

Hepatoprotective and antioxidant potential of *Spirulina fusiformis* on acetaminophen-induced hepatotoxicity in mice

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Abstract

In the present study, we have evaluated the hepatoprotective and antioxidant effects of *Spirulina fusiformis* (a cyanobacterium-family-Oscillatoriaceae) against acetaminophen-induced hepatotoxicity in mice. For comparison purpose, results were compared with those for silymarin, a standard hepatoprotective drug. Activities of liver marker enzymes (glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, and alkaline phosphatase) and inflammatory mediator tumour necrosis factor-alpha (TNF- α) were estimated in serum, while lipid peroxidation and antioxidant status (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase and glutathione) were determined in liver homogenate. Acetaminophen induction (900mg/kg b.wt) significantly increases the levels of liver marker enzymes, TNF- α , and lipid peroxidation, and caused the depletion of antioxidant status. Treatment of *Spirulina fusiformis* (800mg/kg/b.wt) to acetaminophen challenged mice resulted in decreased liver marker enzymes activity, TNF- α and lipid peroxidation levels with increase in antioxidant status. Our study clearly demonstrate that *Spirulina fusiformis* shows hepatoprotective effect through its antioxidant activity on acetaminophen-induced hepatotoxicity in mice.

Keywords: Blue green algae, Lipid peroxidation, Liver marker enzymes, Tumour necrosis factor- α , Mice.

INTRODUCTION

Acetaminophen (also known as paracetamol) is a commonly used, effective analgesic and antipyretic agent for relief of mild and moderate pain. However, either deliberate overdose or accidental overdose can cause hepatotoxicity (Cover *et al.*, 2005). Acetaminophen (AAP) hepatotoxicity is currently the single most important cause for acute liver failure in the US, and is associated with a significant number of deaths (Bjornsson and Olsson, 2006). An overdose of the analgesic drug, acetaminophen (AAP) can lead to severe liver injury in humans and in experimental animals. Although studied intensely for more than 25 years, the mechanism of this injury is still not entirely clear. Key features of the toxic mechanism include the

formation of a reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is quickly conjugated by hepatic glutathione to yield a harmless product called mercapturic acid. However, after acetaminophen overdose, the capacity for glucuronidation, and sulfation is exceeded with the formation of excess NAPQI via cytochrome *P*-450 2E1. After glutathione is depleted, excess NAPQI binds to hepatic cell proteins and DNA which precedes liver injury (Jaeschke *et al.*, 2003). A conventional hepatoprotective drug used for the treatment of such adverse reactions are often inadequate and presently de-challenge of the offending drug is recommended. Therefore, efforts to explore hepatoprotective effect of any natural products against acetaminophen-induced hepatotoxicity carry a great clinical significance.

Spirulina is blue green algae of the Oscillatoriaceae family which grows naturally in countries which have a warm climate and has been considered as supplement in human and animal food (Rasool *et al.*, 2006). They have been found to be a rich source of vitamins,

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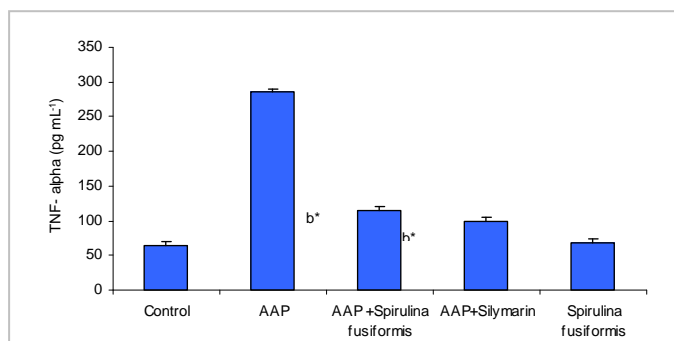


Figure 1: Effect of *Spirulina fusiformis* on the levels of pro-inflammatory cytokine tumour necrosis factor- α in the serum of control and experimental animals.

Each value represents the mean \pm S.D of 6 mice. Comparisons were made as follows: a* - control vs. acetaminophen (AAP); b*- AAP vs. *Spirulina fusiformis* (800mg/kg/b.wt.) + AAP; Silymarin (25 mg/kg/b.wt.) + AAP. The symbols represent statistical significance at: * $p < 0.05$. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.

minerals, essential fatty acids and antioxidant pigments such as carotenoids (Seshadri *et al.*, 1991).

