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Protective Effect of Green Algae Against 7,12-Dimethylbenzanthracene (DMBA)-Induced Breast Cancer in Rats

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Abstract: The present study investigated the chemopreventive effects of the water extract of chlorella on the development and growth of DMBA-induced mammary tumors. Female rats were daily administered vehicle control or chlorella either at 0.5 g or at 1.0 g kg^{-1} body weight starting at age of 35 days and continued to the end of the experiment. At age of 50 days breast tumor was induced by administering DMBA at 25 mg kg^{-1} body weight. Similar DMBA dose was administered to DMBA-alone group at age of 50 days. As a control for chlorella treatment one group (chlorella-alone) was administered chlorella at 1.0 g kg^{-1} body weight starting at age of 35 days and continued to the end of the experiment. Animals were then followed for 15 weeks. Effects of chlorella on the expression of proliferating cell nuclear antigen (PCNA), p53 and estrogen receptor (ER) were investigated in mammary tissues of control and experimental groups using immunohistochemistry. Present data demonstrated that chlorella treatment restored the normal expression levels of PCNA and ER. Chlorella also significantly increased cell death as assessed by the terminal deoxynucleotidyl transferase-mediated triphoshate nick-end labeling (TUNEL) analysis. In conclusion, the protective role of chlorella's water extract against carcinogen-induced breast cancer seems to be mediated through its anti-proliferative and pro-apoptotic properties.

Key words: DMBA, chlorella, breast cancer, PCNA, estrogen receptor, apoptosis

INTRODUCTION

Breast cancer is the most common cancer and cause of death in women and makes up one tenth of all new cancer diagnoses worldwide (Bray *et al.*, 2004; Ray *et al.*, 2007). Development of mammary tumors requires aberrant accretion of cells caused by excessive proliferation, insufficient apoptosis or dysregulation of cellular differentiation (Kumaraguruparan *et al.*, 2006; Hanahan and Weinberg, 2000). Recently, considerable attention has been focused on identifying naturally occurring chemopreventive substances capable of inhibiting, hindering, or reversing the multi-stage carcinogenesis. The main objective of breast cancer chemoprevention research is to advance knowledge in identifying and characterizing entities that might reduce the risk of human population developing cancer. Therefore, it is of interest to explore the possibility of using phytochemicals or other dietary chemicals as chemopreventive agents. Furthermore, the study of the biological effects of these phytochemicals at cellular level provides the molecular basis of their anti-disease function. It also helps to establish the platform for generating more potent chemopreventive and even chemotherapeutic agents (Gosslau and Yu-Chen, 2004).

Unicellular green alga, *Chlorella vulgaris*, has been shown to express various pharmacological effects in animals and humans (Hasegawa *et al.*, 2005). Hot water extracts of *Chlorella vulgaris* contain potent biological modifiers for immune responses against tumors, bacteria, viruses and native antigens such as casein (Hasegawa *et al.*, 1999). Chlorella has also been reported to possess anti-oxidative, anti-inflammatory and anti-tumor properties *in vitro* (Guzman *et al.*, 2001). In addition, chemopreventive

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effects of chlorella were reported against cancer such as hepatocarcinogenesis in rats (Takekoshi *et al.*, 2005). Thus, the benefits of the chlorella organism are shown to be very wide-ranging and it has the potential to be used in nutritional fortification (Hasegawa *et al.*, 2005).

Estrogens are known to modulate proliferative activity not only in the classic estrogen responsive tissues (Yager and Liehr, 1996) but also in organs and/or cells of other apparatuses (Fisher *et al.*, 1984; Francavilla *et al.*, 1989; Messa *et al.*, 2000; Barone *et al.*, 2006). They exert their biological activity by binding with two type of receptors: estrogen receptor-alpha (ER-α), the prevalent form in the breast, bone, cardiovascular tissue, urogenital tract and central nervous system and estrogen receptor-beta (ER-β), the prevalent form in the gut (Di Leo *et al.*, 2008; Gustafsson, 1999; Dechering *et al.*, 2000). ER mediates the action of estrogens and plays a major role in the initiation and progression of breast cancer (Pearce and Jordan, 2004).

p53, the protein encoded by the tumour suppressor gene p53, functions as a master regulator of both cell division and apoptosis (Mihara *et al.*, 2003).

