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## **Histological and Immunohistochemical Studies for Evaluation of the Role of Microalgae *Spirulina* sp. Against Cancer in Experimental Animals**

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**Abstract:** The present study aims to study the possible effect of the cyanobacterium *Spirulina* sp. as a protective agent in rats given diethylnitrosamine (DEN) in drinking water. The investigation is supposed to be carried out through histopathological and immunohistochemical examinations for proliferation marker Ki67 in liver tissues. The hepatoprotective role of *Spirulina* shown by the histopathological examination of the liver where only little dilatation in the blood sinusoids, slight vacuolization in the cytoplasm and few pyknotic nuclei were observed. Immunohistochemical evaluation shown reduction of the number of malignant cells and its proliferation in treated group, but the Ki67 expression was high labeling index in DEN induced hepatocarcinoma group. Also, liver Ki67 labeling index (Ki67 LI) in tumor tissues or adjacent non-tumor tissues were higher than that in normal liver tissues, while in tumor tissues it was higher than that in adjacent non-tumor tissues. So, using *Spirulina* seems to have realistic results and rapid curative effect. The development of specific therapeutic strategies based on natural algal products should be considered as an attractive approach. Based on these fascinating possibilities, *Spirulina* offers a great promise for patients with liver injury and Ki67 expression could represent a valuable tool in the understanding of hepatocellular carcinoma.

**Key words:** Hepatocarcinoma, *Spirulina*, Ki67, immunohistochemistry

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### **INTRODUCTION**

Cancer has become an important topic in medicine since it is a major cause of death in both the developed and developing countries and it is now only secondary to that of myocardial infarction (Gruddy, 1991). A great majority of human cancers (about 80-90%) are attributable to environmental factors (Benjamin *et al.*, 1990). However, it is not an easy task to eliminate carcinogenic causes from the environment. While modern surgery has significantly reduced the cancer mortality, the use of additional treatment such as radiotherapy and chemotherapy has resulted in no more than 5% reduction in the number of deaths (Benjamin *et al.*, 1990). Therefore, there is a continuing search for better control and preventive methods in order to reduce cancer mortality and related side effects. Many investigations are now being carried out to discover naturally occurring compounds, which can suppress or prevent the process of carcinogenesis (Thapliyal *et al.*, 2002).

Over the past decade, several epidemiological and case control studies have linked tea consumption, especially green tea, to a reduced risk of cancer in humans (Su and Arab, 2002; Ke *et al.*, 2002). Also, *Spirulina*, blue green microalgae, has been used since ancient times as a source of food because of its high protein and nutritional value. The chemical composition of *Spirulina* indicates that it has phenolic acids, tocopherols and b-carotene, which are known to exhibit antioxidant properties. Experimental studies have demonstrated inhibitory effects of *Spirulina* on oral carcinogenesis (Premkumar *et al.*, 2004).



The WHO has described *Spirulina* as one of the greatest super foods on earth and NASA considers it as an excellent compact food for space travel, as a small amount can provide a wide range of nutrients. In addition, *Spirulina* is accepted as functional food, which is defined as products derived from natural sources, whose consumption is likely to benefit human health and enhance performance (Khan *et al.*, 2005).

Chemical agents inducing hepatocarcinogenesis have been administered either as DEN alone or in combination with acetylaminofluorene (AAF), ortic acid, phenobarbital benzopyrene, N-amyl-N-methylnitrosamine and CCl<sub>4</sub>. The chemical has advantages at inducing hepatocarcinogenesis, as it is able to induce hepatoma within a short time and can be administered by a variety of methods. The administration of carcinogenic substances may bring about changes in enzyme levels arising from clonic proliferation, so it is of some importance to analyze enzyme activity variation quantitatively in order to understand the processes involved (Ha *et al.*, 2001).

Therefore, the present study aims to study the possible effect of the cyanobacterium *Spirulina platensis* as a protective agent for rats given dimethylnitrosamine (DEN) precursors in drinking water. The investigation is supposed to be carried out through histopathological and immunohistochemical examinations of liver.

## MATERIALS AND METHODS

### Animals and Treatment

In all experiments Sprague-Dawley rats (8 week old, 200±10 g) were used and maintained in a temperature controlled room (25±2°C) under 12 h light/dark cycle (dark phase 6 pm to 6 am). Rats were fed with a standard laboratory diet containing 19% crude proteins, 3.8% fiber and 4400 kcal of energy, based on a formula recommended by the WHO and water *ad libitum* (Sabourdy, 1988).

