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Protective role of food supplement *Spirulina fusiformis* in chemical induced hepatotoxicity: A Bromobenzene model in rats

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Abstract

The present study evaluated the efficacy of *Spirulina fusiformis* in protecting against chemical induced hepatotoxicity in rats using bromobenzene as the candidate toxin. A single oral dose of bromobenzene (BB) (10mmol/kg b.w.) resulted in significant ($p< 0.05$) decrease in antioxidant levels (catalase, superoxide dismutase, glutathione-s-transferase, glutathione peroxidese, total reduced glutathione), total protein and significant ($p< 0.05$) increase in the levels of serum bilirubin, liver marker enzymes (alanine transaminase, aspartate transaminase and alkaline phosphatase) indicating the induction of hepatotoxicity. *Spirulina fusiformis* (400 mg/kg b.w) was orally administered for 8 days prior to the administration of BB and was seen to protect the above parameters from significant alterations upon challenge with bromobenzene. This was also confirmed by the histological examination of liver tissues. The protective effect of *Spirulina fusiformis* was comparable to that of the standard hepatoprotective drug silymarin.
Keywords: Bromobenzene, Hepatotoxicity, *Spirulina fusiformis*, Antioxidants, Hepatoprotective.

1. Introduction

The chemical challenges to the human body are numerous and can have multiple deleterious effects. The liver is the primary organ tasked with the detoxification of chemicals that find their way into the body through ingestion, inhalation, injection or dermal contact. These chemicals may be from the environment or drugs that are administered to the body. Liver damage can be caused by toxic metabolites of such chemicals and poses a significant health risk. Bromobenzene (BB), an aryl halide is such a harmful chemical and has been found in the environment at low concentrations. It serves as an additive in motor oil and as a crystallizing solvent in various industrial processes. Its molecular formula is C6H5-Br and is a colorless liquid with a pungent odor[1]. Ingestion through food or occupational exposure or dermal contact leads to metabolism of BB in liver thereby causing hepatotoxicity. In the liver, BB undergoes hydrolysis by cytochrome P450 monooxygenases which mediates the production of a highly electrophilic compound, bromobenzene 3,4-epoxide. The enzyme glutathione-s-transferase catalyzes the sequestration of the reactive epoxides through its conjugation to glutathione. When the toxin dose is high, conjugation to the metabolites results in depletion of the hepatic GSH pool, thereby intracellular protection against reactive oxygen species and hazardous xenobiotic metabolites are lost. This leads to a various secondary events like elevated lipid peroxidation levels, local inflammation, ATP depletion, mitochondrial dysfunction, energy imbalance and loss of intracellular calcium store[2].

In comparison with synthetic drugs, naturally available drugs and food supplements provide treatment options with less likelihood of adverse effects. Therefore the exploration of naturally available compounds and products for the treatment of various diseases including hepatotoxicity is of particular interest.

Spirulina, a blue green algae of Oscillatoriaceae family is gaining more attention due to its high protein and vitamin contents, natural bio chelating properties, and especially the presence of β carotene a powerful antioxidant. Many studies have shown the strong antioxidant and free radical scavenging properties of *Spirulina* species in addition to its strong chelating effect[3]. These features can be attributed to the high levels of antioxidants present in *Spirulina* such as Vit B1 and Vit B2, carotenoids and pycocyanin[4]. *Spirulina fusiformis* possesses anticancer, antiviral, anti tumour, antimicrobial[5], anti arthritic, metalloprotective[6] radio protective effect and antioxidant potential[7]. Several investigators have reported that β-carotene of *Spirulina* tend to reduce cell damage, especially damage occurring to DNA molecules, thus playing a role in damaged cells repair and regeneration process[8]. The present study was carried out to evaluate the hepatoprotective effect of *Spirulina fusiformis* on hepatotoxicity induced by bromobenzene in female wistar albino rats. Silymarin, a standard hepatoprotective drug was used for comparison purpose. The integrity of the hepatocytes by studying the liver marker enzymes and their antioxidant status were analyzed.

