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HEPATOPROTECTIVE AND ANTIGENOTOXIC POTENCY OF *SPIRULINA PLATENSIS* ON CARBON TETRACHLORIDE (Ccl_4)-INDUCED LIVER FIBROSIS IN RATS

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ABSTRACT

Background: Spirulina (SP) is a commercial alga well known to contain various antioxidants, especially phycocyanin. Most of the previous reports on antioxidant activity of Spirulina were based on chemical rather than cell-based assays. The primary objective of this study was to assess the potential heptoprotective of Spirulina *Platensis* based on its antioxidant activity and anti-genotoxicity against carbon tetrachloride (CCl_4)-induced liver fibrosis in rats.

Methods:

Oral administration of carbon tetrachloride (CCl_4) (3 ml/kg CCl4 dissolved in olive oil (1:1, v/v) five days/week for 6 weeks) induced liver fibrosis in rats. Activities of liver marker enzymes; Alanine transaminase and Aspartate transaminase were estimated, as well as lipid peroxidation and antioxidant status (glutathione peroxidase) were determined in liver homogenate. DNA damage in liver was also evaluated by means of Comet assay. Comet assays and apoptotic cell studies were performed to evaluate the anti-genotoxic effect of SP.

Results: The levels of liver enzymes and lipid peroxidation were increased markedly by CCl_4, inducing DNA damage and depletion of antioxidant status. Treatment of *Spirulina platensis* (500mg/kg/5day/week/6ws) to CCl4 challenged rats resulted in decreased liver enzymes activity, DNA damage and lipid peroxidation levels with increase in antioxidant status.

Conclusion: Our study clearly demonstrates that *Spirulina platensis* showed hepatoprotective effect through its antioxidant activity on CCl4-induced liver fibrosis in rats and its anti-genotoxicity. The potential application of incorporating Spirulina into food products and beverages to enhance their antioxidant capacity and cytoprotective is worth exploring.

Keywords: Spirulina (SP), carbon tetrachloride (CCl4), hepato-protective, Comet assay

INTRODUCTION:

Liver disease is still a worldwide health problem. WHO estimated that (3% of the world's population) about 170 million people, are infected with HCV and 3-4 million persons are newly infected each year [1-3]. Prevalence rates vary widely, ranging from 0.15% in Scandinavia to about 15% in Egypt [4]. Chronic liver infection manifested in about 80% of newly infected patients, cirrhosis progress in (10% to 20%) of those with chronic infection, and over a period of 20-30 years, liver cancer in (1% to 5%) of persons with chronic infection [5]. Furthermore, there is no clear evidence as to whether treatment reduces the risk of liver related morbidity or mortality [6].

Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects. In the absence of a reliable liver protective drug in modern medicine there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders [7]. Many herbal products (Glycyrrhizin, Silymarin, Curcumin) established and confirmed hepatoprotective activity and showed benefits in the treatment of viral hepatitis [8-12].

Spirulina is a blue-green alga (cyanobacterium) that has been consumed as food in many countries; it is presently marketed as a food supplement (nutraceutical) due to its high contents of proteins, g-linolenic acid, vitamins and minerals [13].

Spirulina had been found to possess immune-stimulating and antiviral activities in animals and human volunteers. Spirulina activate macrophages, NK cells, T cells, B cells, and stimulate the production of antibodies and cytokines. [14-18].

A natural sulfated polysaccharide (Calcium spirulan; Ca-SP), isolated from Spirulina platensis had been demonstrated to be a potent inhibitor against several enveloped viruses. Ca-SP was shown to target for both viral absorption/ penetration stages and some replication stages of progeny viruses after penetration into cells [19, 20].

CCl4 produces an experimental damage that histologically resembles viral hepatitis [21]. The toxic metabolite CCl4 radical is produced which is further converted by cytochrome P450 2E1 enzyme to trichloromethylperoxy radical, which binds covalently to the macromolecules producing peroxidative degradation of cellular membrane leading to the necrosis of hepatocytes [21]. Interestingly, the oxidative DNA damage of CCl4 was evaluated by means of the comet assay, which is widely used in genotoxicity testing *in vitro* and also becoming an important tool for assay the genotoxic potential and mutagenicity of many chemicals and natural compounds *in vivo* where as it play important roles in the determination of DNA damage level [22].

