**Hepatoprotective Effect Induced by NaCl-Stressed *Spirulina platensis*: Histopathological, Biochemical and Histochemical Studies**

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**Abstract:** Cyanobacterium *Spirulina platensis* was subjected to different NaCl concentrations (0.02, 0.03, 0.07, 0.14 and 0.27 M). The obtained results showed successive increases in the values of different measured growth parameters (dry weight, total soluble protein and pigment content) up to 0.14 NaCl, thereafter they decreased. The pellets of 0.02M NaCl (control) and 0.14M treated cells were dried and used in suspensions as oral doses for the experimental rats. The hepatoprotective effect of the two *Spirulina platensis* suspensions were studied on paracetamol-induced liver damage in rats by monitoring serum transaminases, alkaline phosphatase, total protein, total bilirubin, histopathological and histochemical alterations. These observations were comparable to the group pretreated with silymarin. Treatment with *Spirulina platensis* suspensions and silymarin has brought back the altered levels of biochemical markers to the near normal levels. The prevention of histological and histochemical deterioration was also evidenced. It was found that *Spirulina* suspension 1 (cultivated under stress conditions) has more hepatoprotective effect than *Spirulina* suspension 2 (cultivated under control conditions). It was concluded that *Spirulina* possess hepatoprotective effect against paracetamol-induced liver injury and stressed *Spirulina* gave better results than normal *Spirulina*.

**Key words:** Stress *Spirulina* · NaCl · Hepatoprotective · Paracetamol biochemistry · Histopathology · Histochemistry

**INTRODUCTION**

*Spirulina* (SP), a blue-green alga, is popularly used as a nutritional supplement as well as in therapeutic applications [1]. *Spirulina* contains proteins, lipids, carbohydrates, some vital minerals, vitamins including β-carotene and a pigmented protein, C-phycocyanin [2]. *Spirulina* is known for its wide-range biological activities and antioxidant properties [3-5], anti-inflammatory [6], antimutagenic [7, 8], antiviral [9, 10], immune enhancing [3, 11], cardioprotective [12, 13] anticancer properties [14, 15] and protection against cisplatin-induced nephrotoxicity [16]. Moreover, hypcholesterolemic effects have been reported in some animal studies [17]. Several reports have indicated that *Spirulina* has a protective effect against many toxicants including mercury [18], D-galactosamine and acetaminophen [19], copper toxicity [20] and CCl₄ toxicity [21], neuroprotection against iron-induced toxicity in SH-SY5Y neuroblastoma cells [22]. On the other hand, *Spirulina platensis* play a role in reducing the toxic effect of cadmium in rats [23] preventing the bioaccumulation of Cd and reversing the Cd-induced oxidative tissue damage in broiler chicken [24].

The aim of the present study is to detect the hepatoprotective role of stressed *Spirulina* against paracetamol-induced liver toxicity.

**MATERIALS AND METHODS**

**Organism:** *Spirulina platensis* (*Arthrospira platensis*) was obtained from the culture collection of Botany Department, Faculty of Science, Mansoura University, Egypt. The medium used for the growth of the Cyanobacterium was Zarrour’s medium [25].

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Culture Technique for NaCl-Stressed *Spirulina platensis*: Different concentrations of NaCl (0, 0.02, 0.03, 0.07, 0.14 and 0.27 M) were added to Erlenmeyer flasks (250 ml) contained 150 ml of Zarrouk's medium stoppered with cotton plugs and sterilized in an autoclave at 1.5 atmosphere for 20 min. After cooling, the Erlenmeyer flasks were inoculated with initial 0.2 O.D (the best optical density obtained in our preliminary experiment) of the pre-culture flasks and incubated at 28°C under continuous fluorescent light of 48.4 μmol m⁻² s⁻¹. The culture flasks were aerated with sterile air mixed with 3% CO₂ to accelerate the cyanobacterial growth; the maximum growth of organism was attained at the 9 day.

**Determination of Growth Parameters**

**Optical Density:** The growth of the cyanobacterium was determined by measuring the optical density of the algal suspension at 560 nm as recommended by Fatma *et al.* [26].

**Dry Weight:** A definite volume of cyanobacterial suspension (20 ml) was centrifuged at 6000 rpm for 10 min. The cells after being precipitated were washed twice with distilled water to eliminate the salts, dried overnight in an oven at 70°C and kept in desiccators for 20 min for cooling and weighed. The data were given as g/100 ml algal suspension [27].

