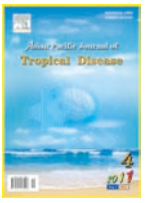
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

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



Document heading doi:10.1016/S2222-1808(11)60073-X

Antimutagenic assay of carotenoids from green algae *Chlorococcum humicola* using *Salmonella typhimurium* TA98, TA100 and TA102

Bhagavathy S[1*], Sumathi P[2], Madhushree M[3]

[1]*Bharathiar University, Coimbatore, Tamilnadu, India*
[2]*Department of Biochemistry, Government Women's College, Krishnagiri, Tamilnadu, India*
[3]*Mohamed Sathak College, PG and Research Department of Biochemistry, Sholinganallur, Chennai, Tamilnadu, India*

ARTICLE INFO

Article history:
Received 10 September 2011
Received in revised form 29 September 2011
Accepted 30 October 2011
Available online 28 December 2011

Keywords:
Chlorococcum humicola
Benzo (a) pyrene
Sodium azide
Daunomycin
Phenyl hydrazine
Salmonella typhimurium
Antimutagenic property
Green algae
Carotenoid extract
Antitumor activity
Mutational inhibition
Ethidium bromide
Hydroxyl amine
Carotenoids
Chemopreventive agent
Nonmutagenic activity

ABSTRACT

Objective: To evaluate the nonmutagenic and antimutagenic properties of the different carotenoids from the selected green algae *Chlorococcum humicola* (*C. humicola*) using Ames mutagenicity assay. **Methods:** Antimutagenicity of fresh water green algae *C. humicola* was tested using its carotenoid extract. From the crude carotenoid extract, the different carotenoids were fractionated by column chromatography and further identified by high performance liquid chromatography (HPLC). In the *in vitro* antimutagenicity assay the total and individual carotenoids were used to check the nonmutagenicity and antimutagenicity effect using the strains of *Salmonella typhimurium* (*S. typhimurium*) TA98, TA100 and TA102 with and without metabolic activation. The *in vivo* antimutagenic studies were carried out using animal model. **Results:** The results showed that the selected green algae were rich in carotenoids. In the crude carotenoid extract, astaxanthin, lutein and β-carotene were found to be the major concentration. α-carotene, zeaxanthin and violaxanthin were also found to be present. In the *in vitro* antimutagenic assay, the total carotenoids showed the maximum inhibition ($P<0.001$) against all three selected strains of *S. typhimurium* with and without metabolic activation. In the separated carotenoids, astaxanthin, lutein and β-carotene showed significant mutational inhibition and exhibited good antitumor activity in the presence of S9 fraction. For direct acting mutagens sodium azide, ethidium bromide and hydroxyl amine with the specified concentration, the total carotenoids gave 63%, 60%, 66% inhibition for TA98, TA100 and TA102, respectively. *In vivo* antimutagenic assay was also confirmed the effective antimutagenic activity of total and separated carotenoids. The results also confirmed that the total and individual carotenoids had no mutagenic activity. **Conclusions:** The overall findings of the present study conclude that the green algae *C. humicola* possess higher concentration of bioactive carotenoids having antimutagenic activity, therefore, it can be used as a chemopreventive agent.

1. Introduction

Mutations are the cause of innate metabolic defects in cellular systems, triggering morbidity and mortality in living organisms. A plethora of synthetic and natural substances, apart from various genotoxic, physical and biological agents are known to act as mutagenic, co-mutagenic and/or carcinogenic agents. There is increasing evidence that mutation in somatic cells is not only involved in the carcinogenesis but can also cause genetic disorders like artherosclerosis, heart disease and several other degenerative disorders. Since, the mutagens are involved in the initiation and promotion of several human diseases, including cancer[1].

The chemical origin of human malignancies was recognized by observations of unusual cancer induction in persons in certain occupational groups. The capacity of the chemicals to cause cancer has been studied in animals. Environmental and life style expenses can modify cancer risk. Individual genetic factor can also influence cancer risk. Since mutations are largely responsible for activating protooncogenes and inactivating tumour suppressor genes, the mutational spectra of chemical and physical

*Corresponding author: Bhagavathy S, Research Scholar, Bharathiar University, Coimbatore, Tamilnadu, India.
Tel: +91-9445283050
E-mail: bhagavathy12@rediffmail.com

carcinogens are of interest to define endogenous and exogenous mutational mechanisms[2].

Cancer is a broad term used for identifying a large number of diseases. It is one of the major diseases that affect nearly 25% of the population in developed countries and nearly 50% of these deaths are due to this disease. Somatic gene mutations are the basic events for the conversion of a normal cell to a mutant cell. This mutant cell converted to malignant cell through several genetic changes. Several chemicals have been implicated in cancer causation. Some of these are sodium azide, ethidium bromide, hydroxyl amine, MNNG, NPD, *etc*[3].

Environmental pollution is associated with increased risk of cancer. Prevention of cancer and other mutation related disease can be pursued by avoiding exposure to recognized carcinogens or mutagens, by favouring the intake of protective factors and fortifying physiological defence mechanism. Moreover, there is an increasing awareness that certain naturally occurring substances in plants and other source have protective effects against environmental mutagens or carcinogens and also endogenous mutagens. Hence, research work related to the discovery, characterization and use of antimutagenic agents is receiving considerable attention. A large number of experimental reports have begun to appear in the scientific literature, wherein increasingly more natural antimutagens have been identified, isolated and found to possess significant mutation chemopreventive properties[4].

Cancer cells are quickly adapted to the toxic environment, keep dividing and forming more cells without a control mechanism. These factors make cancer an extremely difficult disease to treat. The anticancer drugs destroy cells by stopping them from growing or dividing at one or more points in their growth cycle. But the chemotherapy kills the healthy normal cells also. To avoid that herbal therapies are followed. There are several plant products which have been shown to prevent the development of cancer in animals and some of them are in clinical trials[5]. These drugs are called chemopreventive agents which include carotenoids, polyphenols, and flavanoids and so on. More than thousand natural chemopreventive agents have been identified. Chemotherapy drugs work by varying mechanisms to induce cellular death. Some chemotherapy drugs kill cells by inflicting massive free radical damage, while other chemotherapy drugs interfere with different cellular metabolic process in order to eradicate cancer cells depending on the type of cytotoxic drugs used. However, antioxidants may confer protection to cancer cells during active chemotherapy[6].