0.01% DEN (Sigma Chemical Co., USA) was continuously administered to rats via., drinking water for 14 weeks. No differences in the caring conditions of control rats and the DEN treated groups existed, except for the DEN dissolved in the drinking water. Four group used was 15 rats to each (Fig. 1).

### Histological and Immunohistochemical Preparations

Rats were sacrificed to investigate their liver tissues on the same day after weeks 14 of DEN administration. Representative samples of tumors and tissues were histologically examined. The specimens (3-5 mm thick) were fixed in formalin 10% and embedded in paraffin. Slices of 3-4 µm were

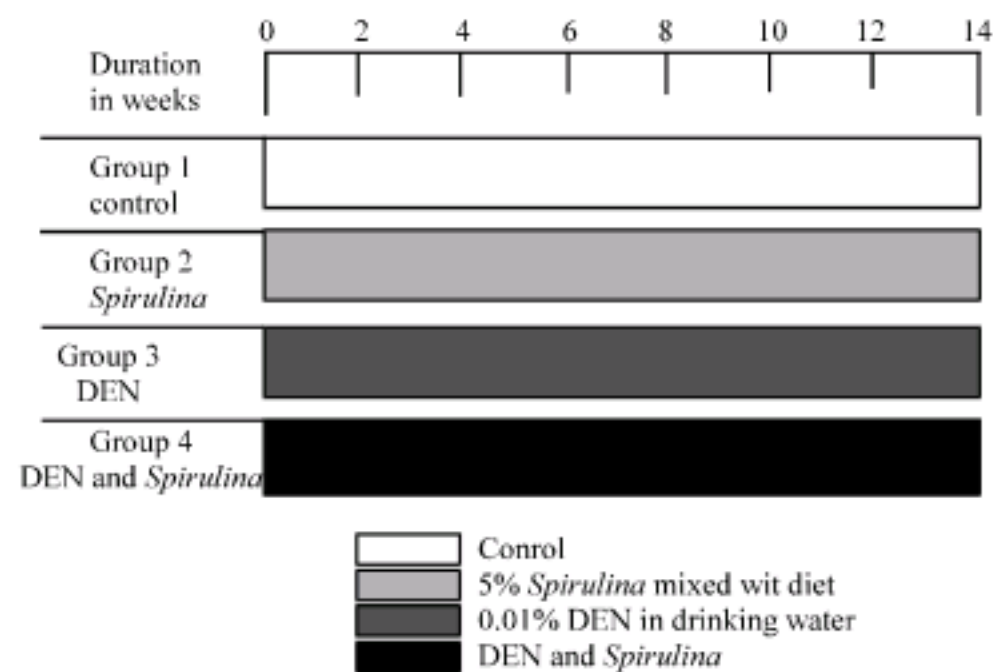


Fig. 1: The different experimental animal groups



stained with hematoxylin and eosin for microscopic examination and other for determination of proliferating cell nuclear antigen by immunostaining technique.

Five-micrometer tissue sections were cut from paraffin blocks, mounted on poly-L-lysine coated glass slides and air-dried overnight at room temperature. Immunohistochemical staining was performed using an avidin-biotin peroxidase complex. Briefly, samples were treated with 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Staining of formalin-fixed tissues requires boiling tissue sections in 10 mM citrate buffer, pH 6.0, (NEOMARKERS' Cat. No. AP-9003), for 10-20 min followed by cooling at RT for 20 min. The slides were preincubated with normal goat serum (1:10) (NEOMARKERS, USA) for 10 min and then with human specific Mouse Monoclonal Ki67 (Clone SP6) antibody (Ab-1, Lab. Vision, NeoMarkers, USA), using antibody dilution at 1:200 for 30 min at RT. The sections were further incubated with biotinylated secondary antibody (NEOMARKERS, USA) for 10 min, followed by incubation with peroxidase-conjugated streptavidin diluted 1:3000 in phosphate- buffered saline for 15 min. The peroxidase reaction was performed using 0.02% 3, 3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide and counterstaining was performed with hematoxylin for 1 min. As negative control, the primary antibody was omitted.

