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RESEARCH ARTICLE

CHEMOPROTECTIVE EFFECTS OF *CHLORELLA VULGARIS* AND *SPIRULINA PLATENSIS* ON COLON CANCER INDUCED BY 1, 2 DIMETHYLHYDRAZINE

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ABSTRACT

Chlorella vulgaris (CV) and Spirulina platenesis (SP) are the single-celled microalgae, which contain high concentrations of whole food nutrients, and they have been shown to inhibit cancer-colony formation. The present study was conducted to investigate the chemopreventive effects of CV and SP against 1, 2-Dimethylhydrazine -induced colon carcinogenesis in rats. rats were divided into five groups. Group1 served as -ve control, Group2 received DMH, and served as +ve control, Group3 received CV, Group4 received SP and Group5 received CV and SP for 5weeks. Group3, 4, 5 were given DMH. Lipid peroxidation (LPO) and oxidative stress markers were examined as well. Immunohistochemistry (Proliferating cell nuclear antigen (PCNA), (Caspase-3), COX2 was performed along with histological analysis. In our study, LPO levels were found to be increased in group DMH and decrease in CV and SP compared to the control group. Antioxidant activity reduced in group 2 as compared to the control and elevated in CV and SP treated groups. Numbers of aberrant cryptic foci in the group CV and SP were decreased as compared to the group 2. CV and SP increased in pro-apoptotic protein caspase-3, a decrease in proliferating protein PCNA and in inflammatory marker COX2. Our study showed that CV and SP have chemopreventive effects by anti-inflammatory, antiproliferating, apoptosis and antioxidant activity in colon cancer induced by DMH in rats.

Key words: Cyanobacteria, Bioactive compounds, Antimicrobial activity, stigonema sp. and spirulina sp.

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INTRODUCTION

In economically developed countries, cancer is the leading cause of death and the second core cause of death in developing countries. Colorectal Cancer (CRC) is the third most popularized cancer worldwide after lung and breast cancers (Gado *et al*., 2014). DMH and its metabolite azoxymethane (AOM) are the agents widely adopted in experimental models of colorectal carcinogenesis in rodents. And they are highly specific indirect colorectal carcinogens that prompt the initiation and promotion steps of colorectal carcinogenesis yielding colorectal tumor lesions in a dose-dependent manner in rats, mice and hamsters. In rats, DMH can produce colorectal tumor lesions in almost 100% of treated animals, despite various strains of rats differ in susceptibility to these carcinogens (Perše and Cerar, 2005). Approximately, 60% of drugs adopted are natural products (Gordaliza, 2007). *Chlorella vulgaris* (CV) and *Spirulina platenesis* (SP) are single-celled \ or improve a specific body function (Ötleş and Pire, 2001).

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Spirulina is a unicellular cyanbacterium, with high nutritional value and wide ranging medicinal applications. And it is considered a rich source of protein, vitamins, minerals, essential amino acids, fatty acids (gamma – linolenic acid – GLA), and antioxidant pigments, such as carotenoids (Abdel-Daim *et al*., 2013). It also contains very potent naturally occurring antioxidant and free radical scavenging agents. *Chlorella* is fresh water, single-celled algae that grows in fresh water and contains the highest amount of chlorophyll of any known plant. And it is also a nutrient-dense super food that contains 60% protein, 18 amino acids (including the essential ones), and various vitamins and minerals (Abdel-Daim *et al*., 2015).

MATERIALS AND METHODS

Chemical: 1, 2-dimethylhydrazine dihydrochloride (DMH), was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).Pure spirulina platensis and chlorella vulgaris were purchased from National Research Center, Cairo, Egypt All kits of biochemical parameter were purchased from Biodiagnostic CO. (Cairo, Egypt).

Animals and experimental design: Male rats weighing (150g -200g) were supplied by Laboratory Animals Unite (Ismailia, Egypt). For induction of colonic cancer, rat will be injected subcutaneously with DMH (40 mg/kg body weight /twice weekly,) for 5 weeks. Treatment with SP and CV were launched before, during the course of DMH, at dose of 500 mg/kg daily for SP (Abdel-Daim *et al*., 2013; Hidalgo-Lucas *et al*., 2014) and 50 mg/Kg body weight daily for CV. Rats were divided into five groups**.** Group1 served as -ve control animals received subcutaneous injection of normal saline, Group2 received DMH, and served as +ve control, Group3 received CV, Group4 received SP and Group5 received CV and SP for 5weeks. Group3, 4, 5 were given DMH**.**

