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Chemopreventive Effect of Chlorella on the Antioxidant System in 7, 12-Dimethylbenz[a]Anthracene-Induced Oxidative Stress in Liver

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Abstract: The present study was designed to evaluate the protective effect of Chlorella against liver oxidative damage induced by oral administration of 7,12-dimethylbenz[a]anthracene (DMBA) to female Wistar rats. Animals of protected groups were orally administered with dried powdered of Chlorella suspended in warm water at two selected doses; 0.5 and 1.0 g kg^{-1} b.wt. Water extract of chlorella tablets (Wakunaga of America Co., Ltd. Mission Viejo, CA, USA) was given to rats two weeks before DMBA administration and was continued for 15 weeks after cancer induction. Administration of DMBA (25 mg/rat) caused a significant increase of lipid and protein oxidations and significantly altered the levels of liver antioxidants. Treatment with chlorella extract has significantly prevented DMBA-induced oxidative changes in liver. Levels of MDA and P.carbonyl were significantly reduced after administration of chlorella extract. Chlorella has also restored normal level of indigenous antioxidants such as CAT, GSH, GST and SOD. The present results also show a high antioxidant activity of chlorella extract. It is therefore concluded that due to its potent antioxidant properties, chlorella modulates the DMBA-induced hepatic oxidative stress that is normally associated with induced-breast cancer in rats.

Key words: Oxidative stress, breast cancer, algae, antioxidants

INTRODUCTION

Oxidative stress is the result of an imbalance between the concentrations of mainly the Reactive Oxygen Species (ROS) (Delimaris *et al*., 2007). ROS normally refers to a group of reactive oxygen metabolites (Karihtala *et al*., 2006) which can increase the oxidative stress in variety of physiologic and pathphysiological processes (Amin *et al*., 2005). ROS directly attacks the cell membranes and nuclei, initiating lipid peroxidation (Arulkumaran *et al*., 2007). Experimental investigations as well as clinical and epidemiological findings have provided evidence supporting the role of ROS in the etiology of cancer (Ray *et al*., 2000). Certain aldehyde such as malondialdehyde (MDA), the end product of lipid peroxidation arising from the free radical generation leading to the degradation of polyunsaturated fatty acids, can cause cross-linking in lipids, proteins and nucleic acids.

Normal healthy cells have developed a defense system called antioxidant, which consist of enzymatic and non enzymatic mechanisms such as SOD, GST, CAT and GSH (Alscher *et al*., 1997). For cells to remain healthy, it is essential that antioxidant defense system continues to compensate for the oxidant forces, any malfunction may lead to disease development (Hanimolgu *et al*., 2007; Delimaris *et al*., 2007).

In addition, ROS are involved in all steps of cancer from initiation to malignant conversion and this is true for all kinds of cancers (Hanimolgu *et al*., 2007). Breast cancer is among the most common malignancies world-wide. Despite the wide rang of treatment available for breast cancer patients, it remains the most common cause of death in women (Ray *et al*., 2007). Therefore, chemoprevention and identifying new phytochemicals and other bioactive molecules have gained more priority in many laboratories nation-wide. Toward that goal, many natural products have been identified for its anti inflammatory, free radical scavenging and anti-tumor activity.

Plants in general and microalgae in particular, are good sources of natural antioxidants. During the photosynthesis levels of molecular oxygen escalate. Oxygen is then easily activated by sunlight ultraviolet radiation into toxic Reactive Oxygen Species (ROS), plants and microalgae have developed a protective mechanism which consists in the preparation of antioxidant compounds able to minimize the concentration of these

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ROS (Rodriguez-Garcia and Guil-Guerrero, 2008; Lu and Foo, 1995). Natural bioactive compounds have the potential to subside the biochemical imbalances induced by various toxins associated with free radicals. They provide protection without causing any side effects and therefore, natural antioxidants have been viewed as promising therapeutic drugs (Prakash and Gupta, 2000; Sabu and Kuttan, 2002; Vijayavel *et al*., 2006; Anbuselvam *et al*., 2007; Guzman *et al*., 2001; Park *et al*., 2005).

The unicellular green alga, *Chlorella vulgaris*, has been shown to express various pharmacological effects both in animals and humans. Hot water extracts of *Chlorella vulgaris* are known as potent anti-tumor remedy against hepatocarcinogenesis in rats (Takekoshi *et al*., 2005). Chlorella has also been reported to possess anti-oxidative, anti-inflammatory and anti-tumor properties *in vitro* (Guzman *et al*., 2001). Because of its phenolic antioxidants, lipophilic or hydrophilic compounds, chlorella is commonly used as a health supplement (Hasegawa *et al*., 2005; Park *et al*., 2005).