2. Materials and methods

2.1 Animals: Female wistar albino rats of weight ranging from 120- 150 grams were selected for the study. They were obtained from animal house, VIT University, Vellore. Animals were maintained under standard laboratory conditions of temperature 27˚C with a 12 hour dark-light cycle. The rats were fed with commercially available pelleted feed from Hindustan Lever Ltd. (Mumbai, India), and water was made freely available. The experimental procedure was approved by the ethical committee (VIT/IAEC/VII th/17) of VIT University, Vellore, India.

2.2 Toxin and Drug: Commercially available *Spirulina fusiformis* was obtained from Acumen pharmaceuticals, Pondichery, India and an aqueous suspension was made in double distilled water and was given orally. Silymarin a standard hepatoprotective drug obtained from Microlabs Ltd (Goa, India) was administered orally after making an aqueous suspension. Bromobenzene was obtained from Sigma Aldrich, India.

2.3 Experimental design: Female wistar albino rats were divided into 5 groups with each group consisting of 6 animals. Animals were orally administered daily for 8 days. Group I, control rats received only coconut oil (0.1 ml) by intragastric intubation. Group II, bromobenzene (10 mmol in 0.1 ml coconut oil) was administered on the 8th day by intragastric intubation. Group III, *Spirulina fusiformis* (400mg/kg/b.wt) was given orally for 8 days followed by bromobenzene (10mmol in 0.1 ml coconut oil) by intragastric intubation on 8th day. Group IV, silymarin (25mg/kg/b.wt) was given orally for 8 days followed by bromobenzene (10mmol in 0.1 ml coconut oil) given intragastrically on the 8th day. Group V *Spirulina fusiformis* extract (400 mg/kg) was administered orally for 8 days. During the course of the experiment, animals were weighed daily. After 19 hours of the last dosage, the rats were decapitated and trunk blood was collected. Tissue sample from liver were obtained for biochemical and antioxidant studies.

The trunk blood collected in the clean dry test tube was allowed to clot followed by centrifuging at 3000 rpm for 10 min at 4°C for serum separation. Separated serum was stored at -70°C for performing liver function tests and serum protein. Plasma sample was obtained by collecting trunk blood in a tube containing EDTA. Liver tissues were homogenized in 5% Phosphate buffered saline (PBS) solution. The homogenate was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant obtained was used for the estimation of antioxidants and enzyme studies. A portion of liver was fixed in formalin for further histopathological studies.

2.4 Biochemical parameters: The levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total and direct bilirubin, in serum were estimated by using commercial kits to the protocol given by manufacturers. plasma ceruloplasmin[9], lipid peroxidation[10], superoxide dismutase (SOD)[11], catalase[12], glutathione peroxidase (GPX)[13], total reduced glutathione (GST)[14], and total protein[15] levels were determined in both plasma and liver tissue homogenate.

2.5 Hisptopathological studies: The livers from 5 groups were sliced and fixed in 10% buffered neutral formalin solution. Following fixation, liver tissue slices were processed using an automated tissue processor and was embedded in wax. The sections were cut to a thickness of 5 µm using Leica microtome RM 2155 and were subjected to Haematoxylin and Eosin staining. The slides prepared were proceeded to histopathological examination using light microscope attached to a digital camera at a magnification of 125x.

2.6 Statistical analysis: All data was expressed as mean ± SD and statistical analysis was performed using ANOVA to determine the significant differences between the groups. It was followed by Student Newman Keul's test: $P<0.05$ was considered statistically significant.

3. Results

In bromobenzene intoxicated group of animals, weight of the liver was significantly ($P<0.05$) increased but it was reversed to near normal values by treatment with *Spirulina fusiformis* and silymarin (Table 1).