Determination Tissue lipid peroxidation and antioxidant enzyme: lipid peroxidation content was evaluated by measurement of MDA and oxidative stress markers were assessed; superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) were estimated in tissue homogenate based on the protocol of the purchased kit from

Immunohistochemistry analysis: The sections were hydrated in 1X phosphate-buffered saline (PBS) for 5 minute. Antigen retrieval was performed by incubating the section in 10mM. Biodiagnostic CO (Cairo, Egypt). Sodium citrate buffer (PH 6.0) at 80°C for 10 minute. The sections were cooled to room temperature for 20 min. All ensuing steps were carried out in room temperature. Following a five min wash with 1XPBS, the endogenous peroxidase was blocked by 3% hydrogen peroxide in PBS for 10 min. The section were washed as before and blocked for 1h in PBS containing 1.5% serum. The slide is then incubated overnight with primary antibodies PCNA, Caspase3Ki67and COX2 all from Thermo Kits at 4°C in humidified chamber. After washing with PBS, the section was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 1:100 dilulion for 30 min at 37°C. The immune reactions were visualized by immersing the slide in 3, 3'diaminobenzidine tetra hydrochloride reagent (Broad spectrum LAB-SA Detection system from Invitrogen cat.no.95-9943-B).The sections counter stained with hematoxylin. All sections were dehydrated, mounted with cover slip and viewed under light microscope. Cells were counted using image J softwere.

Histopathological examination and determination of aberrant cryptic foci: Colon sections were taken immediately from the colon, fixed in 10% buffered formalin, dehydrated in ethanol (50–100%), cleared in xylene, and embedded in paraffin. Sections (4–5 mm thick) were prepared and then stained with hematoxilin and eosin (H&E).

Statistical analysis: The present results were analyzed using Statistical Package for Social Science (SPSS) version (SPSS Inc., Chicago) for windows. Result were expressed as mean ± SE (n=8). One way ANOVA followed by Duncan test were used for analysis. P value less than 0.05 was considered significant.

RESULTS

Lipid peroxidation and antioxidant status in colon tissue of male albino rats: After 6 weeks of experiment, DMH group elucidated lipid peroxidation of colon tissue evidenced significant (P≤0.05%) high level of MAD compared to control rats. While treated groups; *Spirulina platenesis, Chlorella* showed significant decrease in MDA level compared DMH group**.** The tissue SOD, CAT activity and GSH level significant (P≤0.05%) decrease in DMH group compared with control group. Whereas *Spirulina platenesis, Chlorella Vulgasis* and its combination group elevated SOD, CAT activity and GSH level compared with DMH group, There were significant alteration between each other (Table 1 and Figure 1-4).

Table 1.Antioxidant enzyme activities and lipid peroxidation level in the colon tissue of control and treated groups

Parameter	Experimental groups				
	Control	DMH	DMH+CV	DMH+SP	DMH+CV+SP
MDA(nmol/g tissue)	12.94 ±0.85^{d}	52.63±2.28^{a}	39.96±1.40^{b}	28.73±1.66^{c}	15.93±0.69^{d}
GSH (mg/g tissue)	23.21 ±0.73^{a}	12.67 ±0.52^{d}	15.22±0.87^{c}	18.82±1.06^{b}	21.29±1.09^{ab}
SOD (U/g tissue)	9.40 ±0.38^{a}	3.35 ±0.14^{d}	4.70±0.30^{c}	6.68±0.29^{b}	8.58±0.34^{a}
CAT (U/g tissue)	4.29 ±0.13^{a}	1.66 ±0.09^{d}	2.59±0.21^{c}	3.25±0.19^{b}	4.17±0.20^{a}

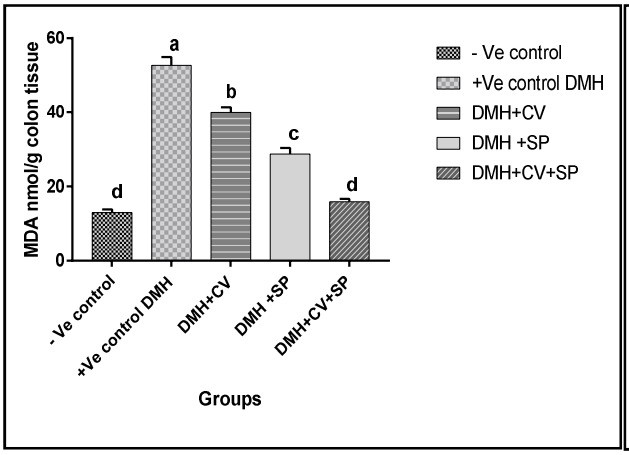

Figure 1. Effect of SP, CV and its combination SP+CV on colon tissue MDA in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

