



Spirulina inhibit the development of diethylnitrosoamine induced premalignant phenotype in rat chemical hepatocarcinogenesis model

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ABSTRACT

Spirulina was shown to exert anti-inflammatory, antioxidant, hepatoprotective properties, and anticancer activity. This study was done to investigate the protective effects of spirulina on DEN induced hepatocarcinogenesis in rats. Forty-five male albino rats were divided into three groups. Group I :(normal control group): rats administered distilled water only. Group II: rats received diethylnitrosoamine (200 mg/kg b.wt/i.p), 2 weeks later rats received (2 ml/kg b.wt) Carbon tetrachloride (CCl₄) orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl₄ injections were repeated once again after 1 month from first DEN injection. Group III: rats received DEN then treated with spirulina platensis at a dose level of (800 mg/kg b.wt/orally) dissolved in distilled water for 6 weeks. DEN induced HCC showed significant increase in hepatic marker enzymes (ALT and ALP), total bilirubin and alpha fetoprotein (AFP) with marked decrease in serum albumin concentration. Also, the results of molecular analysis in liver tissue revealed significant up-regulation in TNF- α gene expression level. Conversely, down-regulation in tumor suppressor gene p53 and Cyp2E1 gene expression compared with control group. Treatment with spirulina platensis to DEN induced HCC protects the liver cells from damage by regulating the biochemical parameters. Spirulina platensis was able to mitigate liver tissue damage induced by DEN through increasing of Cyp2E1 and P53 in addition to decreasing TNF- α gene expression level and ameliorate all serum liver function parameters. The obtained results suggest spirulina platensis can inhibit the proliferation of HCC cells through inducing tumor cell apoptosis via activation of the p53 pathway, improvement of detoxification Enzyme and suppression of inflammation by inhibition of TNF- α overexpression. Spirulina may thus be used as a potentially promising agent to inhibit HCC cell proliferation and may be a novel natural product for the management of HCC.

Keywords: *Diethylnitrosamine, Hepatocellular carcinoma, Spirulina platensis, TNF- α , p53.*

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant neoplasm of hepatocytes and constitutes more than 80% of primary malignant liver neoplasms Satir, (2007). Worldwide, liver cancer is the fifth most common malignancy and the third most

common cause of cancer death (Kung *et al.* 2010).

The major avoidable causes of cancer are smoking, dietary imbalances, chronic infections and hormonal factors which are influenced primarily by lifestyle, other causal

factors in human cancer are excessive sun exposure, viruses (such as human papilloma virus and cervical cancer) and pharmaceuticals (such as phenacetin, some chemotherapy agents, diethylstilbestrol, and estrogen). (Gold et al. 2002). Many hepatocarcinogens such as aflatoxins, acetylaminofluorene3 and diethylnitrosamine have been successfully used to develop hepatocarcinogenesis in animals (Mukherjee et al. 2009).

Diethylnitrosamine (DEN) is a potent hepatocarcinogenic nitrosamine, present in cheddar cheese, cured and fried meals, alcoholic beverages, cosmetics, agricultural chemicals and pharmaceutical agents, ground water having high level of nitrate (Mahmoud and Abdul-Hamid, 2012).

Carbon tetrachloride (CCl₄) is classified as a possible human carcinogen based on inadequate evidence of carcinogenicity in humans but sufficient evidence in animals. However, there are major deficiencies in the available cancer studies. Animal studies suggest that the carcinogenicity of carbon tetrachloride is secondary to its hepatotoxic effects, indicating a possible threshold (Provincial, 2010). A Numbers of modern drugs have been isolated from natural sources and many of these isolations were based on the uses of the agents in traditional medicine. Spirulina is a blue-green alga used as a dietary supplement. It is rich in proteins, carotenoids, polyunsaturated fatty acids, vitamin B complex, vitamin E, and minerals. Additionally, it possesses other potent antioxidants such as spirulans, C-phycocyanin, and allophycocyanin (Piero Estrada et al. 2010). Spirulina phycocyanin has been shown to possess anti-inflammatory, antioxidant, and hepatoprotective properties (Roy et al. 2007). In addition, different studies demonstrated the potential anticancer activity of spirulina in different experimental models (Ismail et al.2009). Therefore, this

work aimed to investigate the potential chemopreventive effect and apoptotic properties of spirulina platensis on DEN induced hepatocellular carcinoma in rats via evaluation of some serum and molecular liver biomarkers analysis particularly detoxification enzyme (CYP 2E1), tumor suppressor gene P53 and pro-inflammatory cytokines TNF- α gene expression in hepatic tissues.