The rat mammary gland is a widely used model for studying the pathogenesis, therapy and chemoprevention of breast cancer (Amin and Buratovich, 2007; Nandi *et al.*, 1995; Russo *et al.*, 1990; Welsch, 1985). Rat mammary gland carcinomas resemble human breast cancer in histopathogenesis, pathological features and hormone-dependence. The present study investigated the effect of chlorella on proliferation, apoptosis and expression of estrogen receptors in breast epithelium in a DMBA-induced mammary tumour in rats model system (Amin *et al.*, 2005). Immunohistochemistry was used to detect expressions of cell proliferating marker, PCNA, ER and p53. Apoptosis was also determined by terminal deoxynucleotide transferase dUTP nick labeling (TUNEL) staining.

MATERIALS AND METHODS

Chemicals

DMBA and olive oil were purchased from (Sigma-Aldrich, St. Louis MO, USA). Chlorella was purchased as tablets from Wakunaga of America CO., LTD. Mission Viejo, CA, USA.

Animals and Experimental Protocol

Five-week-old female Wistar rats (100-150 g body weight) were divided into five groups of seven rats each. Rats were housed in a pathogen-free environment and under constant environmental conditions (photoperiod, temperature, air humidity, food) at the animal house of the Faculty of Medicine in UAE University. Female rats were daily administered vehicle control or chlorella either at 0.5 g or at 1.0 g kg^{-1} body weight starting at age of 35 days and continued to the end of the experiment. Breast tumor was then induced by administering DMBA- dissolved in 1 mL vehicle (0.5 mL of olive oil and 0.5 mL of saline) (Amin *et al.*, 2005; Costa *et al.*, 2002) at 25 mg kg^{-1} body weight at age of 50 days. As a control for chlorella treatment one group (chlorella-alone) was administered chlorella at 1.0 g kg^{-1} body weight starting at age of 35 days and continued to the end of the experiment. Similar DMBA dose was administered to DMBA-alone group at age of 50 days. Animals were then followed for 15 weeks post DMBA treatment. The chlorella was obtained in the form of supplement tablets of 100% pure broken cell wall chlorella powder. Before oral administration, chlorella tablets were ground and suspended in warm water. The rats were palpated for tumor detection twice weekly throughout the hundred and twenty-day experimental period. At the end of the experimental period, all rats were alive which were anesthetized with diethyl ether and sacrificed by decapitation. The mammary tissues were dissected out and were then fixed in 10% buffered formalin. Doses of chlorella used in this study were confirmed to be most suitable and effective in tested rats according to preliminary experiments. This study was conducted after the approval of the Animal Research Ethics Committee of UAE University, UAE.

Histology

Histological examination of rat mammary tissues from rats of control, DMBA-alone and chlorella +DMBA treated groups were performed as described by Samy *et al.* (2006). Briefly, small pieces of mammary tissue, fixed in 10% phosphate-buffered formalin, were embedded in paraffin. The blocks were cut to obtain 5 μm thick sections and stained with hematoxylin-eosin sections were examined using a Leica DMRB/E light microscope (Heerbrugg, Switzerland).

TUNEL Assay

Apoptosis was assessed in deparaffinized sections using TUNEL technique as per manufacturer's protocol (Chemicon International, Temecula, CA, USA). This method detects the DNA fragmentation associated with apoptosis by labeling 3-OH DNA termini with digoxigenin nucleotides, a process facilitated by terminal deoxynucleotidyl transferase. The labeled fragments are then allowed to bind to anti-digoxigenin antibody conjugated with peroxidase. Color was developed by adding sufficient peroxidase substrate to specimens.

Immunohistochemistry

Sections of 5-6 μm were cut and mounted for immunostaining. Sections were deparaffinized in xylene, rehydrated and rinsed in 0.1 M phosphate buffered saline (PBS, pH 7.2). Endogenous peroxidase was blocked by incubating tissue sections in 3% hydrogen peroxide for 20 min at ambient temperature to diminish non-specific staining. Sections were rinsed in PBS and further nonspecific binding was blocked by additional incubation of tissue sections with normal goat serum for 20 min and diluted in PBS for ten min at room temperature. With the removal of excess normal sera, the sections were incubated at 4°C overnight with primary antibodies. All primary antibodies were diluted in 0.1 BSA and 0.01% sodium azide dissolved in PBS. Subsequent to incubation, sections were washed in PBS (3×5 min), incubated for 1 h at 4°C with avidin-biotinylated peroxidase complex (1/400; Dako, Glostrup, Denmark), rinsed in PBS as above. Following PBS washes, the sections were rinsed in acetate buffer for ten min and the peroxidase reaction developed in a 0.05% solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB: (Sigma-Aldrich, St. Louis MO, USA)), 0.03% hydrogen peroxide and imidazole in Tris-HCl buffer (pH 7.6). Sections were counterstained with hematoxylin. The number of immunoreactively stained cells in each section was calculated as explained below.