Specific inhibition of these steps can significantly inhibit the cancer causation. Apoptosis is also an important mechanism of cellular defense in reducing the risks, so the natural products which can induce the apoptosis may inhibit the malignant cell formation. Numerous studies have been carried out in the last four decades in order to identify compounds that might protect humans against DNA damage and its consequences. There are continued efforts all over the world to explore the rich biodiversity of edible as well as medicinal plants and other edible non-toxic plants in pursuit of the mist effect phytoantimutagens. These bioactive compounds belong to a variety of different chemical groups such as phenolics, pigments, allylsulfides, glucosinolates, tannins, anthocyans, flavonoids, polysterols, protease inhibitors and phytosterols. Many of these substances elicit, apart from their antimutagenic and anticarcinogenic properties, additional beneficial effects, such as activation of immune system and /or protection against cardiovascular diseases[1].

Some preparations obtained from these plants have shown anti-inflammatory, insecticidal, anti-hypertensive, antidiabetics, immunomodulatory and antimutagenic effects, and possess the property to inhibit potential benzo (a) pyrene [B(a)P] induced DNA-damage, and neurotoxicity. They also generate oxidative stress by changing thiol cellular status, and may be used as pest control agents[7,8]. Large numbers of plant species are a source of biologically active compounds which include polyphenols, different types of carotenoids such as α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene and zeaxanthin. Various natural carotenoids, besides β-carotene were proven to have anticarcinogenic activity and some of them showed more potent activity than β-carotene. Lutein is the major carotenoid present in human blood. It is covalently bound to one or more fatty acids present in some fruits and flowers. Lutein improves visual function in some patients with retinal degeneration[9]. Compounds from plant could act as protective agent with respect to human carcinogenesis, acting against the initiation, promotion or progression stages of the process[10]. *In vitro* and *in vivo* studies have shown that carotenoid supplementation is associated with decreased DNA damage. Among these lutein is the most important carotenoid. It has specific biological functions in decreasing cancer development, enhancing immune function and protecting against age related muscular degeneration. Dietary lutein also reduced the mammary tumour growth and development. Infection with bacteria and viruses may develop into cancer. Important mechanisms by which infectious agent may induce carcinogenesis include the production of chronic inflammation, the transformation of cells by insertion of oncogenes and inhibition of tumour suppressors, and the induction of immune suppression[11].

In spite of the proven pharmacological properties for several medicinal plants, several analyses should be made including the genotoxic activity, since their constituents can cause harmful changes in the DNA. The risks associated with genotoxic activity are significant when such alternative treatments are applied without criteria, without due attention to correct botanical identification, and not considering the correct part of the plant to be used or even the method of preparation and administration[12]. Therefore,

the present study has been conducted in order to evaluate the nonmutagenic and antimutagenic effects of carotenoids from green algae *Chlorococcum humicola* (*C. humicola*) by the Ames test, using TA98, TA100, and TA102 strains of *Salmonella typhimurium* (*S. typhimurium*).

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 and Algal Biotechnology, Vivekantha Institute, Chennai, India. Algal culturing was carried out with 100 mL Bold's basal medium[13], supplemented with sterile compressed air and kept under fluorescent light (20 μmol/m^2/s) with 16 h light period and at (25±2 ℃) 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. Extraction of total carotenoids

Total carotenoids were extracted from 10 g of algal sample with ethanol until all the pigments were removed, filtered through a sintered glass filter (porosity 3; pore size 20-30 μ). 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, was 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) and dried over Na_2SO_4. The unsaponifiable residue was dissolved in a little ether and then in 10 mL of petroleum ether (b.p. 40-60 ℃) (Delia, 2001). The total carotenoid content was estimated spectrophotometrically at 450 nm using a Perkin-Elmer Spectrophotometer and calculated using the formula.

$$\text{Total carotenoids } (\mu g/g) = \frac{A_{Total} \times \text{volume (mL)} \times 10^4}{A \times \text{sample weight (g)}}$$

2.3. Identification of carotenoids

A part of total carotenoids dissolved in light petroleum was subjected to column chromatography on aluminium oxide grade III (100 mm × 10 mm column). For removal of neutral lipids, the column was washed twice with light petroleum. To ensure that no residual water got into the absorbent, anhydrous sodium sulphate topped 1 cm of the column. About one bed volume of petroleum ether was passed through the column and the volume was adjusted so that the solvent flow was about two to three drops per second. Once petroleum ether was added to the column, the top of the column was kept covered with solvent at all times until chromatography was complete. With the dropper pipette, the carotenoid petroleum ether solution was added into the column and the sample layer was let go down almost to the surface of the sodium sulphate layer. The column was developed by adjusting the mobile phase so as to isolate the desired carotenoids as quickly and efficiently as possible. Astaxanthin was eluted with acetone, lutein with 25%-30% acetone in petroleum ether, β-carotene with 2% acetone in petroleum ether, α-carotene with petroleum ether, zeaxanthin with 40%-45% acetone in petroleum ether and violaxanthin with 15%-18% acetone in petroleum ether. As acetone affects the absorption of carotenoids in petroleum ether, the acetone from β-carotene, lutein and zeaxanthin was removed by washing with water in a separating funnel. The petroleum ether carotenoid extraction was dried with anhydrous sodium sulphate. Aliquots from each isolate were dried under nitrogen and were used to verify the purity by HPLC[14].

$$\text{Carotenoid fractions } (\mu g/g) = \frac{A_{fractions} \times \text{volume (mL)} \times 10^4}{A \times \text{sample weight (g)}}$$

2.4. HPLC analysis of carotenoids

HPLC analysis of carotenoids was carried out using an HPLC (Shimadzu 10AS, Kyoto, Japan) reverse phase 25 cm, 4.6 mm, 5 μm, C18 column (Wakosil 11 5C 18RS) with an isocratic solvent system consisting of dichloromethane:acetonitrile:methanol (20:70:10, v/v/v) at a flow rate of 1.0 mL/min. All the carotenoids were monitored at 476 nm with a UV-visible detector (Shimadzu, Kyoto, Japan). The peak identification and λ_{max} values of these components were confirmed by their retention times and characteristic spectra of standard chromatograms recorded with a Shimadzu model LC-10AVP series equipped with a SPD-10AVP photodiode array detector. They were quantified from their peak areas in relation to the respective reference standards.