Each section was counted manually at high power (x400) after identifying at low power (x100) the representative areas with the highest concentration of stained cells according to the recommendation of Cohen *et al.* (1993), about 1000 cells/slide were counted in each of five microscopic fields from well-labeled areas to determine the average of Ki67 labeling index (Ki67 LI). Ki67 LI was expressed as number of labeled cells (positive for Ki67) as a percentage of the total number of cells counted in each specimen. All identifiable staining was regarded as positive. The results are expressed as mean plus or minus standard deviation (Ki67 LI = Mean $\pm$ SD) the positive results for proliferation marker Ki67 is brown nuclear stain.

#### **Statistical Analysis**

Statistical analysis for obtained results was carried out with the aid of the SPSS computer software program.

### **RESULTS**

The present results demonstrated the normal polyhedral hepatocytes with granular cytoplasm. Each cell has a centrally located nucleus with one or two nucleoli in addition to a number of chromatin particles. Sometimes, binucleated hepatocytes are seen. The hepatocytes arranged in cords or strands forming a network around the central vein. The liver strands are alternating with narrow blood sinusoids also radially extending along the liver lobules. The boundaries of the sinusoids composed of a single layer of fenestrated endothelial cells and kupffer cells (Fig. 2A). *Spirulina* treated rats showed normal liver tissue as previously seen in normal liver of control rats (Fig. 2B). Liver of rats treated with DEN in their drinking water showed obvious fatty degeneration with displacement of the nucleus. Hydrophobic degeneration (oedema) was also seen in severe injured hepatocytes. The nuclei of the hepatocytes were apparently hyperchromatic and displayed some features of pyknosis. Inflammatory lymphocytic infiltration was clearly visible in these rats, also tumor in some area was arranged in cords and in others in a duct-like pattern (Fig. 2C-E). Examination of liver section of these rats shown the role of *Spirulina* in the protection of liver against the effect of the carcinogen. Little dilatation in blood sinusoids, slight vacuolization in the cytoplasm of the hepatocytes, few pyknotic nuclei and active kupffer cells were seen in the liver of this group (Fig. 2F).

In the immunostain investigation, the liver sections of control and *Spirulina* treated rats stained with Ki67 showed very weak positive stained nuclei indicating the mild cell division of some