Semiquantitative Analysis of Immunoreactive Cells

A semiquantitative procedure used to count the number of immunoreactive cells is described by Weaver *et al.* (2007) and Weaver and Liu (2008). Briefly, the immunocytochemically stained mammary tissues of the four different treatments, including control chlorella-alone, DMBA-alone and chlorella + DMBA treated groups, were assessed for the degree of staining for each primary antibody. A total of 100 cells were scored per site. Three sites per section were randomly selected from each of the sections representative of each parameter. This yielded 21 sampled sites per treatment (n = 7). The total number of immunoreactively stained cells recorded for each treatment is presented as a percentage of the total number of cells counted.

Statistical Analysis

SPSS (version 10) statistical program (SPSS Inc., Chicago, IL, USA) was used for statistical analysis; all data are expressed as group Mean±SE. ANOVA was used to detect differences between various groups. If a significant difference was found between the means of the treated and control groups, Dunnett's t test was applied.

RESULTS

All tumors observed at autopsy were encapsulated and of solid consistency. There was no evidence of acute toxicity after the administration of DMBA or chlorella extracts. No mammary tumor development was seen in the control group during the experimental period. First tumors were recorded 100 days post DMBA administration in all groups and then the incidences increased time-dependently throughout the experiment. Histologically, mammary tumors in all DMBA-treated groups represented benign lesions that ranged from florid epithelial hyperplasia to fibroadenomas (Fig. 1). The extent of changes for all tested markers was listed in (Table 1). Breast tissues of the group treated with chlorella-alone appeared normal. Chlorella preparations associated with the prevention and treatment of cancer has been shown to be mediated via the immune system rather than its direct toxicity against tumors (Kralovec *et al*., 2005).
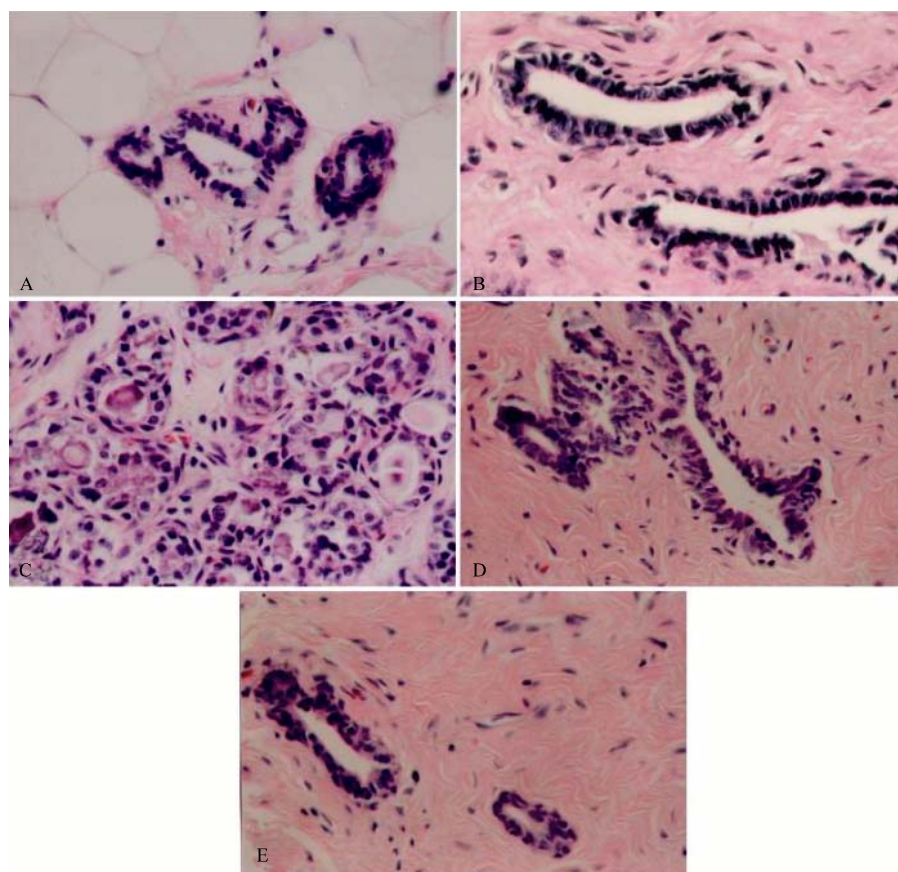