2.5. Determination of in vitro antimutagenicity

2.5.1. Direct acting mutagens

To identify the nonmutagenicity and antimutagenicity of the carotenoid extracts, the Ames antimutagenicity assay was carried out in the presence of standard mutagens according to the method of Moren and Ames[15] using *S. typhimurium* strains TA98, TA100 and TA102 procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The standard mutagens used as positive controls in each experiment were daunomycin (6 μg/plate) for TA98, sodium azide (1 μg/plate) for TA100, and phenyl hydrazine (20 μg/plate) for TA102. The specific dose (100 μg/plate) of total and individual

carotenoids dissolved in dimethyl sulfoxide (DMSO) was used per plate for the nonmutagenicity and antimutagenicity assay. For the mutagenicity assay, the bacterial culture was diluted to 1×10^9 cells/mL approximately. A volume of 100 μL of this diluted culture and 500 μL of Histidine-Biotin enriched KCl were added to 2 mL of molton top agar. A volume of 0.1 mL of overnight culture and 0.1 mL of test compound were added in succession to 2 mL of molton top agar. The contents were mixed gently and thoroughly, poured over the basal agar and spreaded evenly. After the top agar solidified, the plates were inverted and incubated at 37 ℃ for 48 h. At the end of 48 h, the number of histidine-revertant colonies was counted[16].

2.5.2. Mutagens requiring activation

Antimutagenic assay against mutagen that requires metabolic activation [B(a)P - 1 μg/plate] was carried out as follows. Liver microsomal fraction (S9) was prepared from Sprague Dawley rat (200 g). The rat was treated with 0.1% phenobarbital in drinking water for 4 days[17]. After overnight fasting, the animal was killed by decapitation, the liver was removed and the homogenate was prepared asceptically[18]. The activation mixture was prepared by mixing 50 μL of the S9 fraction, containing 0.25 mL phosphate buffer (0.2 M, pH 7.4), 20 μL NADP (0.1 M), 2.5 μL glucose-6-phosphate (1 M) and 10 μL of 1.65 M $MgCl_2$ and 0.4 M KCl and the total and fractions of the carotenoids were mixed with the mutagens at a given concentration poured onto minimal agar plates and incubated for 48 h at 37 ℃. After incubation, number of revertants was counted. Percent inhibition of mutagenicity was determined by the following formula:

$$\text{Inhibition (\%) of mutagenicity} = \frac{(R1 - SR) - (R2 - SR)}{(R1 - SR)} \times 100$$

Where R1 is the number of revertants without carotenoid extract, R2 the number of revertants with carotenoid extract and SR is the spontaneous revertants.

2.6. Determination of in vivo antimutagenicity

Male Wistar rats were divided into three groups. Group I animals were given distilled water for 30 days. Group II animals were left free without any treatment. Group III animals were fed with different types of carotenoid extracts orally (100 mg/kg b.w.) for 30 days. On the 31st day B(a)P (10 mg/rat i.p.) was administered to group II and group III animals. The urine was collected from the animals for 24 h in metabolic cages. The urine thus collected was filtered using Whatman No. 1 filter paper, and 20 mL of urine was passed through Amberlite XAD-4 column (40 mm × 10 mm) to concentrate the mutagen[19]. The weakly anionic components adsorbed were eluted with 10 mL acetone. The eluents were evaporated to dryness at 60 ℃ and the mixture was stored at -20 ℃ and reconstituted in 1.5 mL DMSO just before the antimutagenicity assay[20]. *S. typhimurium* strains TA98 and TA100 were used for the assay. Fresh *Salmonella* culture (1×10^9 cells/mL) and 0.1 mL of urine concentrate were mixed with 2 mL top agar containing 0.2 mL of 0.5 mM histidine-biotin and poured on minimal glucose agar plate. The revertants were counted after incubation for 48 h at 37 ℃. The urine collected from animals treated with B(a)P alone, was processed similarly and kept as control plate. The assays were done in triplicates. The percent inhibition of mutagenicity was calculated by the above formula.

2.7. Statistical analysis

The results were presented as the average and standard error of the three experiments with triplicate plate/dose/experiment. The data were further analyzed for statistical significance by two way ANOVA, SPSS 12 software.

3. Results

3.1. HPLC analysis of carotenoids

HPLC analysis of *C. humicola* carotenoids which were separated within 21 min through a C18 column showed the available carotenoids and xanthophyll pigments (Figure 1). The purity calculated as the percentage of the carotenoid's peak area relative to total area was 91%-97% for astaxanthin, 95%-98% for violaxanthin, 97%-100% for lutein, 92%-96% for zeaxanthin, 90%-97% for β-carotene and 92%-96% α-carotene. The relative percentages of xanthophylls such as violaxanthin (4%), astaxanthin (17%), lutein (41%), zeaxanthin (2%), α-carotene (12%), and β-carotene (22%), were determined. The collected aliquotes of column chromatography were further confirmed by their retention time and absorption spectra of respective reference standards, as shown in Figure 2. Among the carotenoids eluted, astaxanthin and its esters formed the major proportion of carotenoids followed by lutein, β-carotene, violaxanthin, zeaxanthin, and α-carotene.