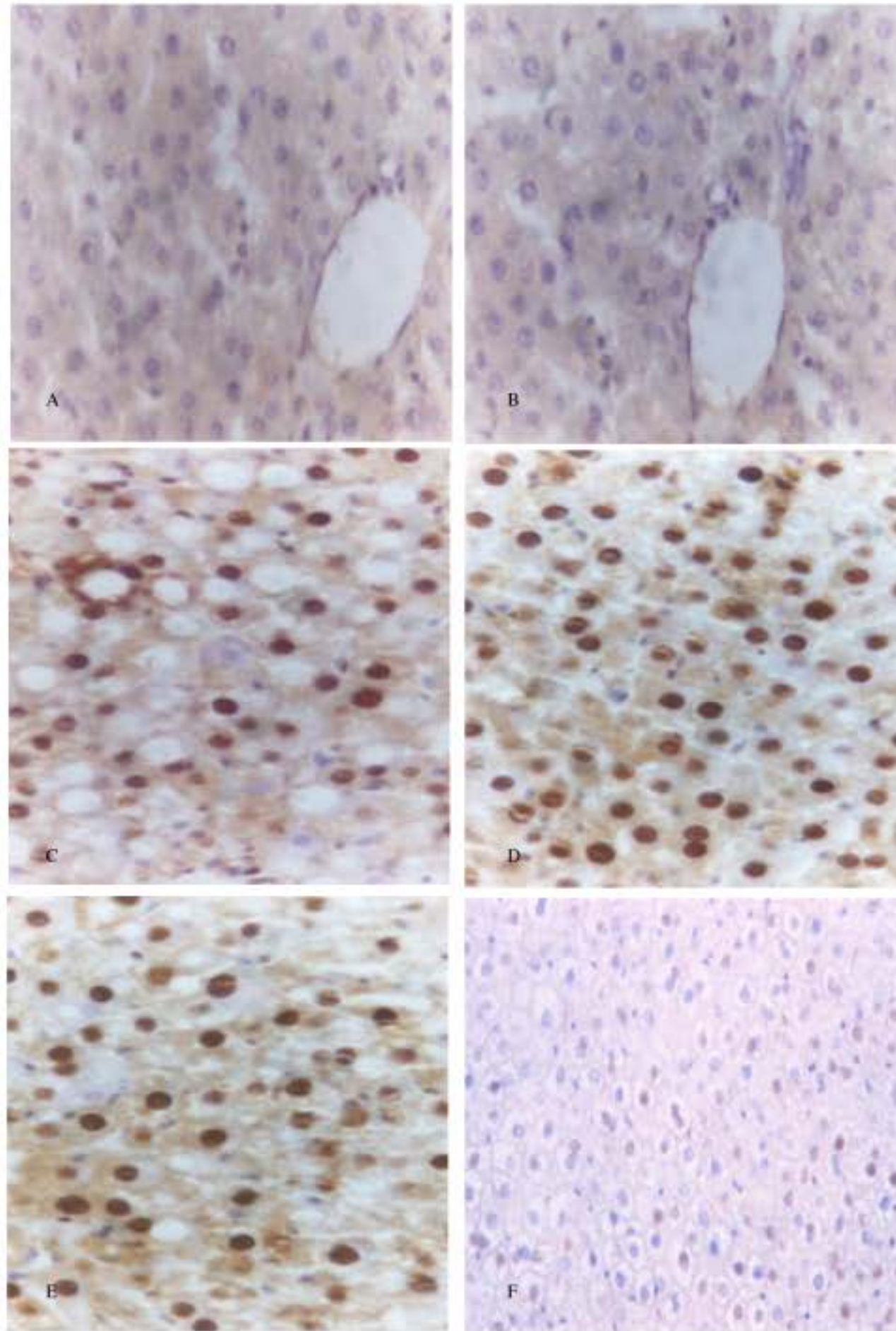


Fig. 2: Photomicrographs of liver from different groups. (A) The normal liver. (B) The liver of *Spirulina* treated animals. (C, D and E) The hepatocellular carcinoma with high proliferation. (F) DEN and *Spirulina* treated animals show the similar future as in normal with some different (show CV, KC, HC, BS, HD, N, DBS, BN, PN, FD and LI mean central vein, kupffer cell, hepatic cell, blood sinusoid, hydrophobic degeneration cell, nucleus, dilated blood sinusoid, binucleated cell, pyknotic cell, fat droplet and lymphocytes infiltration, respectively

hepatocytes (Fig. 3A, B). However, sections in liver of rats treated with DEN were showed strong positive stained nuclei in most of the hepatocytes (Fig. 3C-E). Moreover, the hepatocytes of rats treated with *Spirulina* and DEN were demonstrated that the positive stained nuclei less than that of the DEN treated animal (Fig. 3F).