Fig. 1: Photomicrographs of mammary tissues of rats treated with vehicle (A, control), chlorella-alone (B, chlorella control), DMBA-alone (C, induced), DMBA + chlorella at 0.5 g kg^{-1} b. wt. (D), and DMBA + chlorella at 1.0 g kg^{-1} b. wt. (E). (A-B) Normal ductal structures of the mammary gland. (C) Florid epithelial hyperplasia of DMBA-induced breast tumor with secondary lumina and increased numbers of active cells both at the basal lamina and away from it. (D-E) Mammary ducts with mild hyperplasia after DMBA-treated rats were fed with chlorella extract. Hematoxylin and Eosin staining, 400X

Table 1: The expression of PCNA, ER, TUNEL and p53 in breast tissues of control, chlorella-alone, DMBA-induced and chlorella-protected groups. The percent of PCNA, ER and p53 positive cells in each section was calculated as described in the materials and methods

Parameter	Control (%)	Chlorella-alone (%)	DMBA-alone (%)	DMBA +Chlorella (0.5 g) (%)	DMBA +Chlorella (1.0 g) (%)
PCNA	2.2±0.05†††	1.1±0.3	14±1***	3.0±0.3†††	3.0±0.2†††
ER	16.0±1.6†††	14.0±1.3	29±1.1***	12.0±1.7†††	14.0±1.8†††
TUNEL	10.0±0.1†††	7.0±0.5*	22±1.8*	31.0±4.0***	46.5±3.5***,†††
p53	4.9±0.3†††	5.1±0.2	12±1.1***	10.2±0.5†	11.0±0.4†

(Means±SD; n = 7), ††† p<0.001, † p<0.05 compared with DMBA-alone group, ***p<0.001, *p<0.05, compared with control group
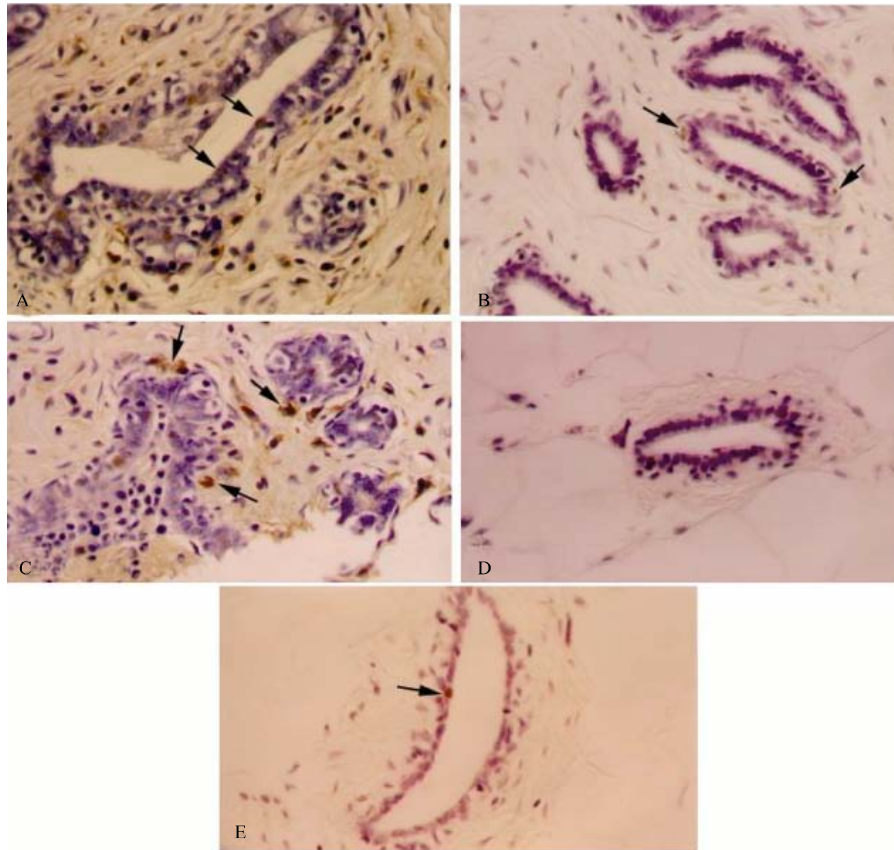

Fig. 2: Immunohistochemical staining for PCNA in mammary tissues of rats treated with vehicle (A, control), chlorella-alone (B, chlorella control), DMBA-alone (C, induced), DMBA + chlorella at 0.5 g kg^{-1} b. wt. (D) and DMBA + chlorella at 1.0 g kg^{-1} b. wt. (E). Chlorella administration decreases the number of proliferating cells in mammary ducts (C-D). Counter stained with hematoxylin. Mouse monoclonal antibody (Dako, Glostrup, Denmark) was used as per manufacturer's protocol. All images, 400X