2. Materials and methods

2.1. Experimental animals:

Forty-five white male albino rats of 6- 8 weeks old and weighing 150-180 gm housed in separated metal cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The animals fed on constant ration and water was supplied ad- labium .

2.2. Chemicals and antioxidant:

All chemicals were of analytical grade and obtained from standard commercial suppliers. The antioxidant and chemicals used in the present study were:

2.2.1. Diethylnitrosamine (DEN) and Carbon tetrachloride (CCl₄) were Purchased from SIGMA Chemical Co. (St. Louis, MO, USA) .

Induction of Hepatocarcinogenesis:

Hepatocellular carcinoma was induced in rats by I.P. injection of DEN in normal saline (200 mg/kg b.wt), 2 weeks later rats received (2 ml/ kg b.wt) CCl₄ orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl₄ administration were repeated once again after 1 month from the first DEN injection. (Hassan et al.2014).

2.2.1. Spirulina platensis was purchased from National Research Centre, Giza, Egypt. Spirulina platensis powder was dissolved in distilled water and administered orally at a dose level of 800 mg/kg body weight/day (Bharali et al. 2003).

2.3. Experimental design:

After acclimatization to the laboratory conditions, the animals were randomly divided into three groups (15 rats each) placed in individual cages and classified as follow :

Group I: Control Normal group:

Consisted of 15 male rats fed with ordinary diet only without any treatment during the entire experimental period.

Group II: DEN- induced hepatocarcinogenesis group:

Consisted of 15 male rats received DEN in normal saline (200 mg/ kg b.wt) by I.P injection, 2 weeks later rats received (2 ml/ kg b.wt) CCl₄ orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl₄ administration were repeated once again after 1 month from the first DEN injection.

Group III: DEN+ spirullina treated group:

Consisted of 15 male rats received DEN in normal saline (200 mg/kg b.wt) by I.P injection, 2 weeks later rats received (2 ml/ kg b.wt) CCl₄ orally at 1:1 dilution in corn oil as a promoter of carcinogenic effect. DEN and CCl₄ administration were repeated once again after 1 month from the first DEN injection then administered orally with spirullina platensis powder dissolved in distilled water at a dose level of (800 mg/kg body weight/day).

2.4. Sampling:

2.4.1. Blood samples:

Twenty-four hours fasting after the last dose of the drugs treatment administration, rats were anaesthetized under diethyl ether anesthesia. Blood samples were collected by ocular vein puncture in dry, clean tubes and allowed to clot for 30 minutes and serum was separated by centrifugation at 3000 r.p.m for 15 minute. Serum was taken by automatic pipettes and collected in dry sterile tubes, then kept in deep freeze at -20 °C until use for subsequent biochemical analysis. All sera were analyzed for determination of the following parameters: AST, ALP, total bilirubin, albumin and AFP .

2.4.2. Liver tissue for molecular analysis:

Briefly, liver tissues were cut, weighed and minced into small pieces, about 0.5 g of liver tissues were collected from all animals groups, put in Eppendorf tubes and were immediately kept in liquid nitrogen and stored at -80°C till RNA extraction. The molecular analysis of the relative gene expression in liver tissues evaluated by reverse transcription polymerase chain reaction (RT-PCR) were: (TNF- α , p53 and Cyp2E1 gene).

2.5. Analysis:

2.5.1 Biochemical analysis

Serum ALT and ALP activities, total bilirubin, albumin and AFP concentrations were determined according to the method described by Schumann et al. (2002), EL-Aaser and EL-Merzabani, (1975), Young, (1997) and Doumas et al. (1971) and Engall, (1980), respectively.

