	49.00±4.32	–	–	1.00±0.05	0.00±0.00	1.00±0.04	2.00±0.17	–
B(a)P	20.30±1.22	11.30±0.91	6.40±0.52	6.60±0.52	5.00±0.42	29.30±2.00	58.60±4.71	–
Carotenoids (100 $\mu$ g/mL)	45.40±3.20	1.30±0.09	0.00±0.00	1.20±0.06	0.00±0.00	2.50±0.28	5.00±0.51	–
Carotenoids (200 $\mu$ g/mL)	47.20±3.71	0.00±0.00	1.60±0.08	0.00±0.00	0.00±0.00	1.60±0.09	3.20±0.27	–
Carotenoids (300 $\mu$ g/mL)	48.50±3.86	0.00±0.00	0.00±0.00	1.00±0.03	0.00±0.00	1.00±0.07	2.00±0.18	–
Carotenoids (100 $\mu$ g/mL) + B(a)P	36.60±2.11	6.20±0.66	2.10±0.24	2.60±0.06	2.50±0.10	13.40±1.14	26.80±1.18**	31.80±2.01
Carotenoids (200 $\mu$ g/mL) + B(a)P	37.90±2.31	5.20±0.52	2.60±0.18	2.50±0.07	1.80±0.09	12.10±1.04	24.20±1.20**	32.60±2.17
Carotenoids (300 $\mu$ g/mL) + B(a)P	39.70±2.16	3.20±0.25	2.60±0.19	2.20±0.05	2.30±0.17	10.30±1.01	20.60±1.10**	38.00±2.18

Del: Deletion; AC: Acentric; DC: Dicentric; TC: Tricentric; \*\*:  $P < 0.001$ .**Table 4**

Sister chromatid exchange by total carotenoid treatment on lymphocyte culture.

Treatment	SCE	PRI	MI (%)
Control	2.50±0.28	2.26±0.19	8.27±0.95
B(a)P	8.62±0.61	1.20±0.18	1.32±0.08
Carotenoids (100 $\mu$ g/mL)	2.30±0.16	2.15±0.12	9.91±1.91
Carotenoids (200 $\mu$ g/mL)	2.52±0.17	2.27±0.09	9.99±1.08
Carotenoids (300 $\mu$ g/mL)	2.37±0.20	2.29±0.08	10.42±1.91
Carotenoids (100 $\mu$ g/mL) + B(a)P	4.79±0.29**	1.89±0.02*	5.29±0.41**
Carotenoids (200 $\mu$ g/mL) + B(a)P	4.26±0.29**	1.92±0.07*	5.28±0.41**
Carotenoids (300 $\mu$ g/mL) + B(a)P	3.25±0.26**	1.97±0.08*	5.26±0.61**

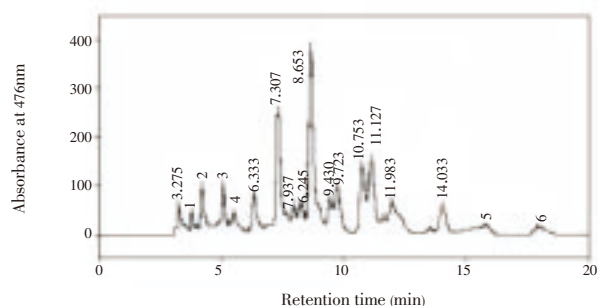
PRI: proliferative index; MI: mitotic index; \*\*:  $P < 0.001$ ; \* $P < 0.01$ .**Table 5**

Micronucleus frequency by total carotenoid treatment on lymphocyte culture (mean±SD).