Antiproliferative Effect of Chlorella

Cell proliferation is regulated by multiple mechanisms. Proliferative cell nuclear antigen (PCNA), a nuclear protein present in proliferating cells, is essential for cell replication and acts as a marker for cellular proliferation (Malkas *et al.*, 2006). Levels of PCNA both in control and chlorella-alone groups did not show any significant variations. DMBA significantly induced (536%) the PCNA expression

compared to control PCNA levels. Treatment with chlorella extract significantly decreased (78.6%) the expression of PCNA in the DMBA + chlorella rats (p<0.001) compared to tumor tissue from animals treated with DMBA-alone (Table 1, Fig. 2). Furthermore, the lactiferous ducts in untreated-DMBA tissue were filled with proliferated cells; however, in the chlorella treated-groups these ducts were mostly clear of the proliferating cells.

Chlorella Modulates Expression of Estrogen Receptors

ER is another marker that is used extensively in breast cancer diagnosis and prognosis. Distribution of ERα in mammary tissues of control, chlorella-alone and DMBA-alone groups is shown in Fig. 3A-C, respectively. ER-immunoreactivity was considered positive only when a strong dark brown stain was detected within the nuclei of epithelial cells of mammary ducts. In control and chlorella-alone samples, some epithelial cells showed consistent nuclear immunoreactivity (16 and 14%, respectively) but occasional and weaker intra-cytoplasmic positivity was detectable as well
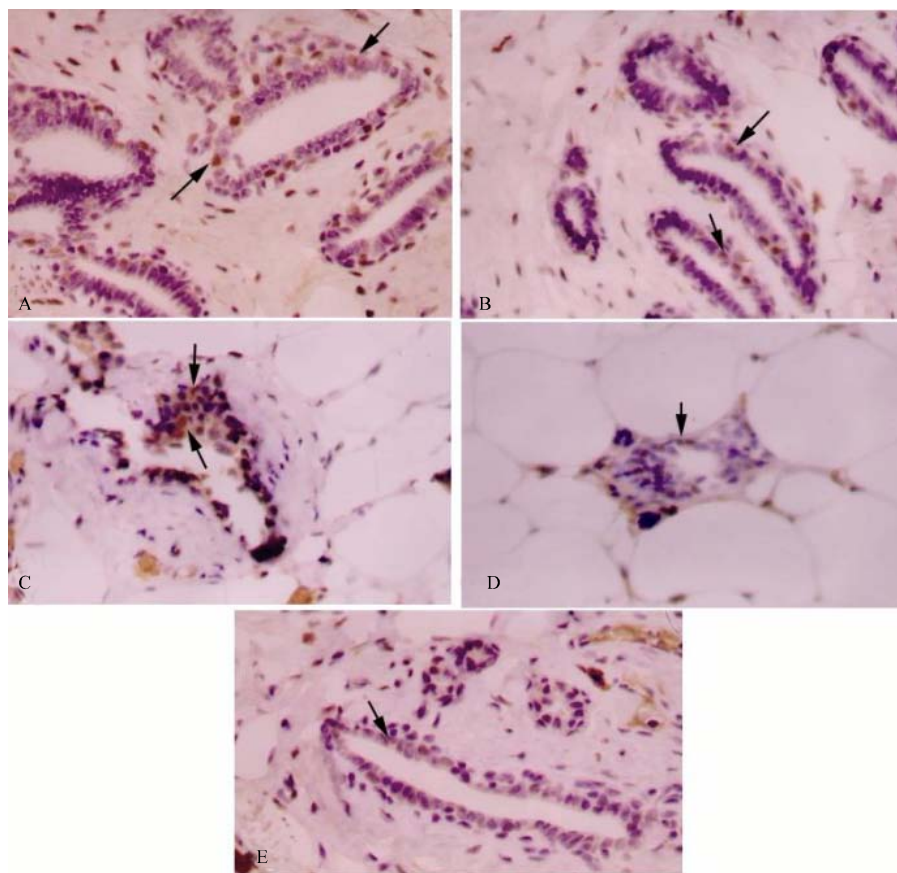

Fig. 3: Immunohistochemical staining of ER in mammary tissues of rats treated with vehicle (A, control), chlorella-alone (B, chlorella control), DMBA (C, induced), DMBA + chlorella at 0.5 g kg^{-1} b. wt. (D) and DMBA + chlorella at 1.0 g kg^{-1} b.wt. (E). Chlorella treatments decrease the number of ER-positive cells in tumor masses (D-E). Counter stained with hematoxylin. Rabbit polyclonal antibody (Dako, Glostrup, Denmark) was used as per manufacturer's protocol. All images, 400X

(Table 1, Fig. 3). In DMBA-alone group, a significant increase (81%) of ERα immunoreactivity was observed. Pretreatments with chlorella abolished (51.7-58.6%) the DMBA-induced upregulation of ERα in mammary tissues (Fig. 3D-E).