In the present study, a polycyclic aromatic hydrocarbon known as 7,12-dimethyl-benz[a]anthracene (DMBA) was used to induce mammary tumor in rats. DMBA acts as a potent carcinogen by generating various reactive metabolic intermediates leading to oxidative stress (Anbuselvam *et al*., 2007). Metabolism of DMBA results in the formation of oxy-radicals "$O_2^{\bullet -}$, $^1O_2$, H_2O_2, $^{\bullet}OH$," which bind covalently to nucleophillic sites on cellular macromolecules thereby eliciting cancerous responses (Giri *et al*., 1995). The aim of this study is to assess the chemopreventive effect of chlorella in modulating the antioxidant system in liver of drug-induced rats. Histopathological analysis and more molecular insights into chlorella's protection against DMBA-induced cancer in breast tissues are underway.

MATERIALS AND METHODS

Chemicals: This study was conducted at biology department of UAE University, 2006-2007. Each tablet contains 500 mg of 100% pure broken cell wall chlorella powder. Chlorella was purchased as health supplement tablets from Wakunaga of America Co., Ltd., Mission Viejo, CA, USA. Apoptag plus peroxidase *in situ* Apoptosis Detection Kit was purchased from Chemicon International Inc., Temecula, CA, USA. O-diamisidine, 2, 4-dinitophenylhydrazine, thiobarbituric acid, Folin reagent, epinephrine, SOD enzyme, H_2O_2 and bovine albumin were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were obtained from local commercial suppliers.

Animals and experimental protocol: Six to seven week-old female Wistar rats (100-150 g body weight) were obtained from the Animal House, UAE University. They were maintained on standard pellet diet and tap water *ad libitum* and were kept in polycarbonate cages with wood chip bedding under a 12 h light/dark cycle and room temperature 22-24°C. Rats were acclimatized to the environment for one week prior to experimental use. Rats were randomly divided into five groups (n = 7) and were subjected to the following treatments: Control animals received an equivalent volume of olive oil based on body weight. Rats of the protected groups were orally administered Chlorella extracts daily for two weeks prior DMBA treatment and until the end of treatment. Animals of these groups were orally administered with dried powdered of Chlorella suspended in warm water at selected doses (0.5 and 1.0 g kg^{-1} b.wt.). The carcinogenic dose of DMBA (25 mg/rat) was based on previous cancer studies of DMBA in rodents (Costa *et al*., 2002). At the end of the 120 day-experimental period, rats were anesthetized with diethyl ether and sacrificed by decapitation. Animals were starved overnight before sacrifice. This study was conducted following the guidelines of the Animal Ethics Committee, UAE University.

Sample preparation: Following diethyl ether anesthesia, rats were sacrificed and liver tissues were removed. For histopathological examination, the left lobe of the liver was immediately fixed in 10% buffered formalin. For biochemical studies, the right lobe of the liver was homogenized in ice-cold KCl (150 mmol L^{-1}). The ratio of tissue weight to homogenization buffer was 1:10. From the latter, suitable dilutions were prepared to determine the levels of protein carbonyl, malondialdehyde (MDA), total antioxidants and total proteins. To assess the activity of Superoxide Dismutase (SOD), glutathione-s-transferase (GST) and catalase (CAT), suitable dilutions were made in different buffers.

Biochemical parameters: MDA is the most abundant individual aldehyde resulting from Lipid Peroxidation (LP) breakdown in biological systems and is commonly used as an indirect index of LP (Sorg, 2004). Determination of MDA, as described by Uchiyama and Mihara (1978), is based on its reaction with thiobarbituric acid to form a pink complex with absorption maximum at 535 nm.

CAT activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm and expressed in units/mg of protein as described by Aebi (1983).

Reduced glutathione (GSH) was determined as described in Moron *et al*. (1979). The sample (1.0 mL) was

precipitated with 1.0 mL of TCA and centrifuged at 1200×g for 20 min. To 0.5 mL of supernatant 2.0 mL of 5, 5`-dithiobis-(2-nitrobenzoic acid) (DTNB) was added and the colour developed was read immediately at 412 nm using a spectrophotometer. Glutathione-S-Transferase (GST) was measured with 1-chloro 2, 4-dinitrobenzene as the substrate and the enzyme activity was expressed as μmoles of CDNB-GSH conjugates formed per minute per milligram protein (Hoilg *et al*., 1974).