In addition, several studies have shown that *Spirulina* species exhibit various biological activities such as antitumour (Mittal *et al.*, 1999), antimicrobial (Hayashi *et al.*, 1996), strengthening immune system (Qureshi *et al.*, 1996), metalloprotective (Sharma *et al.*, 2007), and anti-arthritic effect (Rasool *et al.*, 2006). However, the hepatoprotective effect of *Spirulina fusiformis* against acetaminophen-induced hepatotoxicity has not yet been reported to the best of our knowledge. This study was, therefore, undertaken to examine the hepatoprotective effect of *Spirulina fusiformis* on acetaminophen-induced hepatotoxicity in mice. For comparison purpose, results were compared with those for silymarin, a standard hepatoprotective drug.

MATERIALS AND METHODS

Animals

Swiss albino mice, 25-30g, of either sex were obtained from the Tamil Nadu Veterinary College, Chennai, India. They were acclimatized for a week in a light and temperature -controlled room with a 12 hr dark-light cycle and fed with commercial pelleted feed from Hindustan Lever Ltd. (Mumbai, India) and water was made freely available. The animals used in this study were treated and cared for in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Culture, Chennai, India.

Drug and Chemicals

The commercially available *Spirulina fusiformis* (a fine dark blue-green spray-dried powder) was obtained from, RECON Ltd, Bangalore, India and was dissolved in 2% gum acacia solution to give aqueous suspension. This aqueous suspension of *Spirulina fusiformis* was used as an oral dose of 800 mg/kg/b.wt, orally. Silymarin, a standard hepatoprotective drug (25mg/kg/b.wt) obtained from the SRS Pharmaceuticals, Mumbai, India was administered intraperitoneally. Acetaminophen, thiobarbutric acid, 5, 5'-Dithiobis-p-nitrobenzoic acid, reduced glutathione, and bovine serum albumin were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used were standard laboratory reagents of analytical grade and purchased locally.

Experimental protocol

In this experiment, mice were randomly allocated into 5 groups, each consisting of six animals. All animals were made to fast 24 h before the experiment. The first group, the control group, received saline. The second group, acetaminophen group, was treated with a single dose of acetaminophen (900 mg/kg/b.wt. i.p.) (Goksel Senker *et al.*, 2006). Acetaminophen was first dissolved in water at 70° C, and then cooled to 37° C before administration. The 3rd, group, (*Spirulina fusiformis* + acetaminophen) were given *Spirulina fusiformis* extract (800mg/ kg/b.wt.) orally 30 mts after the single injection of acetaminophen (900 mg/kg/b.wt. i.p.). The 4th group (Silymarin+ acetaminophen) was given Silymarin (25 mg/kg/b.wt.i.p.) 30 mts after the single injection of acetaminophen (900 mg/kg/b.wt. i.p.). The fifth group, the *Spirulina fusiformis* group, received *Spirulina fusiformis* (800mg/kg/b.wt) orally suspended in 2% gum acacia. The mice were decapitated at 4 h after acetaminophen injection; the trunk blood was collected, the serum was separated and stored at -70° C. Tissue samples from the liver were obtained for biochemical analysis.

Biochemical Parameters

The activities of alkaline phosphatase, alanine and aspartate aminotransferases in serum were estimated by using commercial kits (Span Diagnostics, India). In the hepatic tissue samples, lipid peroxidation was determined by the procedure of Ohkawa *et al* (1997). Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of oxidative stress. Superoxide dismutase was assayed according to the method of Marklund *et al* (1974). The unit of enzyme activity is defined as the enzyme required to give 50% inhibition of pyrogallol auto-oxidation. Catalase (CAT) was assayed by the method of Sinha (1972). In this method, dichromate in acetic

Table 1: Effect of *Spirulina fusiformis* on liver functional markers in acetaminophen-intoxicated mice in serum.