Treatment	MN	NDI	% Reduction
Control	1.17±0.18	1.23±0.08	–
B(a)P	8.72±0.73	1.10±0.10	–
Carotenoids (100 $\mu$ g/mL)	0.81±0.02	1.30±0.11	–
Carotenoids (200 $\mu$ g/mL)	1.29±0.10	1.24±0.09	–
Carotenoids (300 $\mu$ g/mL)	1.82±0.09	1.25±0.06	–
Carotenoids (100 $\mu$ g/mL) + B(a)P	3.09±0.18*	1.10±0.12	74.57
Carotenoids (200 $\mu$ g/mL) + B(a)P	3.07±0.17*	1.16±0.16	74.83
Carotenoids (300 $\mu$ g/mL) + B(a)P	3.27±0.15*	1.18±0.09	72.19

NDI: nuclear division index; \*:  $P < 0.05$ .

were generated from 10  $\mu$  L injections of 50 ng/ $\mu$  L solutions. The carotenoid levels were as follows astaxanthin–17%, violaxanthin–4%, zeaxanthin–2%, lutein–41%,  $\beta$ -carotene–22%, and  $\alpha$ -carotene–12%. Higher concentration of lutein and  $\beta$ -carotene was found in the selected green algae *C. humicola*.

**Figure 1.** HPLC profile of carotenoids from *C. Humicola* extract.

1: violaxanthin; 2: astaxanthin; 3: lutein; 4: zeaxanthin; 5:  $\alpha$ -carotene; 6:  $\beta$ -carotene.

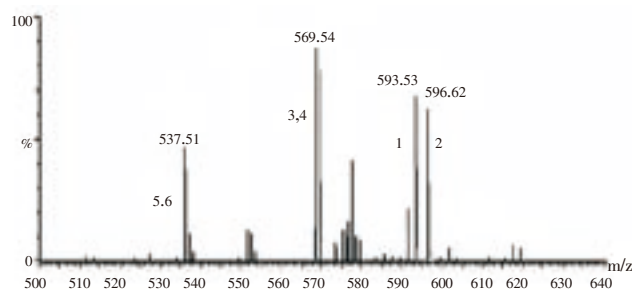
All these spectra were characterized by  $[M+H]^+$  ions.

Additional ions corresponding to  $[M+H-H_2O]^+$  were observed for astaxanthin, violaxanthin, lutein and zeaxanthin. Astaxanthin and zeaxanthin showed significant  $[M+H-H_2O]^+$  ions in addition  $[M+H]^+$  ions.  $\alpha$ -carotene and  $\beta$ -carotene compounds did not contain hydroxyl or epoxy functional group. These two compounds showed  $[M+H]^+$  ions. The presence of trace amount of violaxanthin, astaxanthin and zeaxanthin was found comparing with lutein and carotenes.

### 3.3. Chromosomal aberrations

The structural chromosomal aberrations and their percentage in human lymphocytes on various treatments were also presented in Table 3 and Figure 3. Numbers of chromosomal aberrations of the cells treated with three different doses (100, 200, 300  $\mu$ g/mL) of carotenoids were not statistically different from the negative control. The positive controls B(a)P at concentrations of 25  $\mu$ g/mL were significantly increased in the number of abnormal metaphases and the total number of structural chromosomal aberrations, respectively, when compared with the negative