Proapoptotic Effects of Chlorella

As there was a significant reduction in the tumor size by the chlorella treatment, effects of different doses of chlorella on the tumor growth were examined using TUNEL assay. TUNEL assay was used to identify apoptotic cells of mammary ducts. Brown staining, indicating TUNEL-positive nuclei was visible in breast tissues of control, chlorella-alone and DMBA-alone animals (Fig. 4). It was interesting to notice a decrease in TUNEL-positive cells in animals treated with chlorella-alone (7% versus 10% in control). However, TUNEL-positive cells were significantly (p<0.001) increased in the DMBA-alone group (120%) compared to the control group (Fig. 4). Pre-administration of chlorella prior to and concomitant with DMBA treatment has retained the high number of TUNEL-positive cells in the DMBA-alone group (Fig. 4). It is worth mentioning here however that compared to DMBA-alone group, lower dose of chlorella caused much less increase of cell death (41%) compared to the higher dose (111%).
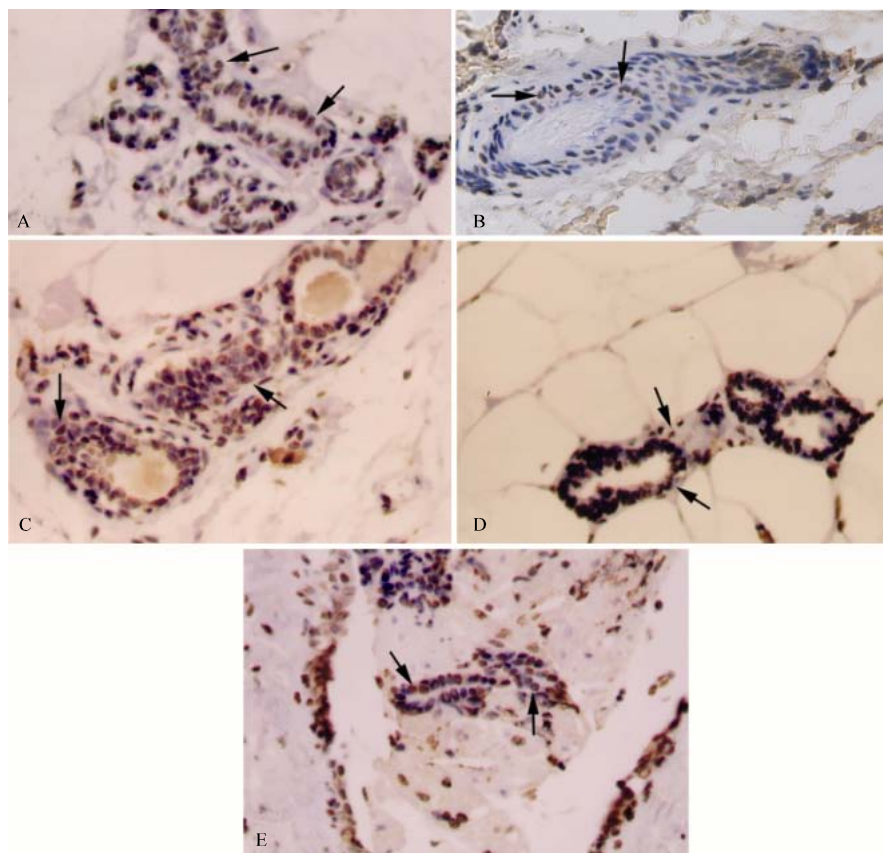