To the best our knowledge, there are few published data on genoprotective activity of Spirulina, therefore the aim of the present work was to investigate the potential heptoprotective of Spirulina *Platensis* based on its antioxidant activity and anti-genotoxicity against carbon tetrachloride (CCl4)-induced liver fibrosis in rats, a different approach for evaluation the possible genoprotective effect of SP on DNA damage. For this purpose, the comet assay was used for measuring DNA damage.

MATERIALS AND METHODS:

The protocol of the experiments was approved by College Research Ethics Committee.

Animals:

Swiss Albino rats weight between 150 to 200 gm were obtained from the Medical Experimental Research Centre (MERC) in Mansoura Faculty of Medicine to be used throughout the present study. During the experimental period these rats were kept in a well-ventilated animal house and under the control managerial and environmental conditions. These animals were divided in five groups of ten animals each. CCl4 (3 ml/kg body weight) was administered orally to rats groups (IV and V) which is well documented to induce hepatic fibrosis in rats. The duration of the experiment was five days/week for six successive weeks.

Group I: This group received normal saline (0.9%NaCl) orally served as negative control.

Group II: Animal in this group received olive oil (1.5 ml/kg body weight/day) orally.

Group III: Each animal received orally Spirulina platensis (500 mg/kg body weight / 0.5 ml drinking water).

Group IV: This group received orally CCl4 (3 ml/kg body weight/day) diluted with olive oil (1:1) as a solvent for the CCl4.

Group V: Animals were pretreated with Spirulina (500 mg/kg body weight/ 0.5 ml drinking water) orally 30 min before the oral CCl4 (3 ml/kg body weight/day).

At the end of experimental period, rats were slightly anaesthetized by diethyl ether (Sigma Chem. Co., St Louis, Mo. U.S.A.) and liver was carefully excised from each rat and immediately immersed in a saline solution (0.9% NaCl). Liver homogenates (10%) were prepared in 0.01M Tris-HCl buffers (pH 7.5). The homogenate were centrifuged at 4000 r.p.m for 15 min, and the resultant supernatants were frozen at -20°C for hepatic parameters assay.

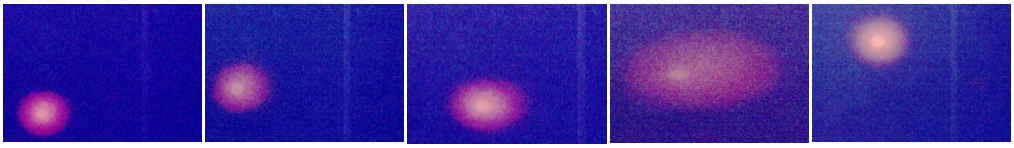
Biochemical Assays: Assessment of aspartate transaminase (AST) and alanine transaminase (ALT), alkaline phosphatase (ALK), and gamma glutamyl transpeptidase (GGT) calorimetrically using diagnostic kits purchased from Bio- Diagnostic, Egypt.

Biomarkers of Oxidative Stress: Activities of superoxide dismutase (SOD), catalase (CAT), reduced Glutathione (GSH), and Glutathione peroxidase (GPx) was assayed using kit purchased from Bio- Diagnostic, Egypt (CAT NO. GP 2524).

Comet assay

The single-cell gel electrophoresis (comet assay) allows detection of DNA fragmentation in single cells, and was initially used for DNA damage estimation [23]. Slides were prepared in duplicate and 100 cells were screened per sample (50 cells from each slide) in a fluorescent microscope (ZEISS, Germany). According to the migration of the fragments ,the nucleus was classified visually into : class 0 (no damage); class 1 (little damage with a short tail length smaller than the diameter of the nucleus); class 2 (medium damage with a tail length one or two times the diameter of the nucleus); class 3 (significant damage with a tail length between two and a half to three times the diameter of the nucleus); class 4 (significant damage with a long tail of damage greater than three times the diameter of the nucleus) figure 1. Representative Comet images of isolated human lymphocytes in different groups.