In liver tissues, the SOD enzyme activity was determined according to the method described by Sun and Zigman (1978). This method is based on the ability of SOD to inhibit the auto-oxidation of epinephrine at alkaline pH to adrenochrome and other derivatives, which are easily monitored in the near-UV region of the absorption spectrum.

Protein carbonyl (P. carbonyl) contents were determined according to the method of Reznick and Packer (1994). This method is based on spectrophotometric detection of the end product of the reaction of 2, 4-dinitophenylhydrazine with P. carbonyl to form protein hydrazones at 370 nm. The results were expressed as nano moles of carbonyl group per milligram of protein with molar extinction coefficient of 22, 000 L M^{-1} cm^{-1}.

The total protein contents of liver tissues were determined according to the Lowry`s method as modified by Peterson (1977). Absorbance was recorded using a Shimadzu recording spectrophotometer (UV-160) for all measurements. For the histological examinations, small pieces of heart tissue were fixed in 10% neutral phosphate-buffered formalin. Hydrated tissue sections, 5 μm in thickness, were then stained with hematoxylin and eosin. The sections were examined under a Leica DMRB/E light microscope.

Statistical analysis: SPSS (version 10) (SPSS Inc., Chicago, IL, USA) was used to carry out a one-way Analysis of Variance (ANOVA) on our data. When significant differences were detected by ANOVA, analyses of differences between the means of the treated and control groups were performed using Dunnett's t-test.

RESULTS

Effects on lipid oxidation: Levels of MDA was significantly elevated ($p<0.001$) in liver tissues of DMBA-treated rats compared to control values. Concurrent treatment with chlorella and DMBA prevented the elevations of this oxidative stress marker. The higher dose showed more effectiveness in restoring normal MDA levels (Fig. 1). Chlorella alone, however, exhibited no significant effect on the MDA level when compared to control group.

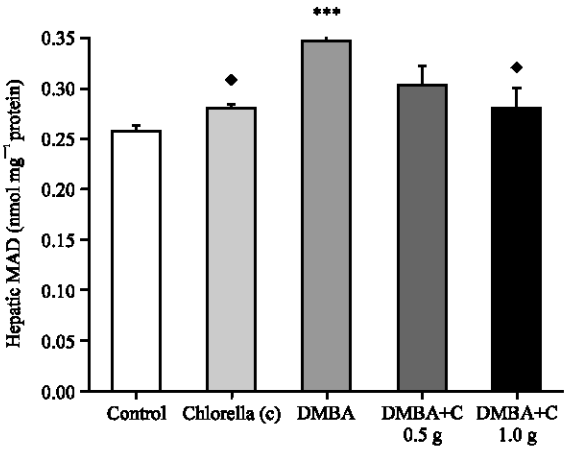

Fig. 1: Effects of Chlorella on MDA activity in livers of rats treated with vehicle (control), Chlorella alone (c), DMBA alone (DMBA), DMBA+ Low dose of Chlorella (DMBA+C 0.5 g) and DMBA+ high dose of chlorella (DMBA+C 1.0 g). Each column represent the mean±SEM (n = 7). ***$p<0.001$ vs. control; ◆$p<0.05$ vs. DMBA-treated group

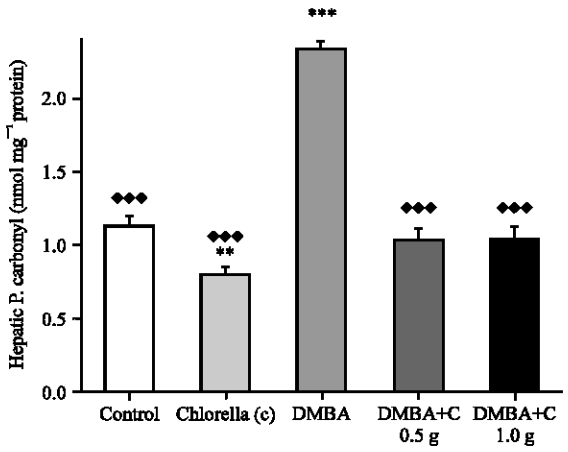

Fig. 2: Effects of Chlorella on P. carbonyl contents in livers of rats treated with vehicle (control), Chlorella alone (c), DMBA alone (DMBA), DMBA+ Low dose of Chlorella (DMBA+C 0.5 g) and DMBA+ high dose of chlorella (DMBA+C 1.0 g). Each column represent the mean±SEM (n = 7). **$p<0.01$, ***$p<0.001$ vs. control; ◆◆◆$p<0.001$ vs. DMBA-treated group