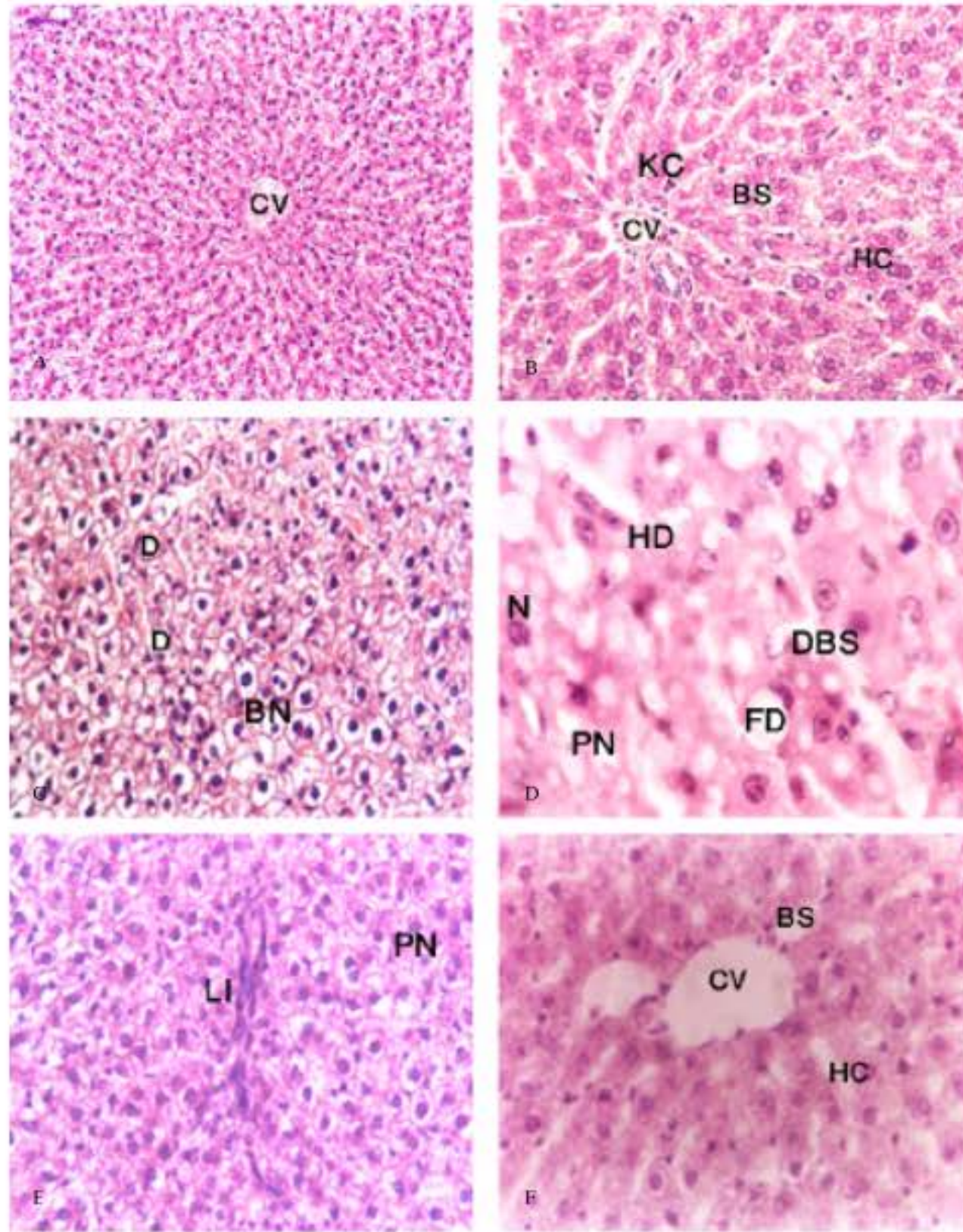


Fig. 3: Immunohistochemical staining photomicrographs show rat liver sections in different groups were stained with anti-Ki67 with Avidin-Biotin technique. (A) Normal liver with very weak immunostaining. (B) Also, the group of *Spirulina* treated animals with very weak reaction. (C, D and E) DEN group show the strong positive brown staining with the proliferating marker Ki67. (F) DEN and *Spirulina* treated animals show weak positive with Ki67

Table 1: Liver Ki67 labeling index as a result of carcinogenicity of DBN precursors and/or *Spirulina* treatment

Group	<i>Spirulina</i> and DEN	DEN group	<i>Spirulina</i>	Control
$\bar{x}$	20.324	79.825	6.293	6.942
SD	4.236	9.925	2.924	3.123
SE	0.945	3.812	0.834	0.901
t-test	5.243	18.012	-0.954	-
**	**		***	NS

$\bar{x}$  : Mean value , SD: Standard deviation, SE: Standard error, NS: Non significant, \*\* Significant ( $p \leq 0.01$ ), \*\*\* Highly Significant ( $p \leq 0.001$ )

Table 1 shows the changes in liver Ki67 labeling index were observed. Rats fed on *Spirulina* containing diet were showed a nonsignificant when compared with control group. While, DEN treated



group was displayed a very high significant. Rats protected with *Spirulina* and treated with DEN were shown a high significant increase compared with control rats and a very high significant when compared with DEN treated group.

## DISCUSSION

In recent years, there is an increasing awareness that certain naturally occurring compounds in plants and other sources, have protective effects against environmental mutagens/carcinogens and endogenous mutagens (Premkumar *et al.*, 2004). Recently, it was shown that *Spirulina*, the most powerful food on earth, is rich in proteins, carbohydrates, polyunsaturated fatty acids, sterols and some more vital elements like calcium, iron, zinc, magnesium, manganese and selenium (Clement, 1975; Tantawy *et al.*, 2004). *Spirulina* is the richest natural source of vitamin B<sub>12</sub>, vitamin E, ascorbic acid, tocopherols in the world and contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments (Estrada *et al.*, 2001).