**Estimation of Pigments:**

- Chlorophyll *a* and carotenoids were determined according to the spectrophotometric method recommended by Mckinney [28].
- Phycobiliproteins contents were determined according to the method described by Bennett and Bogorad [29].

**Estimation of Total Soluble Proteins:** After pigment extraction, the cyanobacterial cells were extracted with 1N NaOH in a boiling water bath for 2 h as described by Payne and Stewart [30]. Total soluble proteins were quantitatively determined using the method described by Bradford [31].

**Hepatoprotective Effects of Spirulina**

**Chemicals:** Paracetamol was purchased from Pharco Pharmaceuticals Company, Alexandria, Egypt as tablets (each tablet contained 500 mg of paracetamol under the trade name of paracetamol. Silymarin was purchased from Medical Union Pharmaceuticals Company, Abou Sultan, Ismailia, Egypt as a gelatinous capsule (each capsule contains 140 mg of Silymarin) under the trade name of Hepaticum.

**Animals:** Female rats (Lab Animal House, National Research Center, Cairo, Egypt) of weight 100-120 g were used in this study. The animals were housed in groups of 6 rats in steel cages at 22±2°C with a 12 h light/dark cycle and allowed to acclimatize for a period of 1 week prior to experimental use. Throughout the experiment, the rats were allowed free access feed (rats dietary pellets prepared by Cairo Company of Oil and Soap, Egypt) and water. Experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals and approved by the Ethical Committee of National Research Centre.

**The Experimental Design:** Rats were divided into eight groups, six rat of each. The experimental design was as follows:

**Group 1:** (control): animals were given saline (1 ml/rat) for one week.

**Group 2:** (stressed *Spirulina*): animals given oral dose (1 g/kg b.wt) of *Spirulina* suspension 1 for 7 days.

**Group 3:** (normal *Spirulina*): animals given oral dose (1g/kg b.wt) of *Spirulina* suspension 2 for 7 days.

**Group 4:** Animals given oral dose of silymarin (100 mg/kg b.wt) for 7 days.

**Group 5:** Animals received water for 7 days prior to a single dose of paracetamol (2.5 g/kg b.wt) on the eighth day [32].

**Group 6, 7 and 8:** Rats were administered as groups 2, 3 and 4 and on the eighth day were given a single dose of paracetamol (2.5 g/kg b.wt).

At the end of each treatment, blood samples were obtained in dry tube and left to clot, then centrifuged at 3000 rpm for ten min. Serum was separated and freezeed at -20°C for biochemical analysis. After blood samples were taken, the animals were sacrificed by mild ether anesthesia and liver were obtained and prepared for histopathological and histochemical examinations.
Biochemical Analyses: Glutamate oxaloacetate transaminase (GOT) and Glutamate pyruvate transaminase (GPT) activities were determined according to the method of Reitman and Frankel [33]. Serum alkaline phosphatase (ALP) activity was determined according to calorimetric method of Kind and King [34]. The method described by Jandrusik and Grof [35] was applied for determination of total bilirubin. Serum total protein was determined according to the method described by King and Woolton [36].

Histopathological and histochemical Studies: Liver slices were fixed in 10% formal saline for 24 h. Sections of 6 μm thicknesses were prepared and stained with Haematoxylin and Eosin [37].

Periodic acid Schiff method [38] was applied for visualization of the polysaccharide materials. Mercury-bromophenol blue method [39] was applied for the demonstration of the total proteins. Feulgen reaction [38] was applied for the demonstration of DNA.

Statistical Analysis: All the results obtained in this study were analyzed using analysis of variance (ANOVA) in addition to Student t test in the biochemical results using state software. A P-value of less than 0.05 was taken as statistically significant.

RESULTS AND DISCUSSION

Effect of NaCl Stress on the Growth Parameters of Spirulina platensis: Results presented in Table 1 showed that successive increase in NaCl concentration caused successive increase in dry weight content and growth rate up to 0.14 M NaCl, above this concentration both values were decreased. The highest dry weight value (0.29 g/ml) and growth rate (0.2933 cd⁻¹) were obtained at this concentration (0.14 M NaCl). The same trend was observed in the values of total soluble protein content where the maximum content (496 mg/g dry weight) was obtained at the same concentration. Results in Table 2 showed appreciable increase in chlorophyll a and carotenoids content in response to the successive increase in NaCl concentrations in the culture medium. The pigment contents reached their maximum values at 0.14 M NaCl (8.3 mg/g dwt for chlorophyll a and 3.1 mg/g for carotenoids), above this concentration the values were reduced. The same trend was observed with phycoerythrin, the results showed that the optimum content of (e-phycoerythrin, allophycocyanin and phycoerythrin) was attained at 0.14M NaCl.