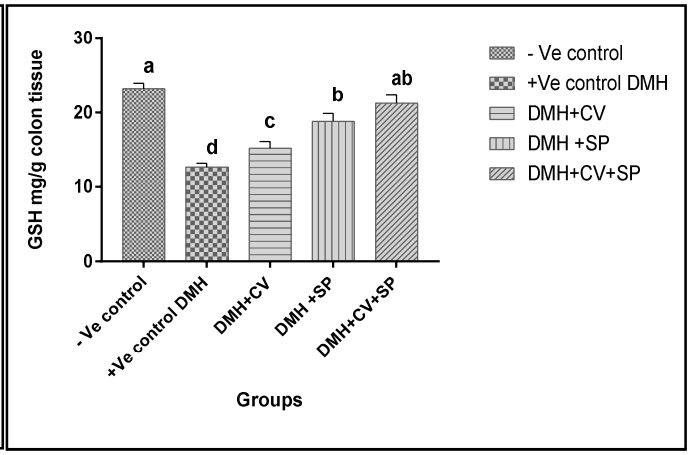

Figure 2. Effect of SP, CV and its combination SP+CV on colon tissue GSH in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

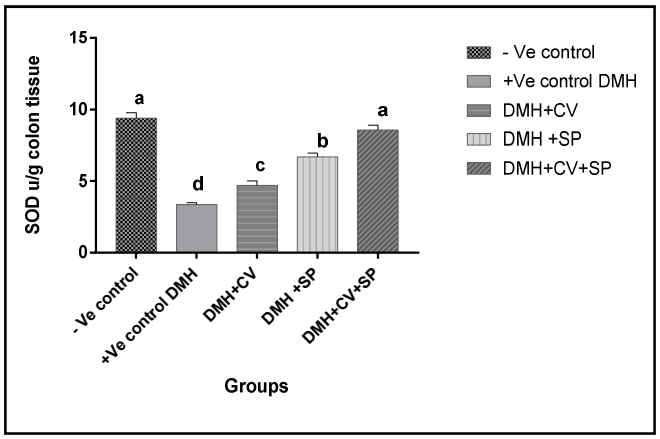

Figure 3. Effect of SP, CV and its combination SP+CV on colon tissue SOD in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

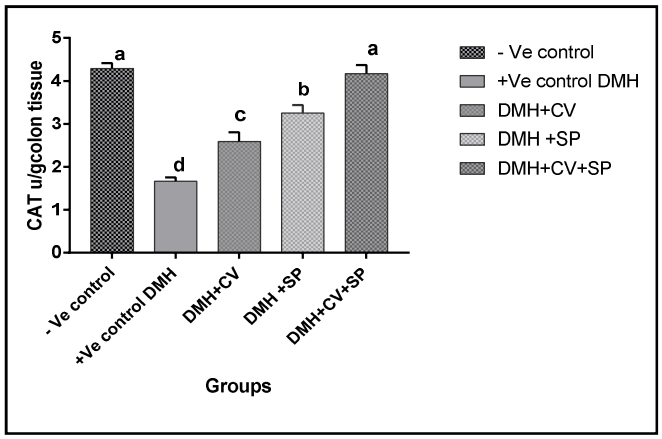

Figure 4. Effect of SP, CV and its combination SP+CV on colon tissue CAT in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

Table 2.Immunohistochemistry stained area in the colon tissue of control and treated groups

Parameter%	Experimental groups				
	Control	DMH	DMH+CV	DMH+SP	DMH+CV+SP
PCNA	6.78 ±1.02^{bc}	24.15±2.77^{a}	10.33±1.29^{b}	6.88±1.06^{bc}	4.17±0.98^{c}
Caspase3	28.02 ±2.16^{b}	9.04 ±1.75^{c}	17.92±3.43^{c}	31.54±1.01^{b}	49.32±2.15^{a}
Cox2	39.98 ±2.47^{b}	54.47 ±1.57^{a}	48.99±1.24^{a}	33.50±2.42^{b}	35.26±3.61^{b}