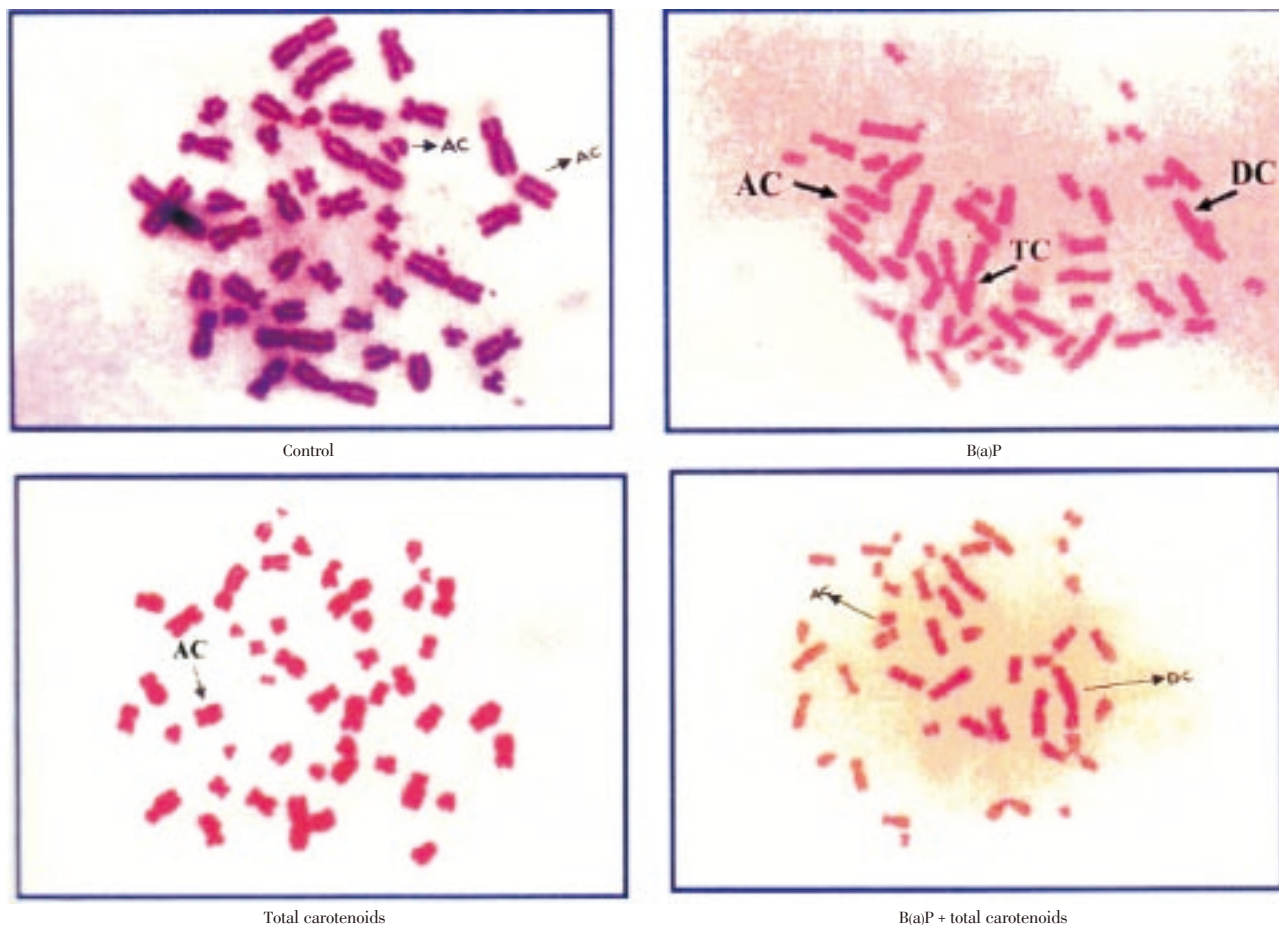
control ( $P < 0.001$ ). The frequency of chromosomal aberrations was significantly decreased by the treatment of carotenoids plus B(a)P relative to the positive control of B(a)P alone ( $P < 0.001$ ) for the percentage of total carotenoids including deletions, acentric, dicentric and tricentric were increased significantly on B(a)P treatment which was effectively protected by total carotenoids with respective on different concentration.



**Figure 2.** LC-MS profile of carotenoids from *C. humicola* extract. 1: violaxanthin; 2: astaxanthin; 3: lutein; 4: zeaxanthin; 5:  $\alpha$ -carotene; 6:  $\beta$ -carotene.

### 3.4. Sister chromatid exchange

The results of SCE were shown in Table 4 and Figure 4.