Our results are in agreement with the results of Abd El-Baky et al. [40] who studied the blue-green alga Spirulina platensis that grown in batch culture at different NaCl concentrations. This species was found to respond to high NaCl level by accumulation of large amounts of commercially important chemicals such as carotenoids and tocopherols. Vonskak et al. [41] studied the response of Spirulina platensis cells to salinity stress. The authors found that Salt-adapted cells have a modified biochemical composition, reduced protein and chlorophyll content and an increased level of carbohydrates. However, Martel et al. [42] found that S. platensis could be adapted to salinity-stress by increasing carbohydrate metabolism in cells. The reduction in the different growth parameters induced by high concentration of NaCl may be due to photoinhibition as suggested by Zeng and Vonskak [43] who mentioned that salinity stress enhances photoinhibition of photosynthesis through a direct effect on PSII reaction center). Much evidence has verified that the reaction center cores both in photosystem II (PSII) and photosystem I (PSI) suffered structural damage including protein degradation during photoinhibition of

<table>
<thead>
<tr>
<th>NaCl concentration (M)</th>
<th>Dry weight (g/100 ml)</th>
<th>Growth rate (cd⁻¹)</th>
<th>Total soluble protein (mg/g dry wt.)</th>
<th>Protein%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.15±0.01</td>
<td>0.10±0.001</td>
<td>436±1</td>
<td>43.6</td>
</tr>
<tr>
<td>0.02 (control)</td>
<td>0.21±0.02</td>
<td>0.26±0.01</td>
<td>451±1</td>
<td>45.1</td>
</tr>
<tr>
<td>0.03</td>
<td>0.24±0.03</td>
<td>0.27±0.001</td>
<td>466±2</td>
<td>46.6</td>
</tr>
<tr>
<td>0.07</td>
<td>0.27±0.01</td>
<td>0.28±0.001</td>
<td>481±1</td>
<td>48.1</td>
</tr>
<tr>
<td>0.14</td>
<td>0.29±0.01</td>
<td>0.29±0.002</td>
<td>496±1</td>
<td>49.6</td>
</tr>
<tr>
<td>0.27</td>
<td>0.28±0.03</td>
<td>0.29±0.001</td>
<td>486±2</td>
<td>48.6</td>
</tr>
<tr>
<td>F value</td>
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<td>905.541</td>
<td>1550</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>0.000***</td>
<td>0.000***</td>
<td>0.000***</td>
<td>-</td>
</tr>
<tr>
<td>LSD</td>
<td>0.0177</td>
<td>0.00744</td>
<td>1.7789</td>
<td>-</td>
</tr>
</tbody>
</table>

Each value is the mean of three reading ± standard deviation
***Highly significant at P< 0.001, **Significant at P<0.01, *Low Significant at P< 0.05
photosynthesis [44-46]. According to the above mentioned results we used the 0.14 M treated S. platensis culture to study the possible therapeutic effect of this organism (stressed and normal one) against paracetamol induced hepatotoxicity in rats. The results show that stressed Spirulina platensis have a significant hepatoprotective role against paracetamol-induced liver injury as compared with the normal Spirulina platensis.

**Hepatoprotective Effects of Spirulina**

**Biochemical Analysis:** The biochemical analyses showed a significant increase in GOT, GPT, ALP and total bilirubin in rats treated with paracetamol. However, a significant decrease of total protein levels in rats treated with paracetamol (Table 3). This elevation in the serum level of hepatic enzymes GOT, GPT and ALP reflected the hepatocellular damage in the paracetamol-induced hepatotoxicity. These results are in agreement with the results of Vandenberghhe [47], Rara et al. [48] and Balamurugan [49] who stated that the extent of paracetamol induced hepatoxic effect is assessed by the level of released cytoplasmic enzymes-ALP, AST and ALT in circulation. When Paracetamol was taken at toxic doses, it becomes a potent hepatotoxin, generating fulminated hepatic and renal tubular necrosis, which is lethal in humans and experimental animals [50, 51]. Treatments with silymarin decreased serum AST, ALT and ALP and restored their values to near normal condition, which indicates the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by paracetamol (Table 3). Our results are in accordance with the findings of Tabassum et al. [52] who reported significant increase in ALT due to paracetamol administration in albino mice. Many studies support our results, which demonstrated the beneficial hepatoprotective effects when treated with silymarin [53-57]. The hepatoprotective activity of silymarin has been attributed to antioxidant and membrane stabilizing activities of the toxicant agents.