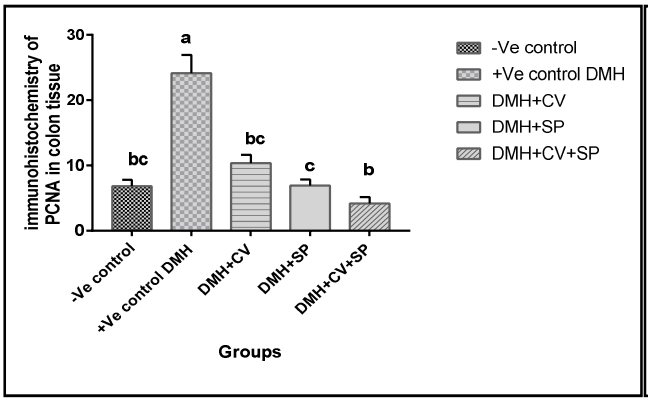

Figure 5. Effect of SP, CV and ts combination SP+CV on immunohistochemistry of PCNA in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

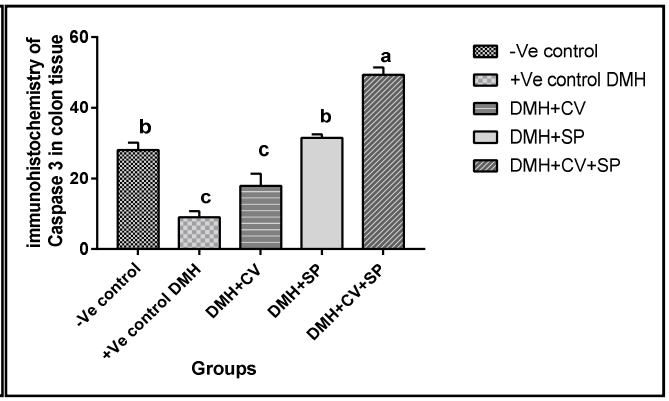

Figure 6. Effect of SP, CV and its combination SP+CV on immunohistochemistry of Caspase 3 in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

Immunohistochemistry results: Photomicrographs and stained area % for immunohistochemictryl for colon tissue specimen in DMH, Spirulina *platenesis, Chlorella vulgaris* and its combination compared to that of normal control showed in (Table 2, Figure 5-15).

Aberrant cryptic foci count

Analysis of the preneoplastic lesions, ACF count was done to monitor cancer initiation. The ACF and MDF count showed significant (P≤0.05%) increase in DMH group compared to control group. While treated groups; *Spirulina platenesis, Chlorella vulgaris* and its combination group showed significant decrease in ACF Count. Compared DMH group The ACF count in the control, DMH and (SP, CV and its combination) groups is showed in (Table 3 and Figure 9). Data are expressed as mean ± SE (*n* = 8).Means with different superscript letters within the same row differ significantly at P < 0.05. MDA (malondialdehyde), GSH (reduced glutathione), SOD (superoxide dismutase), CAT (catalase), DMH (dimethyle hydrazine), DMH-CV (dimethyle hydrazine+ *Chlorella vulgaris*), DMH+SP (dimethyle hydrazine+*Spirulina platensis*), DMH+CV+SP (dimethyle hydrazine+ *Chlorella vulgaris* +*Spirulina platensis*). Data are expressed as mean ± SE (*n* = 8).Means with different superscript letters within the same row differ significantly at P<0.05. PCNA (Proliferating cell nuclear antigen), caspase 3 (cysteine-aspartic acid protease-3), COX2 (Cytochrome c oxidase subunit 2), DMH (dimethyle hydrazine), DMH-CV (dimethyle hydrazine+ *Chloralla vulgrasis*), DMH+SP (dimethyle hydrazine-*Spirulina platensis*), DMH+CV+SP (dimethyle hydrazine-*Chloralla vulgrasis*-+*Spirulina platensis*).

Histopathological result

The histopathological change of colon tissues in DMH group and the *Spirulina platenesis, Chlorella vulgaris* and its combination compared to that of normal control showed in Figure (10).