Parameter	Control	Acetaminophen (900mg/kg/b.wt)	Acetaminophen+ <i>Spirulina fusiformis</i> (800mg/kg/b.wt.)	Acetaminophen+ Silymarin (25mg/kg/b.wt.)	<i>Spirulina fusiformis</i> (800mg/kg/b.wt.)
Alanine transaminase (U/dl)	81.22 ±4.10	172.46± 5.48 a*	95.38±4.41a* b*	94.21±4.61 b*	85±6.53
Aspartate transaminase (U/dl)	43.2±6.16	91.1±4.08 a*	55.45±6.86 a*b*	50.23±5.7 b*	45.5±11.2
Alkaline phosphatase (K.A.units/l)	114.28±4.71	316.95±15.3a*	122.59±5.78 a*b*	120.27±4.13 b*	122.33±11.2

Table 2: Effect of *Spirulina fusiformis* on liver antioxidant enzymes-specific activities and lipid peroxidation levels in acetaminophen-intoxicated mice

Parameter	Control	Acetaminophen (900mg/kg/b.wt)	Acetaminophen + <i>Spirulina fusiformis</i> (800mg/kg/b.wt.)	Acetaminophen + Silymarin (25 mg/kg/b.wt.)	<i>Spirulina fusiformis</i> (800mg/kg/b.wt.)
Lipid peroxidation	1.49±0.59	2.9±0.28a*	1.62±0.04b*	1.62±0.89b*	1.54±0.70
Superoxide dismutase (SOD)	3.2±0.33	1.37±0.16a*	2.855±0.27b*	2.78±0.22b*	3.21±0.17
Catalase (CAT)	51.42±1.16	38.25± 0.61a*	46.92± 0.74b*	47.25± 0.94b*	47.42± 1.39
Glutathione peroxidase (GPx)	24.67 ± 0.61	19.72± 0.52a*	23.67± 0.61b*	23.83± 0.26b*	24.0± 0.32
Glutathione reductase (GR)	20.67± 0.69	15.53± 0.33a*	18.9± 0.37b*	18.92 ±0.59b*	18.32 ± 0.37
Glutathione-S-transferase (GST)	105.25±7.6	80.83±2.40a*	123.0±19.4b*	120.50±11.4b*	113.1 ±11.4
Total reduced glutathione	29.8±0.75	14.75±0.52a*	25.25±0.75b*	26.41±1.43b*	29.33±1.29

acid was reduced to chromic acetate when heated in the presence of H₂O₂, with the formation of perchloric acid as an unstable intermediate. Chromic acetate thus produced was measured colorimetrically at 610 nm. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al.* (1973) based on the reaction between glutathione remaining after the action of GPx and 5,5'-dithiobis-(2-nitrobenzoic acid) to form a complex that absorbs maximally at 412 nm. Glutathione reductase (GR) that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was assayed by the method of Bellomo *et al.* (1987). Glutathione-S-transferase (GST) was assayed by the method of Habig *et al.* (1974). Total reduced glutathione (GSH) was determined by the method of Moron *et al.* (1979). The protein content was determined by the method of Lowry *et al.* (1979) using bovine serum albumin as a standard.

Effect of *Spirulina fusiformis* and Silymarin on TNF- α Production

TNF- α level in serum of control and experimental mice were determined by enzyme-linked immunosorbent

assay kit specific for mouse (ELISA, Cayman Chemicals, USA), according to the manufacturer's instructions.

Statistical Analysis

Results were expressed as mean \pm S.D. and statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student's Newman-Keul's test. P < 0.05 implied significance.

RESULTS

Table 1 shows the activities of alanine transaminase, aspartate transaminase, and alkaline phosphatase in serum of normal, acetaminophen control and treated groups. The activities of alanine transaminase, aspartate transaminase, and alkaline phosphatase in serum were significantly increased in acetaminophen control group compared to normal control group. The levels of the above enzymes were significantly reversed on treatment with *Spirulina fusiformis*.

Table 2 shows the effect of *Spirulina fusiformis* on MDA (index of tissue lipid peroxidation), superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase and glutathione in liver of control and experimental mice. In acetaminophen treated mice, MDA level was increased significantly; whereas superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase and glutathione were found to be decreased when compared to the control group. The administration of *Spirulina fusiformis* to acetaminophen induced mice altered the above changes by regulating the MDA level and anti-oxidant enzymes to nearly that of normal levels.