Statistical analysis: was performed using the Kruskal–Wallis One-way Method of Variance [six independent experiments were analyzed] and followed by Student–Newman–Keuls considering a confidence interval of 95%.



Control Negative **Olive oil** **SP** **CCL_4** **CCL_4+ SP**

RESULTS:

As shown in Table 1, activities of AST, ALT,ALK, and GGT were markedly elevated in CCl4 treated animal groups compared to control group, indicating liver injury by CCl4.Moreover, administration of *Spirulina* at 500 mg/kg body weight, significantly ($p<0.05$) lowered the elevation of enzymes induced by CCl4 in relation to control group.

Antioxidant enzyme activities of liver are presented in table 2. SOD, CAT, GSH, and GPx activities were significantly decreased ($P<0.01$) in the CCL_4 treated rats, as compared with normal control, which were found to attain a near normal level in CCL_4 + SP treated group.

Evaluation of DNA Damage (Comet Assay)

Liver DNA damage was revealed by various comet assay parameters that were provided by the image analysis software including tail length, % of DNA in the tail and tail moment (Table 3, Fig.1). Histogram indicated that tail length for control was 1.86± 0.7 µm then it statistically significant increased ($p<0.05$) for group treated with CCL4 and reached a maximum length (6.18±0.4µm). There were no statistical differences in tail length between oil group and Spirulina group compared to control. The percentage of tail DNA reflecting the proportion of DNA that has migrated from the head. The mean percentage of tail DNA in control is 0.418±0.1% then significant increased ($p<0.05$) was observed for groups 4 and 5.As illustrated the mean tail moment (whose magnitude reflects the frequency of DNA strand breaks per nucleus) for all groups compared to control. The obtained result of this parameter indicated the same phenomena for tail length that Spirulina reduced the degree of damage induced by CCL4.

Table 1: Effect of carbon tetrachloride on activity of liver enzymes in liver tissue homogenate

Groups	AST	ALT	ALP	GGT
Control	36.7±3.6	31.5±3.1	86.7±4.3	4.6±0.3
Olive oil vehicle	42.7±6.3[b]	44.9±12.8[b]	98.8±6.9[b]	6.1±.9[b]
SP	48.7±8.3[b]	51.9±14.6[b]	94.8±10.9[b]	5.8±1.4[b]
Ccl_4 6weeks	248.7±18.7[a]	311.5±28.5[a]	341.6±29.8[a]	29.8±1.9[a]
Ccl_4 + Sp 6 weeks	86.7±7.7[b]	104.9±11.6[b]	112.8±9.9[b]	8.9±1.3[b]

●The results are presented as means ± SE. Level of significance is at P<0.05.

●Oral of CCl4 (3 ml/kg /5 days/ a week for 6 week

●The symbol 'a' represent significance at P < 0.05, where a = compared with control group

●The symbol 'b' represent significance at P < 0.05, where b = compared with Ccl_4 treated group

Table 2: Effect of carbon tetrachloride (Ccl_4) and spirulina (Sp) on activity of antioxidant enzymes in liver tissue homogenate

Groups	SOD	CAT	GPX	GR	
Control	5.8±0.8	56.4±5.8	77.3±8.7	155.9±12.6	
Olive oil vehicle	6.2±0.6[b]	55.2±2.6[b]	73.8±9.3[b]	149.7±11.3[b]	
SP	5.6±0.6[b]	54.2±3.7[b]	79.8±9.2[b]	147.6±12.3[b]	
Ccl_4 6weeks	3.6±0.3[a]	28.6±5.7[a]	45.7±7.4[a]	78.5±6.8[a]	
Ccl_4 + SP6 weeks	5.7±0.6[b]	42±4.6[b]	71.8±8.2[b]	145.8±13.3[b]	

●The results are presented as means ± SE. Level of significance is at P<0.05.