The pretreatment with Spirulina suspension significantly attenuated the elevated levels of the serum markers caused by paracetamol (Table 3). Spirulina seems to preserve the structural integrity of the hepatocellular membrane as evident from the significant reduction in the paracetamol-induced rise in serum enzymes. The decreased serum enzymes may be due to the prevention of leakage of the intracellular enzymes by its membrane stabilizing activity. It has been reported that Spirulina
possess strong antioxidant and free radical scavenging properties [14, 21, 58]. The protective effect of *S. platensis* against paracetamol-induced oxidative can be attributed to the high levels of antioxidants such as vitamins, carotenoids and phyocyanin [59].

**Histopathological Study:** The microscopic examination of the liver tissue in the control, *Spirulina* suspensions 1 (cultivated under salinity stress), *Spirulina* suspensions 2 (cultivated under control NaCl) and silymarin groups showed normal hepatic structure (Figure 1A, B, C and D), respectively. The histopathological observations of the liver of paracetamol-treated rats showed hydropic degeneration of the hepatocytes and loss of cell boundaries and ballooning degeneration (Fig. 2A). In some rats focal necrosis that associated with infiltration of lymphocytes was present (Fig. 2B). On the other hand, portal and sinusoidal dilation and congestion, were seen. Some hepatocytes showed nuclear pyknosis and karyolysis (Fig. 2C). Mitchell [60] support our results in which Paracetamol produced centrilobal hemorrhagic hepatic necrosis if an overdose is consumed. Paracetamol is metabolized to a minor electrophilic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which during paracetamol overdose depletes glutathione and initiates covalent binding to cellular proteins and initiates cell damage [61].

Silymarin-treated rats showed normal histological appearance of the liver (Fig. 2D). In general, antioxidant properties of silybin have been observed in various cellular models, including liver cells [62, 63]. The pharmacological properties of silymarin involve the regulation of cell membrane permeability and integrity, inhibition of leukotriene, reactive oxygen species scavenging, suppression of NF- B activity, depression of protein kinases and collagen production [64]. Histopathological examination of rats treated with *Spirulina* suspension (1 and 2) showed the normal structure of the hepatocytes (Fig. 2 E and F). The protective effect of *Spirulina* against paracetamol induced hepatotoxicity in rats is due to the anti-inflammatory and oxidative properties of its constituent
Fig. 3: Photomicrographs of liver stained with PAS (X 150), A) control rat shows the PAS-positive glycogen particles accumulated at one side of the cytoplasm of hepatocytes (arrows), B) rat received Spirulina suspension 1 shows normal distribution of the PAS-positive glycogen particles in hepatocytes, C) rat received Spirulina suspension 2 shows normal distribution of the PAS-positive glycogen particles in the hepatocytes, D) rat given silymarin shows normal glycogen content in the hepatocytes.

Fig. 4: Photomicrographs of a liver stained with PAS (X 150), A) rat received paracetamol shows depletion of the PAS-positive material in the hepatocytes, B) rat received Spirulina suspension (1) and given paracetamol shows normal distribution of the PAS-positive glycogen particles within the hepatocytes (arrows), C) rat received Spirulina suspension (2) and given paracetamol shows normal distribution of the PAS-positive materials within the hepatocytes (arrows), D) rat given silymarin and paracetamol shows normal distribution of the PAS-positive materials inside the hepatocytes.

Fig. 5: Photomicrographs of liver sections stained with Bromphenol blue (X300): a control (A), Spirulina suspension 1-treated (B), Spirulina suspension 2-treated (C) and silymarin-treated (D) rats show the protein content in the form of irregular particles of various sizes distributing equally in the cytoplasm of the liver cells. The nucleoli are intensely-stained while the ground cytoplasm and nucleoplasm display faint stainability.