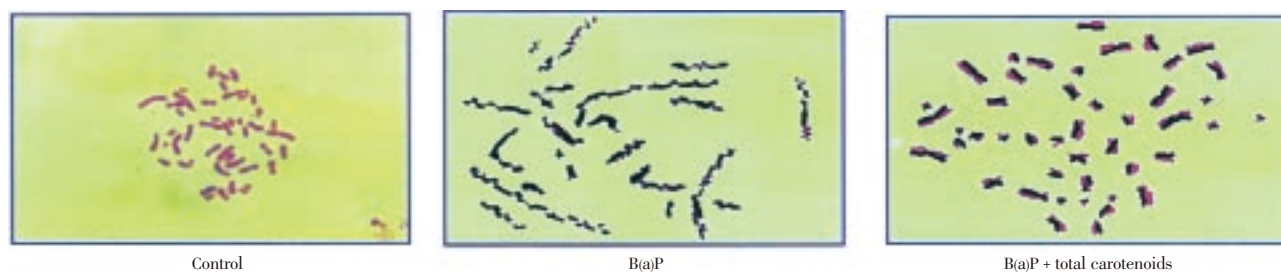


**Figure 3.** Effect of total carotenoids from *C. humicola* on CA in human lymphocytes. AC: Acentric; DC: Dicentric; TC: Tricentric.

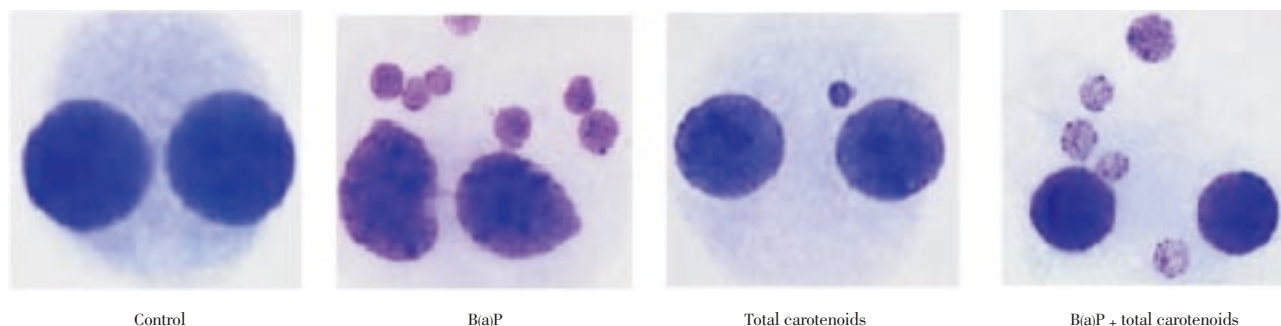
The SCE frequencies of the cells treated with three different doses (100, 200 and 300  $\mu$ g/mL) of total carotenoids were not statistically different from negative control values. The SCE frequencies per cell significantly increased in human lymphocyte cultures treated with B(a)P as positive controls relative to negative control ( $P < 0.001$ ). The SCE frequencies of the cells treated with B(a)P plus total carotenoids significantly decreased relative to the B(a)P positive control ( $P < 0.001$ ).

### 3.5. Micronucleus assay

The results of the micronucleus (MN) assays were shown in Table 5 and Figure 5. The MN frequencies of the cells treated with three different doses of total carotenoids were decreased relative to the negative controls, but not with statistical significance. The MN frequencies were significantly increased in human lymphocyte culture treated with B(a)P as positive controls compared with negative control. The MN frequencies of the cells were significantly decreased by the treatment with total carotenoids plus B(a)P when compared with the positive control B(a)P alone ( $P < 0.05$ ).