2.5.2 Molecular analysis

Total RNA was isolated from liver tissue of rats using RNeasy Mini Kit (Thermo Qiagen, #74104) according to the manufacturer's protocol. Following determination of RNA concentration and purity by Quawell nanodrop Q5000 (USA), 5 mg of total RNA from each sample was reverse transcribed using Quantiscript reverse transcriptase. The produced cDNA was used as a template to determine the relative expression of Tumor necrosis factor alpha (TNF- α), tumor suppressor (P53) and cytochrome P450 2E1 (Cyp2E1) genes using Step One Plus real time PCR system (Applied Biosystem, USA) and gene specific primers. The reference gene, β actin, was used to calculate fold change in target genes expression. The thermal cycling conditions, melting curves temperatures, and calculation of relative expression was done. For the treated groups, assessment of 2- $\Delta\Delta$ Ct determined the fold change in gene expression relative to the control (Livak and Schmittgen, 2001).

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Forward and reverse primers sequence for real time PCR.

Gene	Forward primer (5' ----- 3')	Reverse primer (5' ----- 3')
TNF- α	GCATGATCCGCGACGTGGAA	AGATCCATGCCGTTGGCCAG
p53	ATGGCTTCCACCTGGGCTTC	TGACCCACAACGCACAGGGC
CYP2E1	CTCCTCGTCATATCCATCTG	GCAGCCAATCAGAAATGTGG
β -actin	AAGTCCCTCACCTCCAAAAG	AAGCAATGCTGTCACCTTCCC

2.6. Statistical analysis:

The results were expressed as mean \pm SE using SPSS software program version 16 (SPSS© Inc., USA). The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparison among the groups for testing the inter-grouping homogeneity. Values were considered statistically significant when $p < 0.05$.

3. RESULTS

The obtained results demonstrated in table (1) revealed that, serum ALT and ALP activities, total bilirubin and AFP concentrations were significantly elevated and serum albumin

Table 1. Effect of Spirullina platensis treatment on serum ALT and ALP activities, total bilirubin, Albumin and AFP concentrations in DEN-induced HCC in rats.

Parameters	ALT (U/L)	ALP(U/L)	T. bilirubin(mg/dl)	Albumin (g/dl)	AFP (ng/dl)
Exp. groups					
Group I: Normal control	190.00 \pm 11.55 ^{b,c}	358.00 \pm 27.14 ^b	0.58 \pm 0.02 ^b	3.23 \pm 0.12 ^a	0.73 \pm 0.007 ^c
Group II: DEN	365.00 \pm 14.43 ^a	490.00 \pm 11.55 ^a	1.75 \pm 0.03 ^a	1.80 \pm 0.17 ^b	4.90 \pm 0.47 ^a
Group III: DEN+spirullina	171.67 \pm 14.24 ^c	369.33 \pm 20.50 ^b	0.43 \pm 0.03 ^b	2.93 \pm 0.03 ^a	1.40 \pm 0.2 ^c

Table 2. Effect of Spirullina platensis treatment on liver tissue TNF- α , p53 and Cyp2E1 gene expression level in DEN- induced HCC in rats.

Parameters	TNF- α Fold change mean \pm SEM	p53 Fold change mean \pm SEM	Cyp2E1 Fold change mean \pm SEM
Exp. groups			
Group I: Normal control	1.00 ^d \pm 0.09	1.00 ^a \pm 0.06	1.00 ^a \pm 0.06
Group II: DEN group	7.52 ^a \pm 0.36	0.02 ^d \pm 0.01	0.05 ^c \pm 0.005
Group III: DEN +spirullina	3.56 ^c \pm 0.15	0.57 ^b \pm 0.03	0.22 ^b \pm 0.02

Means within the same column carrying different superscript letters are significantly different at ($P \leq 0.05$).

level was significantly decreased in DEN – induced liver cancer in rats when compared with the control normal group. Spirullina platensis treatment to DEN-induced HCC significantly prevented these changes, resulting in a remarkable protection regarding the same parameters with the ability to restore the value of serum ALT, ALP, total bilirubin, albumin and AFP nearly to the average level of control group when compared with DEN-induced HCC group.