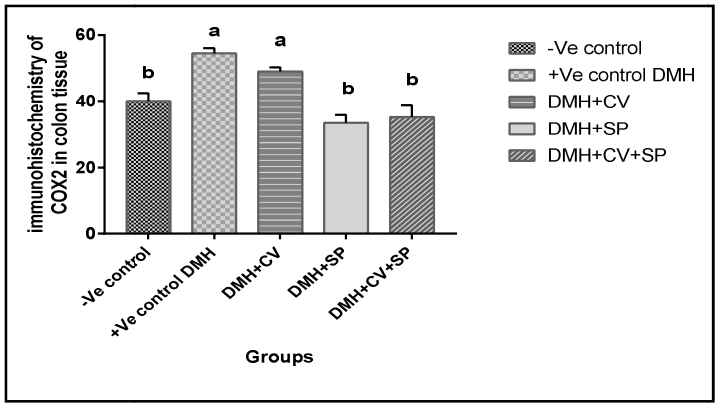

Figure 7. Effect of SP, CV and its combination SP+CV on immune-histochemistry of COX2 in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

Table 2. Immunohistochemistry stained area in the colon tissue of control and treated groups

Parameter %	Experimental groups				
	Control	DMH	DMH+CV	DMH+SP	DMH+CV+SP
PCNA	6.78 ±1.02^{bc}	24.15±2.77^{a}	10.33 ±1.29^{b}	6.88 ±1.06^{bc}	4.17 ±0.98^{c}
Caspase3	28.02 ±2.16^{b}	9.04 ±1.75^{c}	17.92 ±3.43^{c}	31.54 ±1.01^{b}	49.32±2.15^{a}
Cox2	39.98 ±2.47^{b}	54.47 ±1.57^{a}	48.99 ±1.24^{a}	33.50 ±2.42^{b}	35.26±3.61^{b}