●Oral of CCl4 (3 ml/kg /5 days/ a week for 6 week

●SOD Superoxide dismutase, CAT Catalase, GPX Glutathione peroxidase, GR Glutathione reductase

●SOD (U per mg protein) CAT (U per mg protein) GPX (U per mg protein) Gr (U per mg protein)

●The symbol 'a' represent significance at P < 0.05, where a = compared with control group

●The symbol 'b' represent significance at P < 0.05, where b = compared with Ccl_4 treated group

Table 3: Oxidative DNA damage in the liver cell bearing rats in different groups

Group	Tail length µm	% DNA	Tail moment unit
Control	1.86± 0.7	0.418±0.1	0.673±0.04
Olive	1.34±0.5	0.71±0.4	1.356±.05
SP	2.12±0.5	0.967±0.6*	1.924±1.48
Ccl_4 6weeks	6.18±0.4[a]*	2.971±0.5 [a]*	17.445±2.23 [a]*
Ccl_4 + SP 6weeks	3.21±0.6[b]*	1.916±0.4[b]*	5.41902±1.57[b]*

●The results are presented as means ± SE. Level of significance is at P<0.05.

** Statistical significant*

●The symbol 'a' represent significance at P < 0.05, where a = compared with control group

●The symbol 'b' represent significance at P < 0.05, where b = compared with Ccl_4 treated group

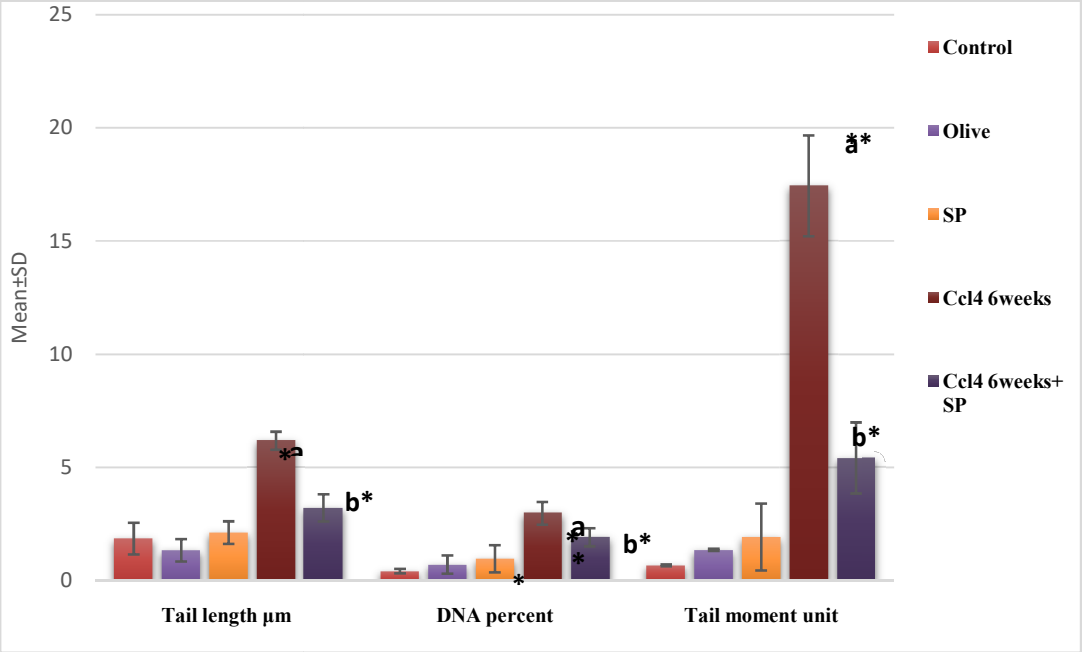


Figure 1: Comet assay parameters (tail Length um, DNA percent, Tail moment unit) for all tested groups
*** Statistical significant**
●The symbol 'a' represent significance at P < 0.05, where a = compared with control group
●The symbol 'b' represent significance at P < 0.05, where b = compared with Ccl_4 treated group

DISCUSSION

Liver is considered key organ of metabolism and it is constantly talented with the task of detoxification of xenobiotic, environmental pollutants and chemotherapeutic agents are constantly donated by liver. Toxic liver fibrosis induced by CCI4- which is well known model for hepatic fibrosis has been extensively performed [24]. CCl4 through lipid peroxidation, oxidative stress of liver cells and consequently decrease in the antioxidant ability of the cells caused an aggressive cellular damage in those cells in which destruction of membranes occurred and enzymes were released from damaged cells [25].