The obtained qPCR results presented in table (2) revealed a significant up-regulation of TNF- α gene expression level in liver tissue of DEN-induced liver cancer in rats. This expression was significantly downregulated after treatment with Spirullina platensis. However, a significant downregulation of p53 and Cyp2E1 gene expression levels were observed in liver of DEN induced HCC in rats as compared to the normal control group. This expression was significantly upregulated following treatment by Spirullina platensis when compared with DEN group.

4. DISCUSSION

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis (Alison, 2005). *N*-Nitrosodiethylamine (DEN) causes a wide range of tumors in all animal species and such compounds are hazardous to human health. The formation of reactive oxygen species (ROS) is apparent during the metabolic biotransformation of DEN resulting in oxidative stress. Oxidative stress leads to carcinogenesis by several mechanisms including DNA, lipid and protein damage, change in intracellular signaling pathways and even changes in gene expression (Balamurugan and Karthikeyan, 2012). Also, DEN is a potent hepatic carcinogen agent (Mahmoud and Abdul-Hamid, 2012). On the other hand, Carbon tetrachloride (CCl₄) produced hepatocellular adenomas and carcinomas in rats, mice and hamsters in oral studies and in rats and mice by inhalation exposure (Manibusan, 2010). DEN and CCl₄ is hydroxylated principally by the ethanol inducible CYP2E1 (cytochrome P450 system) in liver (Verna et al. 1996; Weber et al. 2003). The obtained results revealed that, serum ALT and ALP activities, total bilirubin and AFP concentrations were significantly elevated and serum albumin level was significantly decreased in DEN/CCl₄ – induced liver cancer in rats. DEN+CCl₄ administration induce extensive necrosis and inflammatory infiltration, clusters of hepatocytes, bile duct proliferation and marked atypia (Abd EL- Hamid et al. 2013) or caused hepatic damage by those two toxicants which reflects instability of liver cell metabolism that led to leakage of these enzymes to circulation. (Hassan et al. 2014). Similarity, Hemieda et al. (2016) showed that treatment with DENA/CCl₄ elevate the values of serum ALT, AST and ALP activities and total bilirubin level and markedly decreased

serum total proteins and albumin concentrations. Also, Hashem et al. (2016) confirmed that administration of DEN/CCl₄ significantly increased liver weight, relative liver weight, AST, ALT and ALP while, body weight, total protein, albumin and A/G ratio were markedly decreased. Furthermore, Liver is the main site of DEN metabolism, the generation of ROS in the liver is recognized as an important contributor in DEN-induced damage (Faten et al. 2014). CCl₄ is biotransformed by cytochrome P450 (CYP) enzyme system in the endoplasmic reticulum to produce trichloromethyl free radicals (CCl₃[•]). Then CCl₃[•] leads to elicitation of lipid peroxidation (LPO) and destruction of Ca²⁺ homeostasis, resulting in cell death (Talib, 2012). Moreover, Borai et al. (2017) displayed that a significant elevation in serum AST, ALT and ALP enzymes activities were observed in DEN-treated group as compared to control normal rats indicating that DEN could induce a damaged effect on liver tissues. The elevation in enzymes activities is due to the rupture in the architecture of cell membrane and the leakage and liberation of enzymes into the serum as a result of carcinogenesis, necrosis and toxicity. Also, ALP indicates alteration in biliary flow. Therefore, during carcinogenesis, these enzymes could be used as biomarkers for HCC response to therapy according to (Tork et al. 2015). Furthermore, Vandenberghe, (1996) reported that hypoalbuminemia may result from liver disorders, which are accompanied by a reduction in albumin synthesis. Albumin is a key component of serum proteins. Also, liver toxicity resulted in decrease serum albumin level (Adams et al. 2005). The results of the present study are in agreement with this finding and demonstrate the decreased functional ability of CCl₄-injected rat livers (Saravanan et al. 2006).