**Figure 4.** SCE on treatment of total carotenoids from *C. humicola* in human lymphocyte culture.



**Figure 5.** Effect of total carotenoids from *C. humicola* on micronucleus in human lymphocyte.

#### 4. Discussion

Polycyclic aromatic hydrocarbons appear to be significant contributors to the genotoxicity and carcinogenicity of air pollution present in the environment for humans, and the most extensively studied member of this class of chemicals is B(a)P. Populations exposed to environmental air pollution show increased levels of PAH–DNA adducts and it has been postulated that another contributing cause of carcinogenicity by environmental air pollution may be the production of ROS following oxidative stress leading to oxidative DNA damage<sup>[21,22]</sup>. B(a)P itself is unreactive, but it is converted to a highly reactive electrophile by enzymes involved in drug metabolism<sup>[23]</sup>. Therefore, they are routinely used for evaluating increased sensitivity to DNA cross–linking agents in studies using a variety of cytogenetic endpoints<sup>[24]</sup>.

In the present study, we investigated potential bioactive compounds available in the selected green algae *C. humicola*. Primarily the phytochemical analysis of *C. humicola* extract was compared with spectral data in different types of solvents. All the absorption maxima of the spectral data coincide with the previous published data. It is generally agreed that all taxonomic groups of algae have specific sets of pigments, which vary in concentration from one species to another. It is reported that there is a fixed relationship, in green algae, and total carotenoids (70%–90%)<sup>[25]</sup>. The  $\alpha$ -carotene and  $\beta$ -carotene were found to be lower level in *C. humicola* that may be related to the conversion of these compounds to lutein. This may be the reason for the higher concentration of lutein existence among total carotenoids in *C. humicola*. The results were correlated with Ranga Rao *et al* who reported that those carotenoids were from *Botryococcus braunii*<sup>[17]</sup>. The present results confirmed that *C. humicola* is rich in carotenoid pigments, and the

variation of their concentration values depends on a multiple factors.

The genotoxic/antigenotoxic, cytotoxicity and chemoprotective effect of total carotenoids were analyzed against B(a)P by CA, SCE, and MN. Because CA, SCE and MN values were significantly increased in culture treated with only B(a)P, our results agree with available data. For the determination of cytotoxicity, the MI, PRI and NDI ratios were also evaluated. Only B(a)P treatment decreased MI, PRI and NDI ratios relative to control, as expected. Moreover, in these tests total carotenoids did not show any genotoxic effect as the sole treatment for human lymphocytes in all these genotoxicity assays. MI, PRI and NDI ratios were decreased with high dose total carotenoid treatment, especially at a dose of 300  $\mu$ g/mL. Consequently, we suggest that carotenoids had a relatively low cytotoxic effect on human lymphocytes *in vitro*, even in high doses. Selected carotenoid doses (100, 200 and 300  $\mu$ g/mL) significantly decreased B(a)P induced DNA damage compared with positive control in all three tests ( $P < 0.001$ ,  $P < 0.01$  and  $P < 0.05$ ). While carotenoids decreased DNA damage induced by B(a)P in the CA and MN tests. The increase of SCE frequency in the treatment of B(a)P plus total carotenoids may have been a synergistic effect of total carotenoids and B(a)P. Consequently, the results of CA, SCE and MN tests show that carotenoids have a strong antigenotoxic and chemoprotective effect against B(a)P induced DNA damage and a moderate antigenotoxic and chemo–protective effect against B(a)P induced DNA damage. In our opinion, the antigenotoxic and chemo–protective effect may have originated from the anti-oxidative capacity of carotenoids *in vitro* and *in vivo* which was reported earlier.

Since micronuclei are the result of chromosome breaks or disturbances of the mitotic spindle and chromosomal

aberrations result from clastogenic events with and without chromosomal rearrangements, these parameters are usually considered to be clear evidence for mutagenicity. On the other hand, SCE may not represent actual damage to chromosomes, but could instead be considered as a result of damage repair. Thus, the different dose–response effects obtained for the different end–points tested indicate that B(a)P primarily induce clastogenic events while the SCE–inducing activity becomes significant at high concentrations. This observation could eventually be linked to the shapes of the dose–response curves for chromosomal aberrations and micronuclei, and could indicate the generation of differently acting metabolites[26].