*Spirulina* has been considered as a supplement in human and animal food (Dillon *et al.*, 1995). Actually, it is being widely studied for its possible antioxidant, antibacterial and antiparasitic properties and for several medical conditions such as allergies, ulcers, anemia, heavy-metal poisoning and radiation poisoning (Hirahashi *et al.*, 2002). *S. platensis* has been also studied for its antiviral properties (Hayashi *et al.*, 1993; Aychunie *et al.*, 1998), which seem to be related to its sulfated polysaccharide named calcium spirulan (Hayashi *et al.*, 1996a, b). The present study showed the protective and anticancer properties of *Spirulina* in liver cells.

Qureshi *et al.* (1996), Pang *et al.* (1998) and Ismail *et al.* (2006) showed that *Spirulina* or its extracts can prevent or inhibit cancer in humans and animals. *In vitro* studies suggest that polysaccharides of *Spirulina* enhance cell nucleus enzyme activity and DNA repair synthesis.

Induction of the hepatocarcinogenic process induced by administration of chemicals provides a system for characterizing alterations in the liver at early stages in the process. Human hepatocellular carcinoma and both spontaneous and chemically induced hepatocellular carcinoma in rodents exhibit considerable similarities with regards to morphology, genomic alterations and gene expression (Feo *et al.*, 2000), despite their different etiologies.

Therefore, investigation of the development of liver cancer in rats and mice might provide valuable insight into the human condition. Moreover, the cytoplasm of the hepatocytes in this study showed different sizes of vacuoles. The interpretation of vacuolar formation has been discussed by Robbins and Angell (1976) who mentioned that the cytoplasmic vacuolization is one of the important primary responses to all forms of cell injury and Ki-67 is a nuclear protein, which is expressed in proliferating cells. Ki-67 is preferentially expressed during late G<sub>1</sub>-, S-, M- and G<sub>2</sub>-phases of the cell cycle, while cells in the G<sub>0</sub> (quiescent) phase are negative for this protein. Also, the present study is in agreement with that of Pardhasaradhi *et al.* (2003) who studied the effects that *Spirulina* on rat histiocystic tumor line. They reported that *Spirulina* is a chemotherapeutic agent that causes apoptosis to tumor cells. This is shown in the present investigation by the reduction of the number of malignant cells and its proliferation in treated group, but the of Ki67 expression was high labeling index in DEN induced hepatocarcinoma group. Also, liver Ki67 labeling index (Ki67 LI) in tumor tissues or adjacent non-tumor tissues were higher than that in the normal liver tissues, while in tumor tissues it was higher than that in adjacent non-tumor tissues. The present conclusion was that using *Spirulina* seems to have realistic results and rapid curative effect. The development of specific therapeutic strategies based on natural algal products should be considered as an attractive approach. Based on these fascinating possibilities, *Spirulina* offers a great promise for patients with liver injury. Also, Ki67 expression could represent a valuable tool in the understanding of hepatocellular carcinoma.