Meanwhile, treatment with spirulina platensis to DEN-CCl₄ induced HCC rats caused a significant decrease in serum ALT and ALP activities, and total bilirubin and increase in albumin level when compared with DEN/CCL₄ induced HCC group. These results were agreement with Salama et al. (2017) who reported that spirulina supplementation in DEN administered rats declined serum ALT and total bilirubin and significantly increased total protein. Also, a decline in serum ALT, AST and ALP activities, total bilirubin level with marked increase in serum total protein and albumin concentrations were noticed in animals receiving spirulina platensis (Amin et al. 2018). Furthermore, Prince et al. (2013) confirmed that decrease in plasma bilirubin after treatment with spirulina platensis indicated its effectiveness in maintaining the normal functional status of the liver. Moreover, Ahmed et al. (2014) reported that supplementation with spirulina versicolor significantly prevented the increase in serum liver function markers (ALT, AST, ALP and total bilirubin). Furthermore, several previous animal studies have suggested that hepatoprotective properties of spirulina are related with its antioxidant and anti-inflammatory properties (mainly due to C-phycoyanin, β -carotene, and vitamin E) and the reduction in liver lipid profile (Al-Mokaddem et al.2016).

Administration of DEN/CCl₄ significantly elevated the serum AFP level when compared to normal rats. Similar data was reported by Borai et al. (2017) and Salama et al. (2017) who recognized that AFP concentration was significantly higher in the DEN-treated group as compared to control normal one. It was reported that in DEN induced hepatocarcinogens caused elevation in AFP level (which is widely used as tumor marker for diagnosis of HCC) associated with the increment in tumor growth and progression (Murugan et al.2015). Similarity, Hashem et

al. (2016) reported that a significant increase in AFP level was shown in DEN+CCl₄ group. The increase in serum AFP concentration has been used as a clinical marker in the diagnosis and monitoring of HCC (Tork et al., 2015). Moreover, Zaazaa et al. (2018) found that an increase in serum AFP level was observed in DEN-induced HCC in rats when compared to control group. Additionally, alpha fetoprotein (AFP) is the most commonly used tumor markers for the diagnosis of hepatocellular carcinoma (HCC) which is a unique immunomodulatory glycoprotein, and normally made by the immature hepatocytes in the fetus (oncofetal). Detection of AFP during monitoring of liver cancer treatment is well accepted in patients with increased AFP level before therapy. It has been recognized that exposure of animals with DEN increases the circulating AFP level (Sadik et al. et al. 2008).

Treatment with spirulina to DEN/CCL₄ induced HCC rats caused a significant decrease in serum AFP level when compared with DEN/CCL₄ induced HCC non-treated group. The results approved by Salama et al. (2017) who indicated that supplementation with spirulina mitigate AFP near to the normal level which is indication of the chemoprevention effect of spirulina. Also, Ismail et al. (2009) stated that the potential hepatoprotective role of spirulina may be associated with its antioxidant constituents such as selenium, chlorophyll, carotene, gamma-linolenic acid, tocopherol, phenolic compounds content and vitamin E and C working individually or in synergy (Garcia-Martinez et al. 2007).

The obtained qPCR results revealed significant elevation of TNF- α gene expression level in liver tissue of DEN/CCL₄ induced HCC in rats as compared to the normal control group. Similarity, Kumar et al. (2016) found that TNF- α level significantly increased in DEN treated animals. Also,

Habib et al. (2008) demonstrated that elevation in expression of TNF- α was observed in rats with liver cancer induced by ethionine. Moreover, Song et al. (2013) showed that DEN-induced HCC increased TNF- α , such tumor necrosis factor alpha (TNF- α) is pro-inflammatory cytokines produced by macrophages and it plays an important role under tumor conditions (Lutsiak et al. 2005). It has been reported that TNF- α is an essential factor in tumor promotion (Reuter et al., 2011). Moreover, Hamid et al. (2017) stated that CCl₄ elevated proinflammatory cytokines TNF- α , IL-6, COX-2 and NF κ B. An increased level of TNF- α was also shown to correlate with hepatic inflammation, necrosis, and hepatic failure (Budhu and Wang, 2006).