Fig. 6: Photomicrographs of liver sections stained with Bromphenol blue (X300): (A) rat received paracetamol shows diminution in the protein content (arrows), (B) rat received Spirulina suspension 1 and paracetamol shows normal distribution of the protein content, (C) rat received Spirulina suspension 2 and paracetamol shows normal distribution of the protein content, (D) received silymarin and paracetamol shows the proteinic content appearing more or less like normal control.
Fig. 7: Photomicrographs of liver sections stained with Feulgen stain (X300): (A) control rat shows normal DNA content in the nuclei of the hepatocytes (reddish color), (B) rat received Spirulina suspension 1 (C) rat received Spirulina suspension 2, (D) rat given shows normal DNA content in the nuclei of the hepatocytes (reddish color)

Fig. 8: Photomicrographs of liver sections stained with Feulgen stain (X300): (A) rat received paracetamol shows a decrease in the DNA content in the nuclei, (B) rat received Spirulina suspension (1) and paracetamol, (C) rat received Spirulina suspension (2) and paracetamol shows normal DNA content in the nuclei, (D) rat given silymarin and paracetamol shows normal DNA content in the nuclei

phycocyanin. Phycocyanin, a biliprotein found in Spirulina, exerts a scavenging action against reactive oxygen species (ROS) as well as anti-inflammatory activity demonstrated in various in vitro and in vivo models [65, 66]. Spirulina has antioxidant and anti-inflammatory agent such as β-carotene, phenolic compounds [3, 59].

**Histochemical Examinations:** In the present study, liver sections of animals of control, Spirulina suspension 1, Spirulina suspension 2 and silymarin showed the normal distribution of polysaccharides (Fig. 3A, B, C, D), protein (Fig. 5A, B, C and D) and DNA (Fig. 7A, B, C and D). Histochemical examination showed that paracetamol treated rats indicated reduction in the glycogen (Fig. 4A), protein (Fig. 6A) and DNA (Fig. 8A) contents of the hepatocytes. The necrotic hepatocytes showed a complete depletion in the glycogen, protein and DNA contents. Our results are in accordance with the findings of Shen et al. [67] and Lawson et al. [68]. The authors stated that in both in vivo and in cultured hepatocytes, liver damage paracetamol overdose may also determine a recognizable fragmentation of nuclear DNA and karyolysis. However, rats treated with silymarin reduced the depletion of the contents of glycogen (Fig. 4B), protein (Fig. 6B) and DNA (Fig. 8B) in the liver cells that induced by paracetamol. These results are in agreement with those obtained by Sonnenbichler et al. [69] in which Silybin has been reported to stimulate RNA polymerase I and consequently ribosomal RNA and protein synthesis, both in the liver and in cultured hepatocytes. The Hepatoprotective role of silymarin may be attributed to that silymarin can potentiate doxorubicin cytotoxicity by inhibiting Pglycoprotein–mediated drug efflux [70]. Silymarin increases the activity of antioxidant enzymes, superoxide dismutase and glutathione peroxidase [71]. The histochemical examinations of rats pretreated with Spirulina (1 and 2) indicated more or less normal abundance of glycogen particles (Fig. 4C and D), protein (Fig. 6C and D) and DNA (Fig. 8C and D) of the hepatocytes, this may be due to that Spirulina possesses antimutagenic properties and may be considered as an effective natural antimutagen agent [72, 73]. The hepatoprotective effect of phycocyanin could be due to the inhibition of some of the cytochrome P450 mediated reactions involved in the formation of reactive metabolites or its ability to act as an efficient radical scavenger or both [74]. The components: vitamin E, phycocyanin, enzyme superoxide dismutase, among other, present in the Spirulina platensis confer great antioxidant activity [75]. The results of Karadeniz et al. [23] are in accordance to our results in which S. platensis and P. ginseng treatments showed marked decrease in lipid peroxidation and increase of endogenous antioxidants levels.
CONCLUSION

All the biochemical, histopathological and histochemical results indicated that *Spirulina* suspension 1 (stressed) has more hepatoprotective effect than *Spirulina* suspension 2 (normal). This may be related to the fact that the first suspension is under stress conditions (0.14 M NaCl) this concentration gave the maximum growth, protein and pigmentation from the NaCl stress experiment result obtained before but suspension 2 is under the control conditions. This may indicate that salinity stress induce more production of the secondary metabolites which have the protective action against hepatotoxicity.

REFERENCES