Table 1: Effect of administration of bromobenzene on liver weight with or without prior administration of *Spirulina fusiformis* in control and experimental rats.

Groups	Liver weight(g)
Group I (Control)	5.19±0.76
Group II (Bromobenzene-10mmol/kg)	6.34±0.26a*
Group III (*Spirulina fusiformis*-400/kg +Bromobenzene- 10mmol/kg)	5.26±0.28b*
Group IV (Silymarin-100mg/kg + Bromobenzene- 10mmol/kg)	5.54±0.38b*
Group V(*Spirulina fusiformis* -400mg/kg)	5.23±0.27b*

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V; b- group II vs group III, IV, V. The symbols represent statistical significance at *$p < 0.05$. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

Table 2: Effect of administration of bromobenzene(BB) on aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), direct and total bilirubin with or without prior administration of *Spirulina fusiformis* in control and experimental rats

Parameters	Group I (Control)	Group II (BB-10mmol/kg)	Group III (*Spi*-500mg/kg +BB)	Group IV (Silymarin-25mg/kg +BB)	Group V(*Spi*-500mg/kg)
AST (U/L)	85.83±2.35	233.62±3.89a*	140.68±4.91a*b*	91.86±4.14a*b*	100.48±6.69a*b*
ALT(U/L)	58.93±4.19	124.19±1.65 a*	62.17±1.56 b*	54.84±1.29 b*	59.91±2.87 b*
ALP(U/L)	94.80±2.76	217.88±9.95 a*	114.22±4.05 a*b*	139.15±2.91 a*b*	102.64±5.71 a*b*
Total Bilirubin (mg/dl)	0.80±0.05	4.069±0.08a*	1.08±0.11b*	0.827±0.04a*b*	0.701±0.03b*
Direct Bilirubin (mg/dl)	0.64±0.03	2.36±0.05 a*	0.62±0.04b*	0.41±0.04 b*	0.44±0.03 b*

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V; b- group II vs group III, IV, V. The symbols represent statistical significance at *p < 0.05. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test.

Table 2 shows the activities of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) in serum of the animals. The activities of these liver enzymes were significantly ($p<0.05$) increased in BB treated groups when compared to the normal control group indicating injury to the liver. In contrast, pre treatment with *Spirulina fusiformis* and silymarin exhibited significantly decreased levels of these enzymes indicating that they counteract the hepatotoxic effect of BB. In BB treated group, there was a significant ($p<0.05$) increase in total bilirubin and decrease in total protein content. These effects were also reversed by pre-treatment with *Spirulina fusiformis* & silymarin (Table 2).