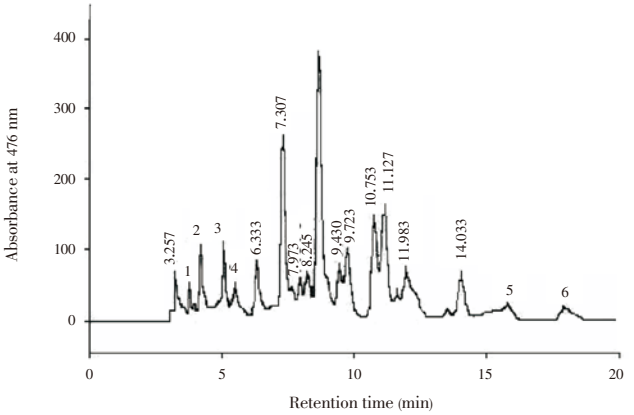


Figure 1. HPLC profile of carotenoids from *C. humicola* extract.
1: violaxanthin; 2: astaxanthin; 3: lutein; 4: zeaxanthin; 5: α-carotene; 6: β-carotene.

Table 1
Effect of the carotenoid extracts of *C. humicola* on *S. typhimurium* spontaneous revertants in the presence (+S9) or absence of (−S9) microsomal fraction (mean±SE) (*n*=3).

Groups	Dose (μg/100 mL/plate)	Number of histidine−revertants					
		TA98		TA100		TA102	
		−S9	+S9	−S9	+S9	−S9	+S9
SR	−	53.00±9.40	55.00±7.50	116.00±5.40	128.00±3.80	255.00±7.50	280.00±3.70
TC	100	51.00±7.50	54.00±5.60	115.00±4.60	125.00±3.70	255.00±6.80	281.00±6.50
Violaxanthin	100	50.00±5.60	52.00±5.60	113.00±4.80	127.00±5.60	254.00±5.60	281.00±5.60
Astaxanthin	100	50.00±9.40	51.00±7.50	114.00±3.50	126.00±7.20	250.00±7.60	279.00±7.50
Lutein	100	51.00±3.70	53.00±7.50	112.00±3.50	125.00±6.20	251.00±5.60	282.00±4.70
Zeaxanthin	100	49.00±2.90	54.00±6.80	114.00±6.80	127.00±4.60	253.00±7.20	282.00±5.40
α−carotene	100	51.00±3.10	53.00±2.90	115.00±3.10	127.00±3.40	252.00±3.70	279.00±5.70
β−carotene	100	50.00±2.80	51.00±3.20	113.00±2.80	123.00±2.80	250.00±6.80	280.00±6.40

SR: Spontaneous revertants in *S. typhimurium* culture; TC: Total carotenoids; +S9: Presence of metabolic activation; −S9: Absence of metabolic activation.

Table 2
Antimutagenic activity of the carotenoid extracts of *C. humicola* against standard mutagens on *S. typhimurium* TA98 (mean±SE) (*n*=3).

Groups	Dose (μg/100 mL/plate)	Number of histidine−revertants		% Inhibition	
		−S9	+S9	−S9	+S9
SR	−	53.00±9.40	55.00±7.50	−	−
TC	100	188.00±7.50***	218.00±3.50***	71.75	62.95
Violaxanthin	100	356.00±3.50***	394.00±2.80**	36.61	22.95
Astaxanthin	100	228.00±6.40***	356.00±3.50***	63.38	31.59
Lutein	100	316.00±7.50***	299.00±4.60***	44.98	40.68
Zeaxanthin	100	343.00±8.40***	412.00±4.60***	39.33	34.55
α−carotene	100	412.00±3.70**	396.00±5.80*	24.90	18.86
β−carotene	100	216.00±4.60***	282.00±4.90***	65.90	48.41
Daunomycin	6	531.00±3.50	−	−	−
B(a)P	1	−	495.00±3.70	−	−

***: $P<0.001$, **: $P<0.005$, *: $P<0.01$ with respect to daunomycin and B(a)P treated group; SR: Spontaneous revertants in *S. typhimurium* culture; TC: Total carotenoids; +S9: Presence of metabolic activation; −S9: Absence of metabolic activation.

Table 3
Antimutagenic activity of the carotenoid extract of *C. humicola* against standard mutagens on *S. typhimurium* TA100 (mean±SE) (*n*=3).

Groups	Dose (μg/100 mL/plate)	Number of histidine−revertants		% Inhibition	
		−S9	+S9	−S9	+S9
SR	−	116.00±8.20	128.00±3.70	−	−
TC	100	315.00±7.60***	288.00±5.60***	60.09	60.28
Violaxanthin	100	432.00±4.50***	436.00±2.80**	44.56	27.19
Astaxanthin	100	396.00±5.60***	416.00±4.90***	50.88	31.91
Lutein	100	352.00±7.20***	399.00±3.30***	58.60	35.93
Zeaxanthin	100	458.00±6.40***	452.00±1.60**	40.00	23.40
α−carotene	100	468.00±8.50***	403.00±2.90***	38.25	34.99
β−carotene	100	346.00±4.60***	398.00±4.80***	59.65	36.17
Sodium azide	1	686.00±5.60	−	−	−
B(a)P	1	−	551.00±9.60	−	−

***: $P<0.001$, **: $P<0.005$, *: $P<0.01$ with respect to sodium azide and B(a)P treated group; SR: Spontaneous revertants in *S. typhimurium* culture; TC: Total carotenoids; +S9: Presence of metabolic activation; −S9: Absence of metabolic activation.

3.2. Quantitative analysis of carotenoids

The total carotenoid content of *C. humicola* was found to be 275 mg/100 g of fresh algal samples. The chromatographic separation yielded violaxanthin (11 mg/100 g), astaxanthin (46 mg/100 g), lutein (109 mg/100 g), zeaxanthin (6 mg/100 g), α−carotene (33 mg/100 g) and β−carotene (61 mg/100 g) of fresh algal sample.