Schabath and his co–workers carried out a case–control study looking at levels of carotenoids and endogenous DNA damage in lymphocytes of bladder cancer patients and healthy controls. They found an increase in DNA damage in cases, with high DNA damage combined with low carotenoid intake being associated with the highest risk[27]. Zhao and colleagues, also used the alkaline comet assay to look at endogenous DNA damage in their intervention study, involving 37 healthy post–menopausal women supplemented with carotenoids (lutein, lycopene and beta–carotene). This study showed a decrease in DNA damage with carotenoid supplementation[28]. Watters and his team found an inverse association between DNA damage in the lymphocytes of 164 healthy subjects and the carotenoid lycopene, using the alkaline comet assay[29].

Carotenoids, the most potent biological quenchers of singlet O<sub>2</sub>, act as chain–breaking antioxidants, and flavonoids inhibit the enzymes responsible for O<sub>2</sub><sup>–</sup> production. β – Carotene and flavonoids have been reported to prevent DNA damage against H<sub>2</sub>O<sub>2</sub> in human lymphocytes assessed by the comet assay[30].

Evaluation on antigenotoxic/antimutagenic activity of the crude ethanolic extracts of *Codium tomentosum* stock house (Chlorophyceae), in human lymphocyte cultures shows the strong evidence on antigenotoxic effect[31]. There are several prospective studies investigating the possible effect of b–cryptoxanthin (and other carotenoids) on risk of various cancers. Toniolo found that levels of b–cryptoxanthin and lutein in serum were linked with reduced risk of breast cancer in a dose dependent fashion[32]. Beta carotene, lycopene, b–cryptoxanthin, zeaxanthin and lutein are reduced in colorectal adenomas, suggesting that mucosal carotenoids could serve as biomarkers for predisposition to colorectal cancer[33]. Women with high circulating concentrations of b–cryptoxanthin and tocopherol may be at a reduced risk of cervical atypical squamous cells of undetermined significance[34].

*In vitro* studies looking at the effects of carotenoids on DNA repair have examined a range of human cell types including leukaemia cells where beta–carotene decreased peroxynitrous acid–induced damage, hepatoma cells where lycopene decreased oxidative lesions, melanocytes where lycopene caused a reduction in UVA–induced damage and

neuroblastoma cells where lutein and zeaxanthin decreased UVA–induced DNA damage[35].

The present study has clearly demonstrated that total carotenoids have significant antigenotoxic activities. In addition, the chemo–protective and antigenotoxic potential of total carotenoids was observed which is of great significance in radioprotection, and may be useful for human pathological condition. Furthermore, the different carotenoids should be isolated, purified and characterized in order to understand the mechanisms underlying its chemo–protective effect and antigenotoxic effects. Therefore, algal species *C. humicola*, as alternative sources of natural antigenotoxic agents, have attracted much attention from biomedical scientists.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

The authors wish to acknowledge Dr. Sivasubramonium V, Head, Post Graduate and Research Department of Plant Biology & Plant Biotechnology, RKM Vivekananda College, Chennai, India for providing the algal cultures throughout the study and Indian Institute of Technology, Chennai, India for carrying out the spectral analysis.