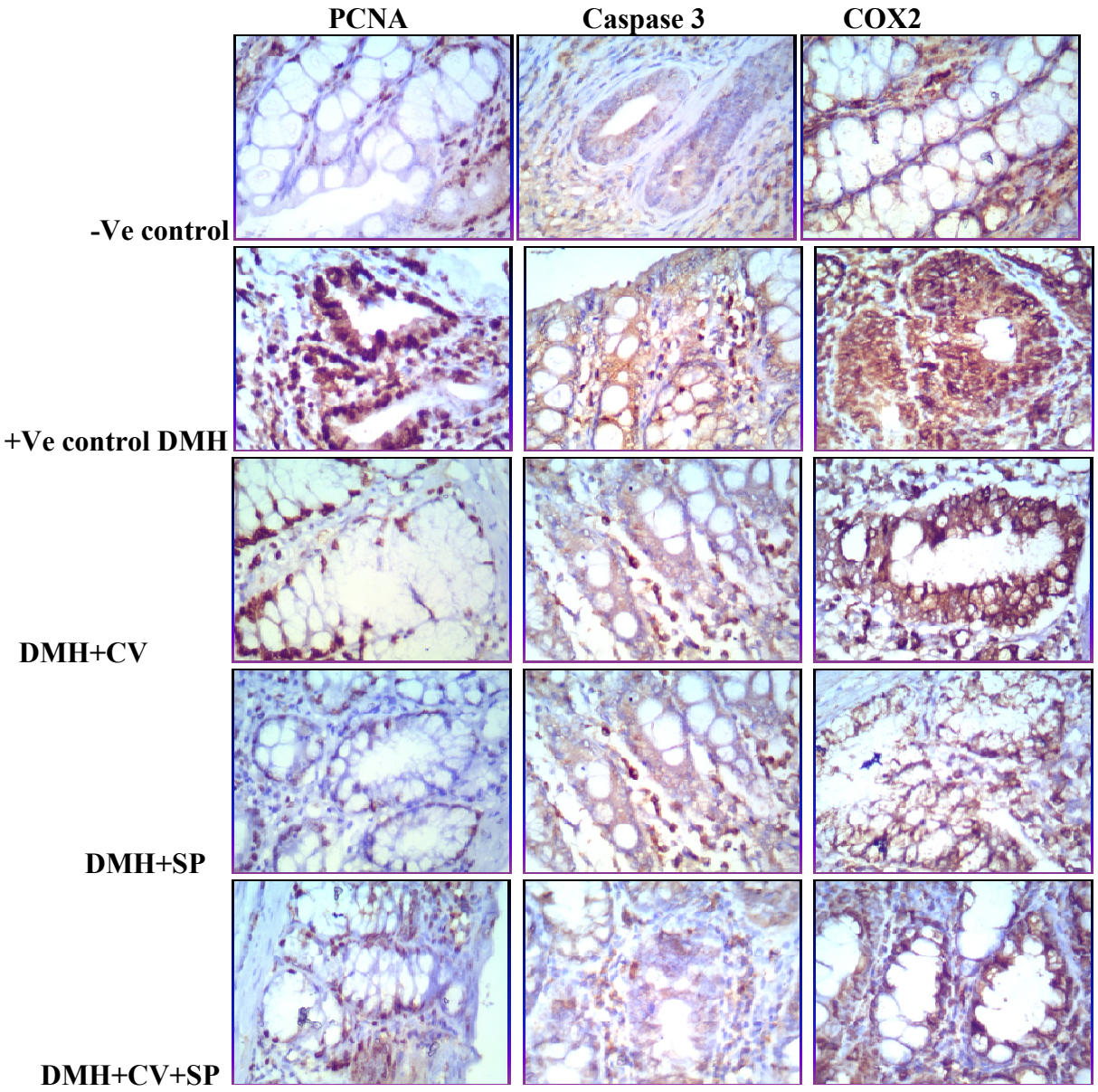

Figure 8. Immunohistochemial staining for PCNA, Caspase 3 and COX2 in colon tissue of control and treated rats

Table 3. ACF and MDF count in the colon tissue of control and treated groups

Parameter	Experimental groups				
	Control	DMH	DMH-CV	DMH-SP	DMH-CV-SP
ACF count	0.50 ±0.22[e]	50.33 ±0.67[a]	32.83 ±1.74[b]	22.67 ±1.33[c]	12.33 ±1.41[d]

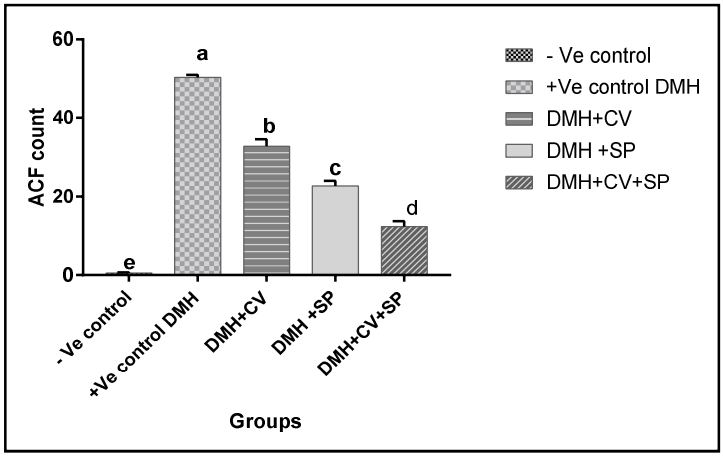

Figure 9. Effect of SP, CV and its combination SP+CV on ACF count in DMH induced colon cancer in rats. Data represent the mean value ± S.E from 8 rats/ group