3.3. In vitro antimutagenicity

To identify the nonmutagenicity and antimutagenicity of the selected algal carotenoids, the Ames *Salmonella* microsome assay was carried out with standard mutagens in the presence and absence of metabolic activation system using the strains TA98, TA100 and TA102. The number of histidine revertants in the culture treated with total and individual carotenoids of the selected green algae *C. humicola* in the presence and absence of metabolic

Table 4
Antimutagenic activity of the carotenoid extract of *C. humicola* against standard mutagens on *S. typhimurium* TA102 (mean±SE) (*n*=3).

Groups	Dose (μg/100 mL/plate)	Number of histidine−revertants		% Inhibition	
		−S9	+S9	−S9	+S9
SR	−	255.00±7.50	128.00±3.70	−	−
TC	100	328.00±6.80***	356.00±6.50***	71.59	65.61
Violaxanthin	100	396.00±3.50***	465.00±7.20*	45.14	16.28
Astaxanthin	100	370.00±6.60***	376.00±4.60***	55.25	56.56
Lutein	100	342.00±4.50***	382.00±5.60***	66.14	53.84
Zeaxanthin	100	420.00±5.20***	464.00±4.90*	35.80	16.74
α−carotene	100	416.00±8.60***	404.00±4.80***	37.35	56.11
β−carotene	100	348.00±4.40***	372.00±3.90***	53.30	58.37
Phenyl hydrazine	20	512.00±5.60	−	−	−
B(a)P	1	−	501.00±3.50	−	−

***: $P<0.001$, **: $P<0.005$, *: $P<0.01$ with respect to phenyl hydrazine and B(a)P treated group; SR: Spontaneous revertants in *S. typhimurium* culture; TC: Total carotenoids; +S9: Presence of metabolic activation; −S9: Absence of metabolic activation.

Table 5
In vivo antimutagenic activity of the carotenoid extracts of *C. humicola* against B(a)P on *S. typhimurium* TA98, TA100, TA102 (mean±SE) (*n*=3).

Groups	Number of histidine−revertants			% Inhibition		
	TA98	TA100	TA102	TA98	TA100	TA102
SR	50.00±5.60	128.00±6.50	249.00±6.80	−	−	−
B(a)P	138.00±8.50	373.00±20.00	614.00±18.60	−	−	−
TC	48.00±4.70	114.00±6.70	251.00±7.20	−	−	−
Violaxanthin	51.00±6.50	125.00±5.40	248.00±6.80	−	−	−
Astaxanthin	50.00±8.70	115.00±8.20	244.00±5.40	−	−	−
Lutein	49.00±1.20	121.00±4.10	253.00±3.20	−	−	−
Zeaxanthin	52.00±4.20	116.00±3.20	249.00±6.40	−	−	−
α−carotene	53.00±3.30	128.00±6.10	243.00±5.40	−	−	−
β−carotene	50.00±7.20	126.00±5.90	250.00±3.10	−	−	−
B(a)P + TC	78.00±4.60**	217.00±4.00**	414.00±3.60**	68.18	58.60	54.79
B(a)P + violaxanthin	82.00±2.50**	268.00±3.90*	426.00±4.80**	63.63	34.88	51.51
B(a)P + astaxanthin	89.00±3.30**	259.00±2.20*	428.00±7.30**	55.68	39.07	50.95
B(a)P + lutein	82.00±2.80**	226.00±1.60**	417.00±6.70**	63.63	54.42	53.97
B(a)P + zeaxanthin	85.00±4.20**	253.00±2.80**	446.00±4.90**	60.28	41.86	46.03
B(a)P + α−carotene	88.00±3.50**	265.00±1.70*	437.00±5.60**	56.81	36.28	48.49
B(a)P + β−carotene	83.00±1.20**	216.00±2.60**	420.00±4.90**	62.50	59.07	53.15

**: $P<0.005$, *: $P<0.01$ with respect to phenyl hydrazine and B(a)P treated group; SR: Spontaneous revertants in *S. typhimurium* culture; TC: Total carotenoids.

activation by phenobarbital was presented in Table 1. The results showed that there was no significant difference between the groups of spontaneous revertants and the carotenoids.

Antimutagenic activity of the carotenoid extracts was determined along with the specific standard mutagen for the individual strains of *S. typhimurium* TA98, TA100 and TA102 (Table 1−4). The results indicated that both total and individual carotenoids effectively reduced the number of histidine revertants, in the presence and absence of metabolic activation. The total carotenoid extract showed the maximum percentage (60%−70%) of mutational inhibition ($P<0.001$) in all the strains of *S. typhimurium* against standard mutagen with and without S9 fraction. Individual carotenoids also showed significant inhibitory effect, but the percentage varied from each of carotenoid while comparing themselves, next to total carotenoids, astaxanthin, lutein and β−carotene showed nearly, 50%−60% reduction of histidine revertants, followed by violaxanthin, zeaxanthin and α−carotene.

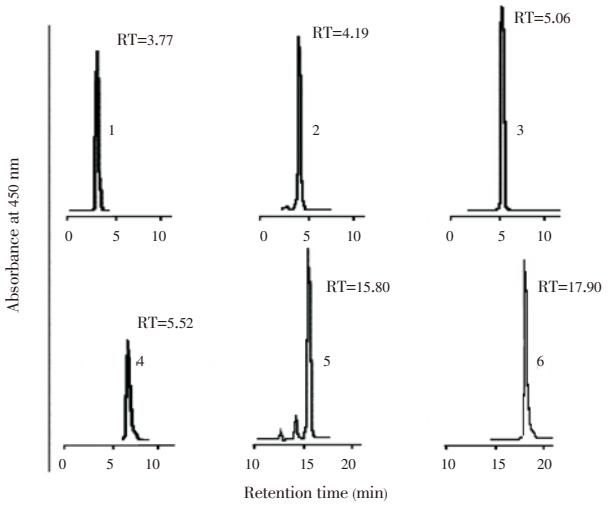


Figure 2. HPLC profiles of carotenoids eluted from *C. humicola*.
1: violaxanthin; 2: astaxanthin; 3: lutein; 4: zeaxanthin; 5: α−carotene; 6: β−carotene; RT: retention time.