On the other hand, treatment with spirulina to DEN/CCL₄ induced HCC rats caused a significant down regulation in TNF- α gene expression. The results are nearly similar with Prince et al. (2013) who found that administration of spirulina altered the levels of TNF- α in the serum and brought them near to normal levels. Also, Amin et al. (2018) reported that treatment of rats with spirulina decrease tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6). The antioxidant properties of spirulina platensis may be attributed to the presence of potent antioxidant components as β -carotene, vitamin C, vitamin E, selenium, and manganese (Mazo et al.2004). Likeness, El-Tantawy, (2015) reported that, spirulina treatment significantly reduced the elevated levels of hepatic TNF- α observed in the lead treated rats. This effect might be attributed to reduction in oxidative stress. Moreover, Spirulina inhibited the expression of inflammation associated genes (TNF- α , and IL-1 β) in LPS, and NF- κ B promoter activity are also blocked by β -carotene, it blocks nuclear translocation of the NF- κ B p65 subunit, which correlates with its inhibitory

effects on I κ B α phosphorylation and degradation (Bai et al. 2005).

The obtained qPCR results revealed significant down-regulation of p53 gene expression level in liver tissue of DEN/CCL₄ induced HCC in rats as compared to the normal control group. Similarly, Loyden et al. (2017) demonstrated that a significant reduction in p53 gene expression was observed in DEN administration group. The p53 protein acts as a central response to cellular stress or DNA damage by inducing cell cycle arrest, apoptosis and other tumor-suppressive actions (Bisteau et al.2014). Moreover, Khan et al. (2016) stated that decrease in p53 expression induced with CCl₄, which explained that CCl₄ acts as a tumor promoter through increasing the intracellular concentration of ROS necrosis/regeneration and cell proliferation and/or may be due to mutation of p53 led to regarding p53 (Farazi et al. 2006).

Treatment with spirulina to DEN/CCL₄ induced HCC rats caused a significant up-regulation in p53 gene expression when compared with DEN/CCL₄ induced HCC group. Similarly, Ying et al. (2015) reported that the induction of C-phycoerythrin which found in spirulina during the apoptosis of HEP-2 cells and found that p53 are up-regulated after treatment with C-PC, thereby promoting the signal transduction of apoptosis and eventually apoptosis. Also, Chen and Wong, (2008) showed that Selenium which found in spirulina treatment upregulated the expression of p53 genes by activating ROS-mediated mitochondrial pathway and p53 phosphorylation to induced apoptosis of A375 human melanoma cells.

The obtained results revealed significant dysregulation of Cyp2E1 gene expression level in liver tissue of DEN/CCL₄ induced HCC in rats as compared to the normal control group. Similarly, Zhang et al. (2013) found that DEN treatment resulted in

significant decreases of the activities of CYP2E1 and CYP1A2. It has been well documented that DEN-induced hepatocarcinogenesis requires metabolic activation by some forms of CYP450, especially CYP2E1. Furthermore, Khan et al. (2016) displayed that significant decrease in CYP 2E1 expression was observed in CCl₄-induced hepatotoxicity. This confirmed that Reactive oxygen species formed during the biotransformation process of CCl₄ are more reactive and toxic than the parental compound. Biotransformation of CCl₄ occurs in the endoplasmic reticulum and the isoenzyme implicated in this process is CYP2E1 (Knockaert et al. 2012).

Treatment with spirulina to DEN/CCL₄ induced HCC in rats caused a significant up-regulation in Cyp2E1 gene expression when compared with DEN/CCL₄ induced HCC non-treated group. Similarly, Savranoglu and Tumer, (2013) demonstrated that Spirulina treatment causes dose-dependent decrease in the amount of both mRNA and protein expression of CYP2E1 isoform when compared to control group. Also, Savranoglu and Tumer, (2013) displayed that spirulina treatment associate with aniline hydroxylase , CYP2E1 activity also significantly decreased in a dose-dependent manner, with nearly 45%inhibition at the highest dose.

5. Conclusion

The obtained results suggest that spirulina platensis led to improvement of liver cells and can inhibit the proliferation of HCC cells which revealed by apparent decrease in serum hepatic marker enzymes (ALT and ALP), total bilirubin and AFP with marked increase in serum albumin. Also, the results of molecular analysis showed up-regulation of TNF- α and down-regulation in p53 and CYP2E1 gene expression level in liver tissues. It could be concluded that, the chemo preventive and apoptotic effect of spirulina platensis as powerful natural agents may be

useful as a potent antioxidants, anti-inflammatory and anti-tumor activities in hepatocellular carcinoma.

6. REFERENCES

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