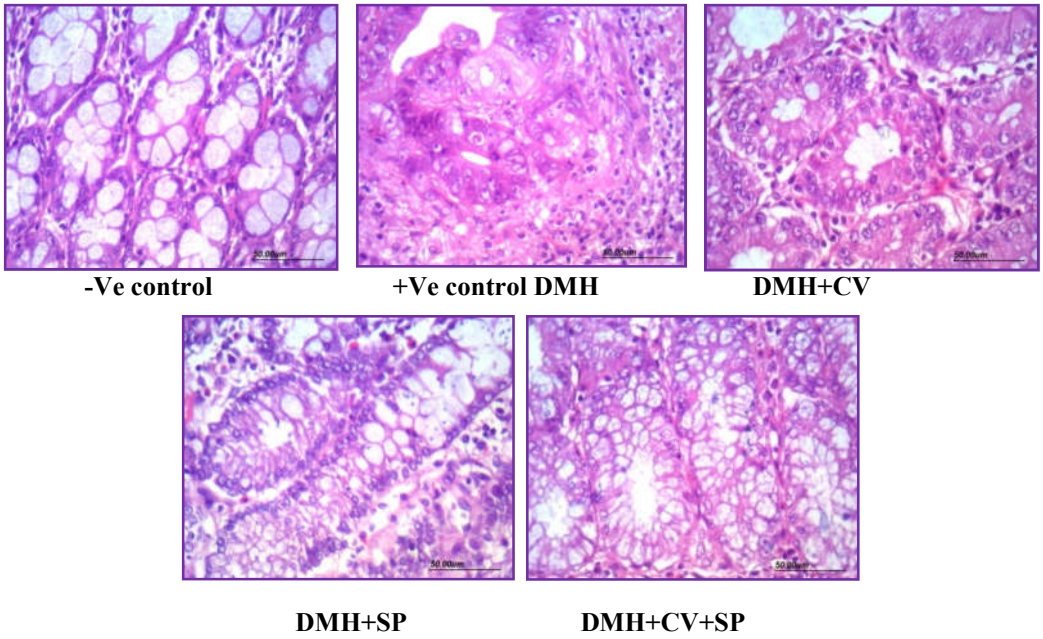

Figure 10. H & E staining for colon tissue. The histopathological change of colon tissues in DMH group and the *Spirulina platenesis, Chlorella vulgaris* and its combination compared to that of normal control showed in this figure

Control groups showed normal colon tissue. DMH group showed marked deorganization of cryptic with marked loss of goblet cells with numerous of ACF and MDF. Also, there is franked carcinoma. SP, CV and its combination showing marked improvement in colon tissue with decrease in ACF. Data are expressed as mean ± SE (n = 8).Means with different superscript letters within the same row differ significantly at P < 0.05. ACF (Aberrant cryptic Foci.), MDF (Mucin Depleted Foci), DMH (dimethylehydrazine), DMH+CV (dimethyle hydrazine+*Chlorella vulgaris*), DMH+SP (dimethyle hydrazine+*Spirulina platensis*), DMH+CV+SP (dimethyle hydrazine-*Chlorella vulgaris*+*Spirulina platensis*).

DISCUSSION

1, 2 Dimethyl hydrazine (DMH), is a potent colon carcinogen, motivating colorectal tumors in experimental animals and is the most widely adopted model of chemically induced colon carcinogenesis. DMH-motivated colon cancer is a multistep process involving a series of pathological alterations, such as formation of (Navarro *et al*., 1997). The biochemical marker chosen; namely MAD, SOD, CAT and GSH are assessed in colon homogenate. Previously, it has proven the role of oxidative stress in colon toxicity motivated by several toxicants, including in DMH (Khan and Sultana, 2011). Lipid peroxidation or MDA development is a core and a close marker of oxidative damage. Soared level of lipid peroxidation product (MDA) has been developed after treatment with DMH (Dudeja and Brasitus, 1990; Sengottuvelan *et al*., 2006). Also, our results also showed tangible increase in the level MDA after DMH treatment, a matter that is consistent with previous reports. SP, CV and its combination treatments remarkably attenuated soared levels of MDA, and as such elimination of free radicals in biological systems is realized through enzymatic and non-enzymatic antioxidants, which act as major

defense systems against free radicals (Nandhakumar *et al*., 2012). In the contrary, reduced glutathione and its oxidized counterpart symbolize a major redox buffer system of the cell. GSH levels in rats treated with DMH showed tangible decrease in our study and previous studies (Khan *et al*., 2012) and the treatment with SP, CV and its combination restored the normal levels of GSH, proving the protective efficacy of them. Dimethylhydrazine treatment produces free radicals in colonic tissue and their level is organized by GSH and other enzymatic antioxidants (such as SOD, CAT, GPx, GR etc.) by scavenging the free radicals. Therefore, DMH treatment reduces level of antioxidant enzymes (Sengottuvelan *et al*., 2006). Also the treatment of SP, CV and its combination significantly brought these levels back near to normal. Oxidative stress and inflammation tumor initiation and have a leading role and were found to affect promotion (Bickers and Athar, 2006). COX-2, an important inflammatory marker, is motivated while inflammation, up-regulated in adenomas and over-expressed in colon cancer (Eberhart *et al*., 1994; Sano *et al*., 1995). We noted that DMH-treated rats have increased expression of COX-2 and showed administration of SP, CV and its combination inhibited the expression of COX-2.Cell proliferation and apoptosis are counterparts that aberrant cryptic foci (Hamiza *et al*., 2012). It has shown that antioxidants could inhibit prolifieration of cancer cells (Chinery *et al*., 1998). It was noted tumor cells yield more peroxides when they proliferate actively after inoculation of tumor which, in turn, could affect many vital organs' functions, which indicated the intensification of oxygen-free radical production have the same responsibility of maintaining normal tissue homeostasis (Wang, 2004).