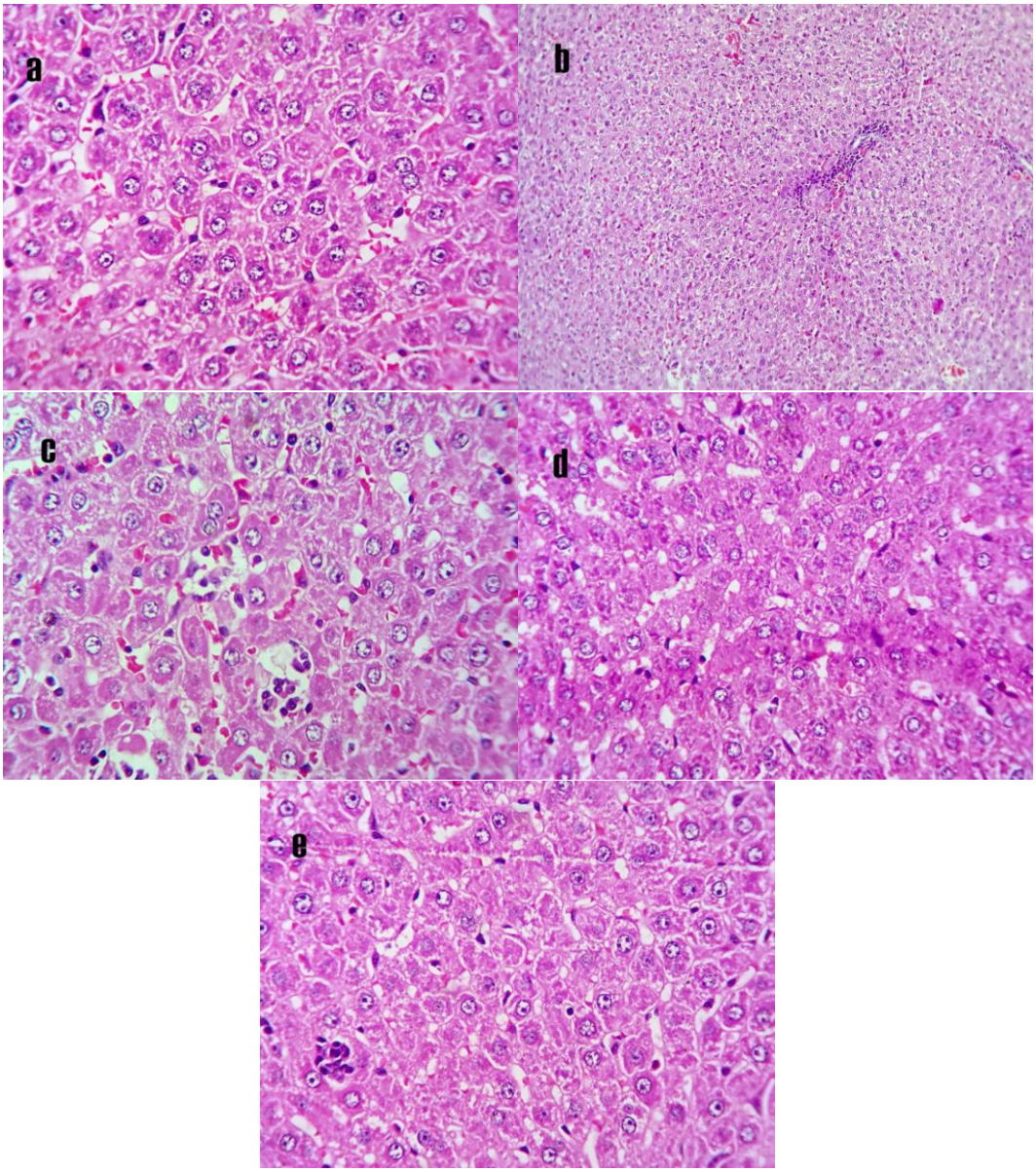
Table 3: Effect of administration of Bromobenzene(BB) on antioxidant status, Lipid peroxidation and plasma ceruloplasmin with or without prior administration of *Spirulina fusiformis* (Spi) in Plasma and Liver homogenate of control and experimental rats