Fig. 1 shows the levels of pro-inflammatory cytokine tumour necrosis factor- α in the serum of control and experimental animals. The level of tumour necrosis factor- α in the acetaminophen treated mice were systemically overproduced in the serum, while the elevated level of tumour necrosis factor- α was found to be decreased in *Spirulina fusiformis* administered mice treated with acetaminophen.

DISCUSSION

In the assessment of liver damage by paracetamol, the determination of enzyme levels such as SGPT and SGOT is largely used. In the present study, the rise in the serum levels of alanine transaminase, aspartate transaminase and alkaline phosphatase in acetaminophen treated mice 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 (Sallie *et al.*, 1991). *Spirulina fusiformis* seems to preserve the structural integrity of the hepatocellular membrane as evident from the significant reduction in acetaminophen-induced rise in serum enzymes in mice. The decreased serum enzymes level in acetaminophen-induced liver damage by *Spirulina fusiformis* may be due to the prevention of leakage of the intracellular enzymes by its membrane stabilizing activity, which was supported by the limited extent of histological changes (Sabina *et al.*, personnel communication). This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew *et al.*, 1987).

It is established that covalent binding of N-acetyl-p-benzoquinoneimine, an oxidation product of paracetamol, with sulphhydryl groups of protein results in cell necrosis and lipid peroxidation in the liver (Lin *et al.*, 1997). In addition, NAPQI can increase the formation of reactive oxygen species and reactive nitrogen species such as superoxide anion, hydroxyl radical, and

hydrogen peroxide, nitric oxide and peroxynitrite, respectively. Excess levels of reactive oxygen and nitrogen species can attack biological molecules such as DNA, protein, and phospholipids, which leads to lipid peroxidation, nitration of tyrosine and depletion of the antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) that further results in oxidative stress (Hinson *et al.*, 2002). In accordance with previous findings, in our study we observed a high lipid peroxidation with a concomitant decrease in the enzymic antioxidant status including glutathione in the hepatic tissue during acetaminophen toxicity. Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidants present in the liver. Its functions are concerned with the removal of free radical species such as hydrogen peroxide and superoxide radicals (Prakash *et al.*, 2001). In our present study, the decreased level of glutathione has been observed in acetaminophen treated mice, whereas its level was significantly found to be increased in *Spirulina fusiformis* treated acetaminophen-induced mice. Superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-s-transferase 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 (Muruges *et al.*, 2005). In our study, *Spirulina fusiformis* treatment was observed to exhibit hepatoprotective effect as demonstrated by enhanced activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase), glutathione and diminished amount of lipid peroxide against the acetaminophen-induced hepatotoxicity animals. *Spirulina fusiformis* has been reported to be a rich source of Vitamin C and E, β -carotene, enzyme superoxide dismutase, selenium and brilliant blue polypeptide pigment phycocyanin. Several investigators have reported that β -carotene of spirulina may reduce cell damage, especially the damage to DNA molecules, thus playing the role in the repair of regeneration process of damaged cells (Luxia *et al.*, 1996). Most of the active constituents present in the *Spirulina fusiformis* have been reported to be a potent inhibitor of lipid peroxide formation, a scavenger of hydroxyl and superoxide radicals, and to increase the antioxidant enzymes (Kulkarni *et al.*, 1994; El-Demerdash, 2001; Sharma *et al.*, 2007; Bhat *et al.*, 2000). Thus the modifying role of *Spirulina fusiformis* observed in our study may be due to the antiperoxidative action of its components that was reported earlier.

Excess production of acetaminophen metabolite causes the initial hepatic damage and subsequent activation of inflammatory mediator TNF- α , which in turn contribute to tissue necrosis (Blazka *et al.*, 1995). In accordance to

this report, our results demonstrate that acetaminophen increases serum TNF- α , indicating the role of this cytokine in acetaminophen induced hepatotoxicity. However, *Spirulina fusiformis* treatment significantly reduced the elevated TNF- α level in acetaminophen challenged mice.

In conclusion, our results provide strong evidence that *Spirulina fusiformis* has hepatoprotective effect upon acetaminophen-induced hepatic damage in mice and possessed anti-peroxidative activity. However, further pharmacological evidences at molecular level are required to establish the mechanism of the action of the drug which is underway.

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