3.4. In vivo antimutagenicity

Antimutagenicity tests on the urine of animals treated with B(a)P showed that the administration of total and different carotenoid extracts inhibited mutagenicity induced by B(a)P. From the results it was evident that total carotenoids gave the maximum inhibitory effect at 68%, 58% and 54% of mutagenicity induced by B(a)P for TA98, TA100 and TA102, respectively. Astaxanthin, lutein, and β-carotene showed significant reduction ($P<0.005$) against B(a)P induced mutagen in the selected tester strains. Comparing the other carotenoids violaxanthin, zeaxanthin and α-carotene showed inhibition up to 30%-60% ($P<0.005$; $P<0.01$) (Table 5).

4. Discussion

The genomes of all cancer cells carry somatic mutations. These may include base substitutions, small insertions and deletions (indels), rearrangements and copy number alterations together with epigenetic changes. Some of these somatic alterations, known as driver mutations, confer selective clonal growth advantage and are causally implicated in oncogenesis. By definition, these are found in cancer genes. The remainder are passengers which do not contribute to cancer development. However, passenger mutations bear the imprints of the mutational mechanisms that have generated them, unsullied by processes of selection, and thus provide insights into the aetiology and pathogenesis of cancer[21,22].

The Ames assay is commonly used to detect mutagenic and antimutagenic activities and is a widely accepted method for identifying various chemicals and drugs that can cause gene mutations. It has a high predictive value for *in vivo* carcinogenicity and the most common test strains are TA98, TA100 and TA102. Hence, the antimutagenicity of different caroteniods was investigated by Ames mutagenicity assay in the most sensitive *Salmonella* test strains. This test was performed in both the absence and the presence of a rat liver metabolizing system (S9 mix), providing a very sensitive study of potentially mutagenic pathways for the metabolism of these materials. It has been suggested that the use of antimutagen in daily life will be the most effective procedure for preventing human cancer and genetic disease. These compounds interfere with mutagen metabolism or they may act as mutagen scavengers. They may also inhibit either the initiation or promotion phase of the carcinogenic process[23].

In the present investigation we have checked the potential activity of carotenoid extracts for its nonmutagenicity and antimutagenicity using *S. typhimurium* assay. This is mainly based on the reversion of mutant cells by mutagenic agents. From the results it was found that the carotenoid extracts of green algae *C. humicola* could inhibit the revertant formation produced by direct acting mutagens such as sodium azide, daunomycin, phenylhydrazine and B(a)P. The spontaneous revertants by the treatment of carotenoid extracts both in the total and individual on the selected strains of *S. typhimuriun* TA98, TA100 and TA102 showed no significant difference when compared to the number of the spontaneous revertants indicating their nonmutagenicity on the tested strains of *S. typhimurium*. This nonmutagenicity was found in the presence and absence of metabolic activation.

The antimutagenic assay was performed to check the modulatory effect of carotenoids against frame shift mutagen daunomycin in TA98, base pair substitution mutagen sodium azide in TA100 and the oxidative mutagen phenyl hydrazine in TA102 in the presence (+S9) and absence (-S9) of the metabolic activation requiring mutagen B(a)P using Ames *Salmonella* histidine reversion assay. From the results it was evident that there is a significant colony with the standard mutagens in all the three strains whereas the co-administration of carotenoids with the standard mutagens significantly decreased the number of revertant frequency and showed their antimutagenic potential both in the presence and absence of S9 fraction. But the level of significance varies from total carotenoid pigments with the individual pigments. Almost in all the three strains with and without metabolic activation the total carotenoids showed nearly 70%-75% mutagenicity inhibition caused by the standard mutagen. This indicates that the combined carotenoid extracts have potential antimutagenic activity. The *in vivo* B(a)P induced mutagenicity using animal model was also effectively inhibited by the treatment of total and individual carotenoids but the combined carotenoid shows better results.

Xanthophylls are excellent antioxidants with antimutagenic and anticarcinogenic properties. Reports support that pure lutein from marigold flower (*Tagetes erecta*) has been shown to inhibit the mutagenicity, using test strains. The study indicated that lutein can inhibit nitropyrene (1-NP), B(a)P and 2-amino-3-methylimidazo(4,5-f)quinoline[24]. In a study published in the Journal of Dermatological Science, astaxanthin was tested *in vitro* to examine its ability to protect against alterations in human DNA induced by exposure to UVA radiation, and it concludes that astaxanthin can significantly prevent UV induced collagen degradation and the formation of wrinkles. These results suggest that topically applied astaxanthin, which scavenges singlet oxygen effectively, can play an important role in protecting the skin from various photodamages such as lipid peroxidation, sunburn reaction, photo toxicity and photo allergy induced by singlet oxygen[25].

The report from Nishino *et al*[26] supports the present finding indicating that α-carotene and β-carotene can suppress the tumour in skin, lung, liver, and colon. Recently, there is an increasing attention to lycopene as well as zeaxanthin and lutein. In the case of lycopene, there is some evidence for a protective role against prostate cancer[27]. For lutein and zeaxanthin, considerable evidence is available for a role of these xanthophylls in protecting DNA damage. In addition, the important role for zeaxanthin and lutein in the protection against cancer and heart disease

emerged[28]. Zeaxanthin, as mentioned above, protects against the formation of potentially destructive reactive oxygen species in leaves exposed to intense sunlight alone or moderate levels of sunlight in the presence of environmental conditions un-favourable for plant growth[29]. In addition, zeaxanthin serves in photo protection *via* another mechanism that involves a direct inhibition of the oxidation of fatty acids of biological membranes (lipid peroxidation)[30]. Epidemiological studies have identified inverse links between zeaxanthin/lutein and a wide range of human diseases[28,29,31]. However, the underlying mechanisms for these apparent protective effects remain poorly understood.