Parameters	Group I	Group II	Group IV	Group V	Group VI
	(Control)	(BB 10mmol/kg)	(*Spi*-400mg/kg +BB)	(Silymarin-25mg/kg +BB)	(*Spi*-400mg/kg)
Liver					
Catalase (Units/min/mg protein)	62.23±0.95	23.68±0.43a*	58.27±2.09b*	56.43±4.37b*	56.82±3.21b*
SOD (U/mg protein)	65.76±6.26	13.85±1.16a*	65.1±5.04 b*	62.52±4.02 b*	66.80±3.62 b*
Lipid Peroxidation (nmol/mg protein)	1.18±0.07	2.25±0.05a*	1.41±0.01b*	1.36±0.06b*	1.30±0.01b*
GST (nmol/minmg protein)	20.16±0.08	6.05±0.1a*	20.03±0.21b*	18.25±0.19b*	20.39±0.12b*
Reduced Glutathione (mmol/mg protein)	7.67±0.66	3.97. ±0.54a*	6.98±0.59b*	7.54±0.53b*	7.86±0.48b*
Glutathione Peroxidase (µg of GSH utilized/min/mg protein)	29.69±0.20	18.35±0.67a*	27.78±0.24b*	24.28±0.15b*	26.96±0.72b*
Plasma					
Catalase (Units/min/mg protein)	63.89±4.67	41.85±1.76a*	64.53±2.05b*	62.47±2.23b*	54.25±0.63a*b*
SOD (U/mg protein)	58.62±6.18	12.53±2.52a*	63.75±2.35b*	56.78±1.46b*	58.45±2.47b*
Lipid Peroxidation (nmol/mg protein)	2.51±0.01	4.99±0.02a*	2.61±0.01b*	2.58±0.01b*	2.34±0.01b*
GST (nmol/min mg protein)	18.37±0.03	7.51±0.021a*	17.64±0.16b*	16.29±0.16b*	16.15±0.14b*
Reduced Glutathione (mmol/mg protein)	17.16±0.18	6.12±0.647a*	16.16±0.32b*	14.62±0.15b*	14.72±0.17b*
Glutathione Peroxidase (µgof GSH utilized/min/mg protein)	30.42±1.15	20.43±0.97a*	27.75±1.34b*	29.67±1.25b*	29.67±1.58b*
Plasma ceruloplasmin (g/l)	0.69±0.05	1.79±0.27a*	0.67±0.12b*	0.59±0.13b*	0.68±0.23b*

Each value represents the mean ± SD of six rats. Comparisons were made as follows: a-group I vs groups II, III, IV, V; b- group II vs group III, IV, V. The symbols represent statistical significance at *p < 0.05. Statistical analysis was calculated by one-way ANOVA followed by the Student Newman–Keul's test

Table 3 shows the effect of BB, *Spirulina fusiformis* and silymarin on activities of the antioxidants SOD, catalase, glutathione peroxidase, glutathione-s- transferase, and reduced glutathione in both liver and plasma. The levels were found to be decreased in BB treated rats when compared to normal control group, indicating that the antioxidants are being used in counteracting the toxin. It is known that as the innate antioxidant mechanism counteracts the toxic metabolites of BB it leads to their depletion. The pre-treatment of BB treated rats with *Spirulina fusiformis* reversed the antioxidant levels to near normal levels and was comparable to the effect of the standard drug silymarin. In BB treated group, lipid peroxidation and plasma ceruloplasmin levels were found to be high compared to the normal group (Table 3). Its levels were reduced nearly to normal on action with Spirulina and silymarin.

Fig 1(a) Control, normal liver parenchyma.(b) Toxin, Congestion of portal vessels, hepatic sinusoids and central vein with periportal chronic inflammatory infiltrates.(c) Toxin + drug, congestion of central vein, hepatic sinusoids and portal vessels with minimal periportal inflammation.(d) Toxin+ reference drug, normal liver parenchyma with minimal congestion.(e) Drug, normal liver with mild periportal inflammation.



Histopathological examination of liver tissues were performed and the results correlated with the biochemical findings. Microscopic observation revealed a normal histology of liver tissue with regular morphology in the control group, Group I (Fig 1a). Group II, bromobenzene treated group (Fig 1b) showed congestion of portal vessels and central vein with hepatic sinusoids showing an increase in kupffer cells and presence of periportal chronic inflammatory infiltrates. Group III, those pre-treated with *Spirulina fusiformis* (Fig 1c) showed minimal periportal inflammation and congestion of central vein, hepatic sinusoids and portal vessels. Group IV, Silymarin pre-treated group (Fig1d) showed minimal congestion with normal appearing liver parenchyma. Group V, those treated with *Spirulina Fusiformis* alone (Fig 1e) showed normal liver with mild periportal inflammation.