## REFERENCES

- Ayehunie, S., A. Belay, T.W. Baba and R.M. Ruprecht, 1998. Inhibition of HIV-1 replication by an aqueous extract of *Spirulina platensis* (*Arthrospira platensis*). J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol., 18: 7-12.
- Benjamin, H.S.L., P.T. Padma and M.T. Jeffrey, 1990. *Allium sativum*: (garlic) and cancer prevention. Int. J. Vitamin Nutr. Res., 10: 937-948.
- Clement, G., 1975. Production and characteristic constituents of the algae *Spirulina platensis* and *Spirulina maxima*. Ann. Nutr. Aliment., 29: 477-488.
- Cohen, M.B., F.M. Waldman and P.R. Carroll, 1993. Comparison of five histopathologic methods to assess cellular proliferation in transitional cell carcinoma of the urinary bladder. Hum. Path., 24: 772-778.
- Dillon, J.C., A.P. Phuc and J.P. Dubacq, 1995. Nutritional value of the alga *Spirulina*. World Rev. Nutr. Diet, 77: 32-46.
- Estrada, J.E., P.B. Bescós and A.M. Villar Fresno, 2001. Antioxidant activity of different fractions of *Spirulina platensis* protean extract. Il Farmaco, 56: 497-500.
- Feo, F., R.M. Pascale, M.M. Simile, M.R. De Migilo, M.R. Muroli and D. Calvisi, 2000. Genetic alterations in liver carcinogenesis: Implications for new preventive and therapeutic strategies. Crit. Rev. Oncog., 11: 19-62.
- Gruddy, S.M., 1991. Recent nutrition research, implications for foods of the future. Ann. Med., 23: 187-193.
- Ha, W.S., C.K. Kim, S.K. Song and C.B. Kang, 2001. Study on mechanism of multistep hepatotumorigenesis in rat: development of hepatotumorigenesis. J. Vet. Sci., 2: 53-58.
- Hayashi, K., T. Hayashi and N. Mortia, 1993. An extract from *Spirulina platensis* is a selective inhibitor of Herpes simplex virus type 1 penetration into HeLa cells. Phytother. Res., 7: 76-80.
- Hayashi, T., K. Hayashi, M. Maeda and I. Kojima, 1996a. Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. J. Nat. Prod., 59: 83-87.
- Hayashi, K., T. Hayashi and I. Kojima, 1996b. A natural sulfated polysaccharide, calcium spirulan, isolated from *Spirulina platensis*: *in vitro* and *ex vivo* evaluation of anti-herpes simplex virus and anti-human immunodeficiency virus activities. AIDS Res. Hum. Retroviruses, 12: 1463-1471.
- Hirahashi, T., M. Matsumoto, K. Hazeki, Y. Saeki, M. Ui and T. Seya, 2002. Activation of the human innate immune system by *Spirulina*: Augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. Int. Immunopharmacol., 2: 423-434.
- Ismail, M.F., Y.A. Azab, D.A. Ali, E.K. Abd Elhady and S.K. Areida *et al.*, 2006. Ultrastructural studies on the effect of *Spirulina platensis* on experimentally induced bladder cancer in rats. Mansoura J. Biol., 33: 1-14.
- Ke, L., P. Yu, Z.X. Zhang, S.S. Huang, G. Huang and X.H. Ma, 2002. Congou tea drinking and oesophageal cancer in South China. Br. J. Cancer, 86: 346-347.
- Khan, Z., P. Bhadouria and P.S. Bisen, 2005. Nutritional and therapeutic potential of *Spirulina*. Curr. Pharm. Biotech., 6: 373-379.
- Pang, Q.S., B.J. Guo and J.H. Ruan, 1998. Enhancement of endonuclease activity and repair DNA synthesis by polysaccharide of *Spirulina platensis*. J. Chuan Hsueh Pao., 15: 374-381.
- Pardhasaradhi, B.V.V., A.A. Mubarak, A.L. Kumari, P. Reddanna and A. Khar, 2003. Phycocyanin-mediated apoptosis in AK-5 tumor references cells involves down-regulation of Bcl-2 and generation of ROS. Mol. Cancer Ther., 2: 1165-1170.
- Premkumar, K., S.K. Abraham, S.T. Santhiya and A. Ramesh, 2004. Protective effect of *Spirulina fusiformis* on chemical-induced genotoxicity in mice. Fitotherapia, 75: 24-31.



- Qureshi, M.A., J.D. Garlich and M.T. Kidd, 1996. Dietary *Spirulina platensis* enhances humoral and cell-mediated immune functions in chickens. *Immunopharmacol. Immunotoxicol.*, 18: 465-476.
- Robbins, S. and D. Angell, 1976. *Basic Pathology*. 2nd Edn., W.B. Saunders Co., Philadelphia, London.
- Sabourdy, M.A., 1988. *Breeding and Care of Laboratory Animals*. Volume 1, WHO Health Laboratory Technology Unit. Geneva, Switzerland.
- Su, L.J. and L. Arab, 2002. Tea consumption and the reduced risk of colon cancer-results from a national prospective cohort study. *Public Health Nutr.*, 5: 419-426.
- Tantawy, A., T. Borg, L. Eissa, E.M. Marwan, I. Ismail and Y. Azab, 2004. Effect of *Spirulina platensis* tablets on the level of iron and protein in rabbits. *Alex. G. Pharm. Sci.*, 18: 119-123.
- Thapliyal, R., S.S. Deshpande and G.B. Maru, 2002. Mechanism(s) of turmeric-mediated protective effects against benzopyrenederived adducts. *Cancer Lett.*, 175: 79-88.