Fig. 4: Immunohistochemcal staining for TUNEL in mammary tissues of rats treated with vehicle (A, control), chlorella-alone (B, chlorella control), DMBA-alone (C, induced), DMBA + chlorella at 0.5 g kg⁻¹ b. wt. (D) and DMBA + chlorella at 1.0 g kg⁻¹ b. wt. (E). A greater number of TUNEL positive cells were observed in tumors from chlorella treated animals (D-E). Brown staining indicates TUNEL-positive cells. Counter stained with hematoxylin. All images, 400X
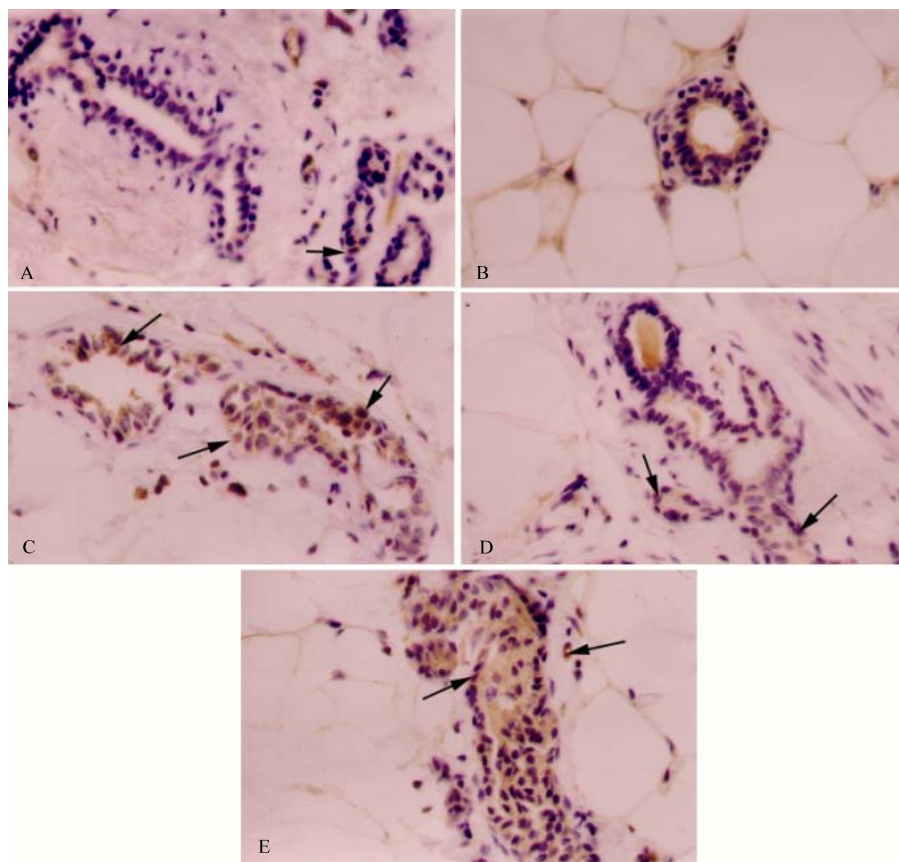
Fig. 5: Immunohistochemcal staining for p53 in mammary tissues of rats treated with vehicle (A, control), chlorella-alone (B, chlorella control), DMBA-alone (C, induced), DMBA + chlorella at 0.5 g kg^{-1} b. wt. (D) and DMBA + chlorella at 1.0 g kg^{-1} b. wt. (E) Brown staining indicating positive immuno-stained cells increased in the DMBA-alone group (B). The concomitant treatment with chlorella before and after DMBA treatment decreased the number of p53-positive cells compared to the DMBA-alone group (D-E). Counter stained with hematoxylin. Mouse monoclonal antibody (Dako, Glostrup, Denmark) was used as per manufacturer's protocol. All images, 400X

Effect of Chlorella on p53 Expression

p53 protein expression was detected in mammary tissues of control, chlorella-alone and DMBA-alone animals (Fig. 5A-C). Similar level of p53 expressing cells were reported in control and chlorella-alone treated animals. Brown staining, indicating positive immunostained cells, was significantly more (144%; $p<0.001$) in the DMBA-alone group compared to the control group (Fig. 5). Treatment with chlorella before and concomitant with DMBA uptake had a slight effect (8.4-15%) on the number of p53-positive cells compared to the DMBA-alone group (Fig. 5).

DISCUSSION

Despite significant advances in the treatment of breast cancer, this disease remains the leading cause of death and the most commonly diagnosed cancers among women (Jemal *et al*., 2005,

2008). Epidemiological data from more than 250 case control and cohort studies shows an inverse relationship between the risk of certain types of cancer and consumption of dietary phytochemicals and fibers (Borek, 2004). Multiple mechanisms have been identified for the anti-neoplastic effects of plants, including antioxidant, anti-inflammatory and anti-proliferative activities, inhibition of bio-activating enzymes and induction of detoxifying enzymes (Le Marchand, 2002). Chlorella's antioxidative, anti-inflammatory and anti-tumor properties were reported by Guzman *et al.* (2001). In addition, chemopreventive effects of chlorella were reported against hepatocarcinogenesis in rats (Takekoshi *et al.*, 2005).