Effects on protein oxidation: Treatment with DMBA has significantly ($p<0.001$) elevated the level of P. carbonyl in liver tissues of DMBA-treated rats compared to control values. Concurrent treatment with chlorella and DMBA prevented the elevations of this oxidative stress marker (Fig. 2). Both concentrations (high and low doses) of chlorella had similar potent recovery effects on this

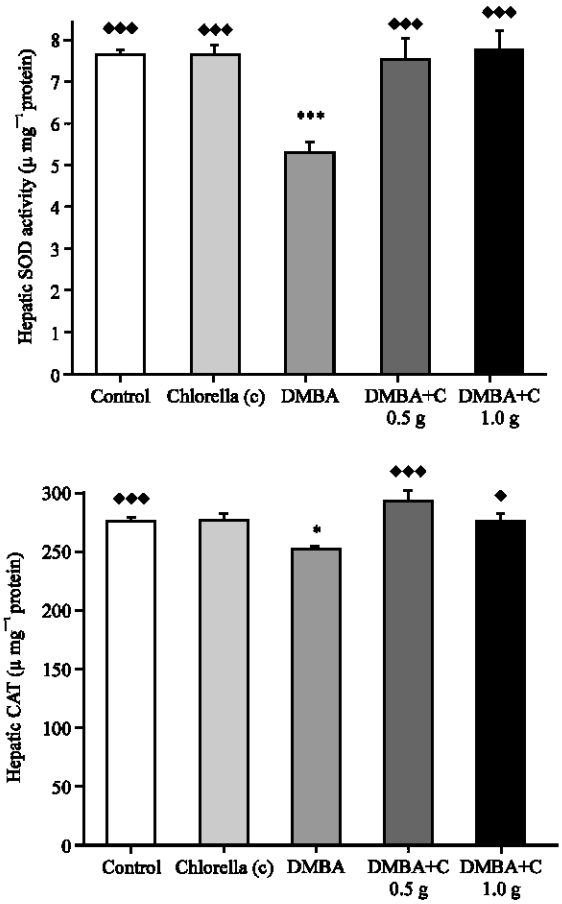

Fig. 3: Effects of Chlorella on SOD and CAT activities in livers of rats treated with vehicle (control), Chlorella alone (c), DMBA alone (DMBA), DMBA+ Low dose of Chlorella (DMBA+C 0.5 g) and DMBA+high dose of chlorella (DMBA+C 1.0 g). Each column represent the mean±SEM (n = 7). *p<0.05; ***p<0.001 vs. control; ♦p<0.05; ♦♦♦p<0.001 vs. DMBA-treated group

oxidative stress indicator. In comparison to the control group, Chlorella alone decreased the activity level of P. carbonyl.

Effects of chlorella on antioxidant defense system: Changes of antioxidants in liver tissues of rats treated with DMBA showed significant (p<0.001) depletion in SOD and CAT (Fig. 3) while increased GSH activities (p<0.001) (Fig. 4) and in TAC (FRAP) content (p<0.001) (Fig. 5). Interestingly, the treatment with chlorella has restored normal levels of SOD and TAC as well as significantly upregulated CAT activity. Treatment with chlorella alone exhibited no significant effect on the enzyme activity of SOD and CAT or on the content of TAC, compared with control group. While DMBA has

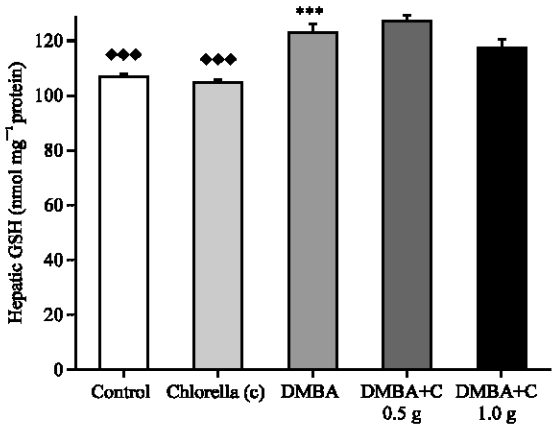

Fig. 4: Effects of Chlorella on GSH activity in livers of rats treated with vehicle (control), Chlorella alone (c), DMBA alone (DMBA), DMBA+ Low dose of Chlorella (DMBA+C 0.5 g) and DMBA+ high dose of chlorella (DMBA+C 1.0 g). Each column represent the mean±SEM (n = 7). ***p<0.001 vs. control; ♦♦♦p <0.001 vs. DMBA-treated group