### References

- [1] Lee SH, Kohn H. Nucleophilic activation of a tetra–substituted mitomycin cyclic bis–disulfide. *Chem Pharm Bull* 2009; **57**(2): 149–157.
- [2] Mantena RKR, Wijburg OLC, Vindurampulle C, Bennett–Wood VR, Walduck A, Drummond GR, et al. Reactive oxygen species are the major antibacterials against *Salmonella typhimurium* purine auxotrophs in the phagosome of RAW 264, 7 cells. *Cell Microbiol* 2008; **10**(5): 1058–1073.
- [3] Cornelli U. Antioxidant use in nutraceuticals. *Clin Dermatol* 2009; **27**: 175–194.
- [4] WCRF/AICR. *Food, nutrition, physical activity, and the prevention of cancer*. Washington DC: American Institute for Cancer Research; 2007.
- [5] Nikoli B, Stanojevi J, Ga BV, Simi D, Vukeyi JK. Effects of vitamin C on DNA damage. *Food Technol Biotechnol* 2006; **44**(4): 449–456.
- [6] Samarth RM, Panwar M, Kumar M, Kumar A. Protective effects of *Mentha piperita* Linn on benzo[a]pyrene–induced lung carcinogenicity and mutagenicity in Swiss albino mice. *Mutagenesis* 2006; **21**(1): 61–66.
- [7] Russell M. The multifunctional carotenoids: insights into their behavior. *J Nutr* 2006; **136**: 2690S–2692S.
- [8] Nishino H, Murakoshi M, Tokuda H, Satomi Y. Cancer prevention by carotenoids. *Arch Biochem Biophys* 2009; **483**: 165–168.