Despite a large number of studies analyzing chlorella's chemoprotective effects *in vitro*, a paucity of data is available on its *in vivo* mode of action. The present research demonstrates, *in vivo*, the anti-breast cancer effect of chlorella utilizing DMBA as a cancer inducer. DMBA, a polycyclic aromatic hydrocarbon, is genotoxic and is capable of forming carcinogen-DNA adducts in human or animal tissues. The development and progression of mammary carcinoma is influenced by estrogens that mediate their effects via. ER. ER pathway is involved in proliferation, angiogenesis and metastasis of cancer cells (Wesierska-Gadek *et al.*, 2008). The estrogen–ER complex induces both genetic and epigenetic changes that influence the expression of a number of genes involved in the regulation of cell proliferation and differentiation (Murphy and Watson, 2002; Shaw *et al.*, 2002). In this study, chlorella has significantly restored the control levels of ER. A number of studies including the present one have demonstrated the overexpression of ER in mammary tumours. ER-positive breast cancers have also been reported to be associated with evasion of apoptosis (Alfred *et al.*, 2001; Baccouche *et al.*, 2003; DeLas Mullas *et al.*, 2005).

Data presented in this study suggest that chlorella's water extract is effective in suppressing the proliferation of mammary tumors as shown by growth inhibition and apoptosis induction. Many of the molecular alterations that accompany carcinogenesis lead to uncontrolled proliferation and growth and the ability of the transformed cells to prevaricate apoptosis. Elevated proliferative activity has been shown not only to be responsible for hyperplastic phenomena but also to facilitate tumour development by enhancing the probability of genomic mutations (Barone *et al.*, 2003). Anticancer effects of some potential phytochemicals are known to be mediated through differential regulation of the cell cycle and subsequent events leading to cell death (Pozo-Guisado *et al.*, 2002; Jackson and Singletary, 2004). Chlorella has a significant antiproliferative effect (Table 1, Fig. 2). Immunohistochemical staining showed a decrease in the PCNA and an increase in apoptosis in tumors from animal treated with chlorella. PCNA is synthesized in the G1 to S phase of the cell cycle. Expression of PCNA is known to be increased in rapidly proliferating tumours (Maga and Hubscher, 2003; Paunesku *et al.*, 2001). A positive correlation between the number of PCNA-positive cells and histopathologic malignancy has been reported in different mammary tumours (Franzen *et al.*, 1997; Funakoshi *et al.*, 2000). Neoplastic cells undergo changes that diminish their susceptibility to apoptosis (Hersey and Zhang, 2003; Johnstone *et al.*, 2002; Kaufmann and Vaux, 2003). Results presented herein shows that chlorella effectively suppresses the proliferation as well as induces apoptosis in DMBA-induced mammary tumors. The antiproliferative effect of chlorella extract has been shown to be associated with apoptosis-inducing activity of chlorella in different human cancer cell lines (Cha *et al.*, 2008). Moreover, higher dose of chlorella has shown more cytotoxic effects in DMBA-induced breast cancer. Other studies have also shown that water extracts of chlorella induced antiprolifrative and apoptotic effects on activated hepatic stellate cells (Wu *et al.*, 2005).

p53 is critical for the prevention of the onset and progression of breast cancer, overexpression of p53 can stop the cell cycle and causes DNA repair and apoptosis (Benz, 2008). Carcinogens-induced DNA damage, if not repaired, can lead to mutagenesis and tumor initiation (Sun *et al.*, 2007; Cox, 1998). Following DNA damage, p53 can be activated leading to the repair of DNA damage or apoptosis. Chlorella is widely known as a health supplements (Mallick and Rai, 1999;

Hasegawa *et al.*, 2000) and is reported to have anti-tumor properties *in vitro* (Park *et al.*, 2005). A growing body of evidence indicates a close correlation between the up-regulation of p53 and apoptosis in cancer cells treated with chemotherapeutic drugs (Gosslau *et al.*, 2005). It is therefore quite intriguing to find that p53 is not required for the antiproliferative and pro-apoptotic action of chlorella in the present study (Table 1, Fig. 5). 2-chloroadenosine (a resistant analogue of adenosine) has been shown to induce apoptosis *in vitro* through the activation of the intrinsic pathway of apoptosis in a p53-independent way (Bastin-Coyette *et al.*, 2008). Resveratrol has also been shown to induce apoptosis independent of p53 (Gosslau *et al.*, 2008). It is therefore possible that p53 may not be the primary target for the proapoptotic action of chlorella extract in this study. Finally, the fact that the proapoptotic action of chlorella is p53-independent, emphasizes that chlorella is potentially useful to be developed into a chemotherapeutic agent against p53-resistant cancer cells.

In conclusion, the present study provides evidence for antiproliferative and pro-apoptotic capacities of chlorella against DMBA-induced-breast cancer in rats. Despite the growing number of studies that suggest a chemopreventive potential of chlorella, human intervention trials will remain to be the ultimate determinant of the aforementioned beneficial nature of this green alga.

ACKNOWLEDGMENTS

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