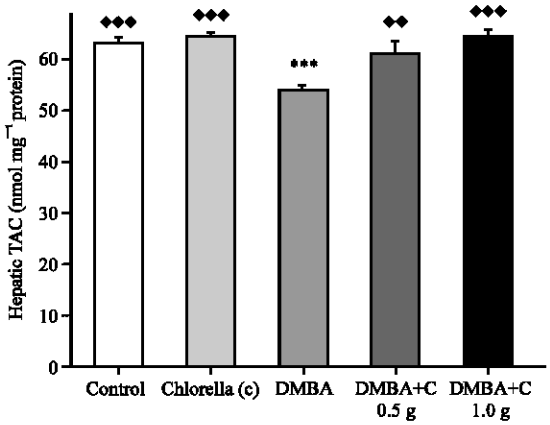

Fig. 5: Effects of Chlorella on TAC in livers using the FRAP assay. Liver tissues were collected from rats treated with vehicle (control), chlorella alone (c), DMBA alone (DMBA), DMBA+Low dose of chlorella (DMBA+C 0.5 g) and DMBA+ high dose of chlorella (DMBA+C 1.0 g). Each column represent the mean±SEM (n = 7). ***p<0.001 vs. control; ♦♦p <0.01; ♦♦♦p <0.001 vs. DMBA-treated group

upregulated GST enzyme activity, only the high dose of chlorella has significantly restored the control level of GST. Chlorella treatment alone did not induce any significant change in the activity of GST enzyme compared to the control group (Fig. 6). Taken together, the present study indicates that administration of algal

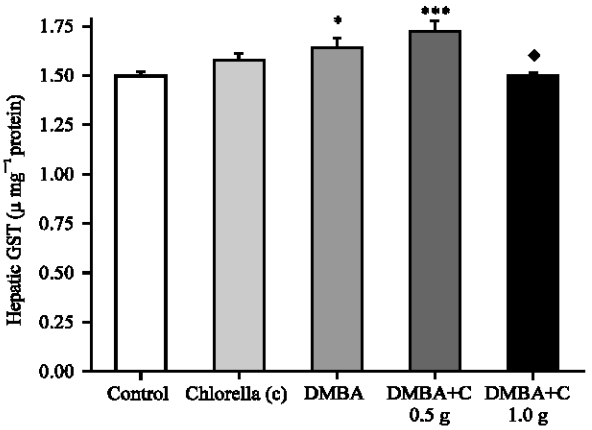

Fig. 6: Effects of Chlorella on GST activity in livers of rats treated with vehicle (control), Chlorella alone (c), DMBA alone (DMBA), DMBA+ Low dose of Chlorella (DMBA+C 0.5 g) and DMBA+ high dose of chlorella (DMBA+C 1.0 g). Each column represent the mean±SEM (n = 7). *p<0.05; ***p<0.001 vs. control; ◆p<0.05 vs. DMBA-treated group

extract resulted in substantial inhibition of oxidative stress in liver tissues of DMBA-treated animals.

DISCUSSION

Animal experimental systems provide powerful tools to study different types of human cancers particularly mammary carcinogenesis. Rat mammary glands show high susceptibility to develop neoplasms that closely mimic human breast cancer once induced with DMBA (Samy *et al*., 2006). Toxic manifestation of DMBA is associated with its oxidative metabolism leading to the formation of reactive metabolites (epoxides and quinines) capable of generating free radicals.

The production of ROS and the peroxidation of membrane lipids are well linked with the initiation of carcinogenesis that further affects the normal biochemical process (Rice-Evans and Burdon, 1993; Davis and Kuttan, 2001). Oxidative stress, induced due to the generation of free radicals and/or decreased antioxidant level in the target cells and tissues, has been suggested to play a crucial role in carcinogenesis (Huang *et al*., 1999). Increased incidence of oxidative stress and lipid peroxidation are implicated in carcinogenic processes (Khanzode *et al*., 2004). Free radicals are involved both in the initiation as well as promotion stage of tumourigenesis and their biochemical reactions in each stage of the metabolic process are associated with cancer development.