- [9] El-Baky A, El Baz FK, El-Baroty GS. Production of carotenoids from marine microalgae and its evaluation as safe food colorant and lowering cholesterol agents. *Am Eurasian J Agric Environ Sci* 2007; **2**: 792–800.
- [10] Steven G, Thomas D, Haffner J, Kroetsch T, Davidson SR, James WE, et al. Effect of short-term lycopene supplementation and postprandial dyslipidemia on plasma antioxidants and biomarkers of endothelial health in young, healthy individuals. *Vasc Health Risk Manag* 2008; **4**(1): 213–222.
- [11] Rao AV, Rao LG. Carotenoids and human health. *Pharmacol Res* 2007; **55**: 207–216.
- [12] Guterre ZDR, Mantovan MS, da Eir AF, Ribeir LR, Jorda BQ. Genotoxic and antigenotoxic effects of organic extracts of mushroom *Agaricus blazei* Murrill on V79 cells. *Genet Mol Biol* 2005; **28**(3): 458–463.
- [13] Wu LC, Ho JA, Shieh MC, Lu IW. Antioxidant and antiproliferative activities of *Spirulina* and *Chlorella* water extracts. *J Agric Food Chem* 2005; **53**: 4207–4212.
- [14] Deslandes E, Pondaven P, Auperin T, Roussakis C, Guezennec J, Stiger V, et al. Preliminary study of the *in vitro* antiproliferative effect of a hydroethanolic extract from the subtropical seaweed *Turbinaria ornata* (Turner J. Argadh) on a human nonsmall-cell bronchopulmonary carcinoma cell line (NSCLC–N6). *J Appl Phycol* 2000; **12**: 257–262.
- [15] Celikler S, Yildiz G, Vatan O, Bilaloglu R. *In vitro* antigenotoxicity of *Ulva rigida* C. Agardh (Chlorophyceae) extract against induction of chromosome aberration, sister chromatid exchange and micronuclei by mutagenic agent MMC. *Biomed Environ Sci* 2008; **21**: 492–498.
- [16] Scholtz B, Liebezeit G. Chemical screening for bioactive substances in culture media of microalgae and cyanobacteria from marine and brackish water habitats—first results. *Pharm Biol* 2006; **44**97: 544–549.
- [17] Rao RA, Sarada R, Baskar V, Ravisankar GA. Antioxidant activity of *Botryococcus braienii* extract in *in vitro* models. *J Agric Food Chem* 2006; **54**: 4593–599.
- [18] Rao RA, Sarada R, Baskar V, Ravisankar GA. Identification of carotenoids from green algae *Haematococcus pluvialis* by HPLC and LCMS and their antioxidant properties. *J Microbiol Biotechnol* 2009; **19**: 1333–1341.
- [19] Kulkarni PS, Ambani LM, Vaidya AB. Modified FPG technique for demonstrating sister chromatid exchange (SCE). *J Postgrad Med* 1982; **28**: 9–12.
- [20] Bonassi S, Fenech M, Lando C, Lin YP, Ceppi M, Chang WP, et al. Micronucleus project: international database comparison for results with the cytokinesis block micronucleus assay in human lymphocytes: effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ Mol Mutagen* 2001; **37**: 31–45.
- [21] Singh R, Sram RJ, Binkova B, Kalina I, Popov TA, Georgieva T, et al. The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. *Mutat Res* 2007; **620**(1–2): 83–92.
- [22] Tarantini A, Maitre A, Lefebvre E, Marques M, Rajhi A, Douki T. Polycyclic aromatic hydrocarbons in binary mixtures modulate the efficiency of benzo[a]pyrene to form DNA adducts in human cells. *Toxicology* 2011; **279**(1–3): 36–44.
- [23] Landvik NE, Gorria M, Arlt VM, Asare N, Solhaug A, Lagadic-Gossman D, et al. Effects of nitrated-polycyclic aromatic hydrocarbons and diesel exhaust particle extracts on cell signalling related to apoptosis: possible implications for their mutagenic and carcinogenic effects. *Toxicology* 2007; **231**: 159–174.
- [24] Singh R, Sram RJ, Binkova B, Kalina I, Popov TA, Georgieva T, et al. The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. *Mutat Res* 2007; **620**(1–2): 83–92.
- [25] Hegazi MMI. Separation, identification and quantification of photosynthetic pigments from three red sea seaweeds using reversed-phase high-performance liquid chromatography. *Egypt J Biol* 2002; **4**: 1–6.
- [26] Canales-Aguirre A, Padilla-Camberos E, Gómez-Pinedo U, Salado-Ponce H, Feria-Velasco A, De Celis R. Genotoxic effect of chronic exposure to DDT on lymphocytes, oral mucosa and breast cancer of female rats. *Int J Environ Res Public Health* 2011; **8**: 540–553.
- [27] Schabath MB, Grossman HB, Delclos GL, Hernandez LM, Day RS, Davis BR, et al. Dietary carotenoids and genetic instability modify bladder cancer risk. *J Nutr* 2004; **134**: 3362–3369.
- [28] Zhao X, Tian H, Ma X, Li L. Epigallocatechin gallate, the main ingredient of green tea induces apoptosis in breast cancer cells. *Front Biosci* 2006; **11**: 2428–2433.
- [29] Watters JL, Satia JA, Kupper LL, Swenberg JA, Schroeder JC, Switzer BR. Associations of antioxidant nutrients and oxidative DNA damage in healthy African-American and white adults. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 1428–1436.
- [30] Astley SB, Hughes DA, Wright RM, Elliott RM, Southon S. DNA damage and susceptibility to oxidative damage in lymphocytes: effects of carotenoids *in vitro* and *in vivo*. *Br J Nutr* 2004; **91**: 53–61.
- [31] Celikler S, Vatan O, Yildiz G, Bilaloglu R. Evaluation of anti-oxidative, genotoxic and antigenotoxic potency of *Codium tomentosum* Stackhouse ethanolic extract in human lymphocytes *in vitro*. *Food Chem Toxicol* 2009; **47**(4): 796–801.
- [32] Toniolo P. Serum carotenoids and breast cancer. *Am J Epidemiol* 2001; **153**: 1142–1147.
- [33] Muhlhofer A. Carotenoids are decreased in biopsies from colorectal adenomas. *Clin Nutr* 2003; **22**: 65–70.
- [34] Goodman MT. The association of plasma micronutrients with the risk of cervical atypical squamous cells of undetermined significance (ASCUS). *Asian Pac J Cancer Prev* 2000; **1**: 227–235.
- [35] Santocono M, Zurria M, Berrettini M, Fedeli D, Falcioni G. Influence of astaxanthin, zeaxanthin and lutein on DNA damage and repair in UVA-irradiated cells. *J Photochem Photobiol* 2006; **85**: 205–215.