On the other hand, studies with human cancer cell lines provided evidence that lutein can stimulate programmed cell death of human breast cancer cells and leukemic cells. Lutein furthermore selectively induces programmed cell death in mouse tumour cells, but decreases programmed cell death in cancer-fighting immune cells (blood leukocytes) of tumour-bearing mice[32]. Up to now it is not known how xanthophylls exert these remarkable and beneficial roles, including opposite effects on programmed cell death in different cell types. The retention of zeaxanthin can be accomplished *e.g.* by knocking out or silencing the enzyme/gene (zeaxanthin epoxidase of the xanthophyll cycle) responsible for zeaxanthin conversion to violaxanthin and/or by over expressing enzymes in earlier portions of the carotenoid biosynthetic pathway[33].

The majority of results (46 out of 68, *i.e.* 67.6%) are indicative of protective effects of carotenoids and vitamin A toward this class of compounds. Thus, the ability of these nutrients to prevent point mutations produced by B(a)P in bacteria, as reported in five studies. The ability of cantaxanthin, β-carotene and its intermediate metabolites 89-apo-b-carotenal and 89-apo-b-carotene methyl ester to inhibit the bacterial mutagenicity of B(a)P is correlated with the ability of the same compounds to inhibit the development of forestomach tumours induced by B(a)P given by gavage in mice[34]. Both β-carotene and retinol were found to decrease binding of B(a)P to DNA in organ cultures of tracheal epithelium, which was accompanied by a stimulation of UDS in these cells. This finding was interpreted by the authors as an inhibition of DNA adducts levels due to an enhancement of DNA repair activity[35]. Retinyl palmitate inhibited the mutagenicity of B(a)P in cultured human cells. The oral administration of retinyl esters inhibited DNA binding of B(a)P metabolites in rat hepatocytes and stomach cells, but not in lung and kidney cells[36]. In studies with B(a)P derivatives, retinol inhibited the S9-mediated bacterial mutagenicity of 6-methylbenzo(a)pyrene and 6-hydroxymethylbenzo(a)pyrene, but failed to affect the direct mutagenicity of 6-acetoxymethylbenzo(a)pyrene[37].

An *in vivo* study showed that β-carotene decreases B(a)P-induced DNA damage in the mouse forestomach mucosa but not the induction of micronuclei in mouse bone marrow cells[38]. In addition to α-carotene, other carotenoids, such as lutein, lycopene, and β-cryptoxanthin, also seem to be promising carotenoids, as these carotenoids showed strong anti-carcinogenic activity in screening tests. Furthermore, these carotenoids may be more suitable in combinational use, rather than in single use, since it has been found that the treatment with mixture of these carotenoids resulted in more effective inhibition than each carotenoid alone[14].

There is a relationship between mutagenesis and carcinogenesis, in that, both show abrupt changes in a single cell, permanent and inherited by daughter cells. Because of this relationship, the mutagenicity evaluation for new drugs and also for medicinal plants should be highly recommended for detecting potential genotoxic compounds[39]. It has been suggested that the use of antimutagen in daily life will be the most effective procedure for preventing human cancer and genetic disease. These compounds interfere with mutagen metabolism or they may act as mutagen scavengers. They may also inhibit either the initiation or promotion phase of the carcinogenic process.

The results of the present study suggest that the green algae *C. humicola* is a rich source of bioactive carotenoids that can effectively fight against different mutagens and should be further focused well for its beneficiary effect.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We are very much thankful to Dr. Sivasubramonium, Director, Department of Plant and Algal Biotechnology, Vivekanda College, Chennai, India for providing the algal cultures throughout the study.

References

[1] Bhattacharya S. Natural antimutagens: a review. *Res J Med Plant* 2011; **5**(2): 116-126.

[2] Hanafy Z. Ginger extract antimutagens as cancer chemopreventive agent against Ehrlisch ascites cancer. *Acad J Cancer Res* 2009; **2**(2): 61-67.

[3] Prabhu N, Elizabath GS, Joice PA, Soumya TS. Effect of *Rosa multiflora* extract on chemical mutagens using Ames assay. *Der Pharma Chemica* 2010; **2**(1): 91-97.

[4] Benfenati E, Benigni R, Demarini DM, Helma C, Kirkland D, Martin TM, et al. Predictive models for carcinogenicity and mutagenicity: frameworks, state-of-the-art, and perspectives. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2009; **27**(2): 57-90.

[5] Burgess DJ. Anticancer drugs. *Nat Rev Drug Discov* 2011; **10**: 338-339.

[6] Madhuri S, Pandey G. Some anticancer medicinal plants of foreign origin. *Curr Sci* 2009; **96**(6): 779-783.

[7] Cotinguiba F, Regasini LO, Bolzani VS, Debonsi HM, Passerini GD, Cicarelli RMB, et al. Piperamides and their derivatives as potential anti-trypanosomal agents. *Med Chem Res* 2009; **18**: 703-711.

[8] Varanda EA. Atividade mutagênica de plantas medicinais. *Rev Ciênc Farm Básica Apl* 2006; **27**: 1-7.

[9] Karikas GA. Anticancer and chemopreventing natural products: some biochemical and therapeutic aspects. *J BUON* 2010; **15**(4): 627-638.

[10] de Morandim-Giannetti AA, Cotinguiba F, Regasini LO, Frigieri MC, Varanda EA, Coqueiro A, et al. Study of *Salmonella typhimurium* mutagenicity assay of (Z)-piplartine by the Ames test. *Afr J Biotechnol* 2011; **10**(27): 5398-5401.

[11] Riccioni G, D'Orazio N, Franceschelli S, Speranza L. Marine carotenoids and cardiovascular risk markers. *Mar Drugs* 2011; **9**: 1166-1175.