So, the present study designed to investigate the potential hepato-protective activity of *Spirulina* against CCl4 induced liver fibrosis. Liver damage induced by CCl4, was estimated by enzyme levels such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALK),and gamma glutamyl transpeptidase (GGT) and it is largely used, that has been attributed to the damaged structural integrity of the liver, because these are normally located in the cytoplasm and are released into the circulation after cellular damage [26]. Chaung *et al*., [27] demonstrated that one of the indices of the degree of cell membrane damage is ALT enzyme, whereas the

indicators of mitochondrial damage is AST, because mitochondria contain 80% of the enzyme.

In the present study, the rise in the levels of ALT, AST, ALK and GGT in CCl4 treated rats are in agreement with the results of [28] and [29].

Moreover, administration of *Spirulina* at 500 mg/kg body weight in the present study, significantly ($p<0.05$) lowered the elevation of enzymes induced by CCl_4. Bhat and Madyastha [30] reported that phycocyanin (the blue pigment) in *Spirulina* reduced the hepatotoxicity caused by CCl_4 – induced free radicals, also this may attributed to the inhibition of reaction involved in the formation of reactive metabolites and its radical scavenging activity. In addition to immune-stimulant activities of SP due to high content of β-carotene, enzyme superoxide dismutase, vitamins or selenium in *Spirulina* producing cyto-protective effects against CCl4 – induced liver damage [31]. Data in Table 2signify increased oxidative stress by CCL_4;itrevealed thatSOD, CAT, GSH, and GPx activities were significantly decreased ($P<0.01$) in the CCL_4 treated rats, which were found to attain a near normal level in CCL_4 + SP treated group.

It is widely accepted that the induction of antioxidant enzymes are a major strategy for protecting cells against a variety of endogenous and exogenous toxic compounds such as ROS and chemical carcinogens [31]. Murugesh *et al.,* [31] indicated that liver antioxidant enzymes including the above mentioned enzymes; SOD, CAT, GSH, and GPx are thought to be the fundamental antioxidant enzymes, for they are closely related to the direct elimination of reactive oxygen species. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. *Spirulina* treatment showed hepato-protective effect as demonstrated by enhanced activities of antioxidant enzymes. Most of the active constituents present in the *Spirulina* have been determined to scavenger hydroxyl and superoxide radicals, and to increase the antioxidant enzymes [33]. So it is reported that some of the active constituents of *Spirulina*such as flavonoids, β-carotene and phycocyanin possess strong antioxidant activity and induce free radical scavenging enzyme system [34].

Figure 1 indicated that *Spirulina* reduced the degree of DNA damage induced by CCL4. The present result was in parallel to Kaji [35] who reported that the polysaccharide content of SP enhanced significantly both the repair

activity of damaged DNA excision and the unscheduled DNA synthesis. Also Bhat and Madyastha [30] found that phycocyanin and phycocyanobilin contents of SP possess strong anti-cyclooxygenase II, antioxidant activity to scavenger peroxidinitrite and reduce OONO-induced oxidative damage to DNA. Most damage is repaired by effective DNA-repair enzymes, but some damage escapes repair, causing permanent damage [36].

In conclusion, Spirulina seems to maintain the hepatocellular membrane structural integrity as evident from the significant reduction in CCl4-induced rise in liver enzymes and increase in hepatic antioxidants activities. So, our results provide strong evidence that Spirulina possess hepato-protective effect through its strong antioxidant activity, and reduction the degree of DNA damage. It is recommended to supplement SP in the diet concurrently with potential genotoxic drugs to exert its beneficial effects. Additional researches are needed to study the possible additional desired effects of SP.

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