MDA is an aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Hence, it is of interest to assess MDA as a marker of oxidative stress. MDA is known to play a role in lipid peroxidation and in the modulation of antioxidants during the progression of breast cancer. There is naturally a balance between the amount of free radicals generated in the body and antioxidant defense system that scavenge them and thereby protect the body against pathogenesis (Kolanjiappan *et al*., 2002). It is evident from the results presented herein that the level of MDA was increased in cancer-bearing animals. On the contrary, reduced level of MDA was observed in chlorella-fed animals denoting chlorella as a potent free-radical-scavenger.

In addition to lipids, proteins are also affected by ROS (Hanimoglu *et al*., 2007). Protein oxidation is also common in ROS-induced carcinogenesis. One indicator of protein oxidation is P.carbony which is considered one of a battery of markers of oxidative stress. Oxidation of protein is the result of tertiary structural alteration that causes protein aggregation and elevated levels of P.carbonyl have been associated with different diseases (Shinall *et al*., 2005). In this study, levels of P.carbonyl are significantly reduced, almost to control levels, after chlorella administration.

Administration of chlorella has also influenced the activities of the antioxidant enzymes involved in scavenging the oxygen free radicals, which initiate lipid peroxidation. These antioxidant enzymes protect tissues from oxidative injury by lowering lipid peroxidation. Increased level of superoxide radicals in tumour cells decreases the antioxidant activity when compare to normal cells (Gupta *et al*., 2004). SOD levels were decreased in the present study in the cancer-bearing animals (DMBA only).

Data from this report also shows that decreased level of CAT observed in DMBA-treated animals may be due to the utilization of antioxidant enzymes in the removal of H_2O_2 by DMBA. Decreased level of CAT activity was measured in patients with breast cancer and benign breast disease conditions (Gonenc *et al*., 2006). The activity of SOD was significantly increased in the rats fed chlorella. The elevated levels of SOD were significantly high in the chlorella treated groups of rat liver than the normal control. SOD is the first line of defense against superoxide and hydrogen peroxide. It functions through dismutating the superoxide (O^{-}_{2}) to hydrogen peroxide (H_2O_2), CAT, then, catalyses the removal of H_2O_2 (Gago-Dominguez *et al*., 2007). SOD is also known to act as an anti-carcinogen inhibitor during initiation and promotion/transformation stages of carcinogenesis (Samy *et al*., 2006).

The non-enzymic antioxidant systems are the second line of defense against free radical damage. GSH is an important non-protein cellular thiol that is essential for cell proliferation (Bhuvaneswari *et al*., 2001). GSH is involved in scavenging the electrophilic moieties involved in the cancer initiation (Sunde and Hoekstra, 1980; Bhuvaneswari *et al*., 2001). An increased activity of GSH in DMBA-treated animals was reported in the present study. GSH serves as a marker for evaluation of oxidative stress and it acts as an antioxidant at both extra cellular and intracellular levels (Comporti *et al*., 1989; Bhuvaneswari *et al*., 2001). In this study, chlorella increased the GSH levels, which clearly suggest their antioxidant property. Chlorella extract has been shown to increase GSH levels by delaying its consumption and therefore strengthening its antioxidant activity (Noctor *et al*., 1998). Similarly, chlorella was able in this study to upregulate the GST levels. GSTs represent a multigene family of isozymes that catalyze the conjugation of GSH to a variety of electrophilic compounds and thereby exert a critical role in cellular protection against ROS (Hayes and Pulford, 1995; Pari and Suresh, 2008).

The phenolic compounds are reported as an index of antioxidant function (Maulik *et al*., 1997). In the present report, total phenolic compound was 2.7 mg TAE/g (data not shown) which is not as high as in other microalgae (Li *et al*., 2007). Chlorophyll and phenolic compounds of chlorella (Miranda *et al*., 2001; Negishi *et al*., 1997; Buratti *et al*., 2001; Park *et al*., 2005) and of other plants (Monagas *et al*., 2006) have been suggested as the active components with antioxidative activity.

The biochemical alterations observed in cancer-bearing animals in the present study may be due to the induction of lipid peroxidation and reduction of the indigenous antioxidant level following carcinogen administration. However, administration of chlorella significantly reversed the alteration to near normal level in cancer-bearing animals. From the present results it can be inferred that chlorella positively modulated the antioxidant activity by quenching and detoxifying the free radicals induced by DMBA. Considering the antioxidant property of chlorella, the bioactive compounds derived from this microalga can be supplemented with anticancer medicines. Further investigations on the chlorella's anticancer mechanisms are currently underway.

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