The proliferation index was examined by immunehistochemial staining for PCNA on colon tissue. (PCNA) a nuclear protein existed in proliferating cell, is vital for cell replication and acts as a marker for cell proliferation (Malkas *et al*., 2006). Expression of PCNA is to be increased in rapidly proliferating tumor (Maga and Hübscher, 2003). CV has a significant anti-proliferating effect and was also reported to inhibit proliferation by reducing PCNA count (Amin, 2009). Also, recent studies showed that the proliferating cell nuclear antigen (PCNA) was significantly linked with tumor growth and that the apparent linkage between PCNA and Ki67 indices was close to significance (Nieto *et al*., 2000).In the current study, the colon of both controlled and SP-treated rats exhibited few PCNA-positive cells in the proliferating stage. In contrast, colon sections of DMH treated rats showed significant hike in the PCNA-positive cells number, which was significantly decreased by SP supplementation. Apoptosis is an important passage in antitumor drug response (Galeano *et al*., 2005Galeano *et al*., 2005; Makin and Dive, 2001). The caspase are aspartate specific cysteine proteases which execute apoptosis and assessing their activity is widely adopted for the detection of apoptosis in various cell types. The current study has revealed an increase in caspase-3 expression in SP, CV and its combination causing cell injury that saw DNA damage, leading to liberation of cytochrome c from mitochondria into cytoplasm and to activation of caspase-3 and apoptosis at the final stage (Roy *et al*., 2007). In this study we determine ACF and up to now, ACF are considered a 'gold standard' of colon carcinogenesis biomarkers (Corpet and Taché, 2002); meanwhile ACF determination is widely adopted to identify potential chemopreventive agents (Wargovich *et al*., 2000). Our study demonstrates that injection of DMH produces histopathological changes in colon; meanwhile DMH-induced ACF have several biological aberrations, foremost of which soared proliferative state and expansion of the proliferative zone. In our present study, increased count was observed in DMH-treated rats. Colon of -ve control (normal cell) showed sections in normal colonic mucosa formed by glands and crypts and regularly arranged to each other and lined by columnar epithelial cells with luminal cytoplasmic mucus secretion (goblet cells) as well as flattened to small rounded basal uniform regular nuclei. The lamina propria in-between glands showed loose connective tissue without inflammatory infiltrate. Sections in colonic mucosa of DMH group (+ve control group) showed marked disorganization of crypts with notable loss of goblet cells and numerous ACF and colonic glands lining replaced by highly dysplastic epithelial cells, showing increased nucleo-cytoplasmic ration, enlarged nuclei with nuclear pleomorphism and hyperchromasia with coarse chromatin. Also some glands are fused and form cribriform pattern (frank carcinoma).

There are many mitotic figures indicating high mitotic activity. The surrounding lamina propria is mildly fibrotic infiltrated by inflammatory cells mainly lymphocytes and plasma cells.Examined sections in colonic mucosa of DMH+SP group exhibited notable improvement with decreased ACF and that gland and crypts are regularly arranged to each other and lined by columnar epithelial cells with preserved luminal cytoplasmic mucus secretion (goblet cells) as well as flattened to small rounded basal uniform regular nuclei. The lamina propria in-between glands showed loose connective tissue with focal inflammatory infiltrate. Also, there were only very few ACF. Sections in colonic mucosa (DMH+CV group) showed mild improvement with residual ACF. Meanwhile, glands and crypts showed moderate irregularities lined by columnar epithelial cells with mostly lost luminal cytoplasmic mucus secretion and rounded to oval basal moderately pleomorphic nuclei with few mitoses. The lamina propria in-between glands showed loose connective tissue with focal inflammatory infiltrate. There were only very few ACF. Examined sections in colonic mucosa (DMH+CV+SP group) showed moderate improvement with decreased ACF. Glands and crypts are regularly arranged to. each other and lined by columnar epithelial cells with preserved luminal cytoplasmic mucus secretion (goblet cells) and flattened to small rounded basal nuclei, showing mild. pleomorphism. The lamina propria in-between glands showed loose connective tissue with focal inflammatory infiltrate. There was a limited number of ACF cell in the examined fields.

Conculsion

Spirulina platensis and *chlorella vulgrasis* are rich with nutrients and active components. They have chemopreventive effects by anti-inflammatory, antiproliferating, apoptosis and antioxidant activity in colon cancer induced by DMH in rats.

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