[12] Bezerra DP, Moura DJ, Rosa RM, Vasconcellos MC, Silva ACR, Moraes MO, et al. Evaluation of the genotoxicity of piplartine, an alkamide of *Piper tuberculatum*, in yeast and mammalian V79 cells. *Mutat Res* 2008; **652**: 164-174.

[13] Bischoff HW, Bold HC. Psychological studies. Some soil algae from enchanced rock and related algal species. *Univ Texas Publ* 1963; **6318**: 32-36.

[14] Rodriguez-Amaya DB, Kimura M. *Harvestplus hand book for carotenoid analysis*. Washington, DC and Cali: International Food Policy Research Institute (IFPRI) and International Center for Tropical Agriculture (CIAT); 2004, p. 40-42.

[15] Maron DM, Ames BN. Revised methods for the *Salmonella* mutagenicity test. *Mutat Res* 1983; **113**: 173-215.

[16] Lima ZP, Santos RC, Torres TU, Sannomiya M, Rodrigues CM, Santos LC, et al. *Byrsonima fagifolia*: an integrative study to validate the gastroprotective, healing, antidiarrheal, antimicrobial and mutagenic action. *J Ethnopharmacol* 2008; **120**: 149-160.

[17] Ames BN, Purtson WE, Yamasaju E, Lee FD. Carcinogens are mutagens: a simple test system combining liver homogenate for activation of bacteria for detection. *Proc Natl Acad Sci* 1973; **70**: 3555-3559.

[18] Lakshmi B, Ajith TA, Sheena N, Gunapalan N, Janardhanan KK. Antiperoxidative, anti-inflammatory and antimutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* occurring in South India. *Teratog Carcinog Mutagen* 2003; **22**: 85-97.

[19] Yamasaki E, Ames BN. Concentration of mutagens from urine by adsorption with the non-polar resin XAD-2: cigarette smokers have mutagenic urine. *Proc Natl Acad Sci USA* 1997; **74**: 3555-3559.

[20] Polosa K, Sesikaran B, Krishna TP, Krishnaswamy K. Turmeric (*Curcuma longa*) induced reduction in urinary mutagens. *Food Chem Toxicol* 1991; **29**: 25-30.

[21] Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature* 2009; **458**: 719-724.

[22] Zhang L, Ren XY, Alt E, Bai XW, Huang SY, Xu ZM, et al. Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis. *Nature* 2010; **464**: 1058-1061.

[23] Pounikar R, Dawande AY. Detection of potential carcinogens by Ames test. *Asiat J Biotechnol Resour* 2010; **1**: 57-64.

[24] Wang Y, King JM, Xu Z, Losso J, Prudente A. Lutein from ozone-treated corn retains antimutagenic properties. *J Agric Food Chem* 2008; **56**(17): 7942-7949.

[25] Lyons NM, O'Brien NM. Modulatory effects of an algal extract containing astaxanthin on UVA-irradiated cells in culture. *J Dermatol Sci* 2002; **30**(1): 73-84.

[26] Nishino H, Murakoshi M, Tokuda H, Satomi Y. Cancer prevention by carotenoids. *Arch Biochem Biophys* 2009; **483**: 165-168.

[27] Ellinger S, Ellinger J, Stehle P. Tomatoes, tomato products and lycopene in the prevention and treatment of prostate cancer: do we have the evidence from intervention studies? *Curr Opin Clin Nutr Metab Care* 2006; **9**: 722-727.

[28] Mares-Perlman JA, Millen AE, Ficek TL, Hankinson SE. The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. *J Nutr* 2002; **132**: 518S-524S.

[29] Demmig-Adams B, Adams WW. Antioxidants in photosynthesis and human nutrition. *Science* 2002; **298**: 2149-2153.

[30] Havaux M, Dall'Osto L, Cuiné S, Giuliano G, Bassi R. The effect of zeaxanthin as the only xanthophyll on the structure and function of the photosynthetic apparatus in *Arabidopsis thaliana*. *J Biol Chem* 2004; **279**: 13878-13888.

[31] Sajilata MG, Singhal RS, Kamat MY. The carotenoid pigment zeaxanthin-a review. *Compr Rev Food Sci Food Saf* 2008; **7**: 29-49.

[32] Chew BP, Brown CM, Park JS, Mixter PF. Dietary lutein inhibits mouse mammary tumour growth by regulating angiogenesis and apoptosis. *Anticancer Res* 2003; **23**(4): 3333-3339.

[33] Demmig-Adams B, Adams WW. Overview of diet-gene interactions and the example of xanthophylls. *Adv Exp Med Biol* 2010; **698**: 17-26.

[34] Al-Attar AM. The meliorative role of β-carotene pretreatment on diazinon-induced enzymological and histopathological changes in Wistar male rats. *Glob J Pharmacol* 2009; **3**(3): 171-177.

[35] Jung KH, Yan BZ, Chillrud SN, Perera FP, Whyatt R, Camann D, et al. College of physicians and surgeon computational toxicology research program research publications predictive models for carcinogenicity and mutagenicity: division of pulmonary, allergy and critical care of medicine. *Int J Environ Res Public Health* 2010; **7**: 1889-1900.

[36] Siems W, Salerno C, Crifò C, Sommerburg O, Wiswedel I. Beta-carotene degradation products-formation, toxicity and prevention of toxicity. *Forum Nutr* 2009; **61**: 75-86.

[37] Yvonne GJ, Helden V, Keijer J, Heil SG, Pico C, Palou A, et al. Beta-carotene affects oxidative stress-related DNA damage in lung epithelial cells and in ferret lung. *Carcinogenesis* 2009; **30**(12): 2070-2076.

[38] Failla ML, Thakkar SK, Kim JY. *In vitro* bioaccessibility of β-carotene in orange fleshed sweet potato (*Ipomoea batatas*, Lam.). *J Agric Food Chem* 2009; **57**: 10922-10927.

[39] Maiani G, Caston MJ, Catasta G, Toti E, Cambrodon IG, Bysted A, et al. Carotenoids: actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. *Mol Nutr Food Res* 2009; **53**: S194-S218.