4. Discussion

It is known that upon entry into the liver BB is metabolized by the Cyt P450 system to BB 3,4 epoxide which then binds to GST thereby lowering its levels. BB is known to cause acute glutathione depletion and reduction of GSH levels in liver and thus intracellular protection against reactive oxygen species and hazardous xenobiotic metabolites is lost. This leads to cell damage like lipid peroxidation and ATP depletion. This has been clearly replicated in the BB treated rats in our current study as well. This rise in the serum levels of SGOT, SGPT and ALP in bromobenzene treated rats indicates damage to the structural integrity of the liver. These marker enzymes are normally located in the cytoplasm and following

cellular damage they are released into the circulation. The damage to structural integrity and the induction of inflammation is also indicated by the rise in the liver weight in the BB treated group along with the concomitant increase in the levels of bilirubin. It is also known that BB treatement causes protein degradation in the subjects. All these characterisitics make BB an ideal chemical for use as a candidate toxin in the study of hepatotoxicity. *Spirulina fusiformis* has been studied for its hepatoprotective potential against toxins such as cadmium and acetaminophen. The dual nature of *Spirulina fusiformis* as both a drug and a food supplement help in making it an ideal candidate for the treatment of hepatotoxicity due to various chemicals. As the pathways of toxicity of various chemicals overlap it shall also be suggested that the hepatoprotective, metal chelating and antioxidant roles of food supplements such as *Spirulina fusiformis* shall also be effective in treating hepatotoxicity due to various chemicals.

It is seen that the liver marker enzyme levels in serum have decreased in the group pretreated with *Spirulina fusiformis*. This may be due to the prevention of leakage of these intracellular enzymes due to its membrane stabilizing activity. The limited extent of histological changes and the decrease in the average liver weight in *Spirulina fusiformis* pre treated groups supports the findings. Lipid peroxidation is one of the important causes of BB induced liver injury which is mediated by the free radical derivatives of BB. Antioxidant activity and the prevention of free radical generation are important for protecting the liver from toxin induced damage. Thus antioxidant enzymes are important in detoxifying xenobiotics by catalyzing their conjugation with reduced glutathione. Hepatic glutathione has an important relationship with lipid peroxidation, as it can bind with free radicals such as hydrogen peroxide and super oxide radicals that may initiate peroxidation. Increased levels of GSH provides a protective response against the toxic effects of chemicals, particularly those which are involved in oxidative stress. A notable characteristic of BB intoxication is that lipid peroxidation develops only when GSH value reaches a threshold. Supporting evidences were derived from previous observations which relate glutathione depletion by toxin intoxication to lipid peroxidation and necrosis. Chronic intoxication of BB resulted in significant depletion of GSH level in liver, whereas its level was found to be significantly ($P<0.05$) reversed to near normal in *Spirulina fusiformis* treated BB induced rat.

The antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-s-transferase are related to direct elimination of reactive oxygen species. BB intoxication results in decreasing antioxidant enzyme levels due to the effect of BB epoxides on the antioxidant system, but these levels were found to be restored on pretreatment with *Spirulina fusiformis*, resulting in reduced levels of lipid peroxidation. This suggests that *Spirulina fusiformis* may be able to protect against the hepatocellular membrane damage through its free radical scavenging property. Many of the active constituents present in *Spirulina fusiformis* such as phycocyanins, phycobilins were reported to be potent inhibitor of lipid peroxidation and scavengers of hydroxide and superoxide radicals thereby increasing the antioxidant enzyme. Thus the protective role of *Spirulina fusiformis* seen in the study may be due to the antiperoxidative action of its components.

The reference drug silymarin is an already proven drug for its antioxidant activity as it scavenges the free radicals that induce lipid peroxidation and influence the enzyme system that is associated with GSH and SOD. In conclusion, strong evidence is provided from the results on hepatoprotective effect of *Spirulina fusiformis* upon bromobenzene induced liver damage, and this is suggested to be through inhibiting CYP dependent monooxygenases activity and enhancing the activity of hydrolase enzyme which detoxifies the toxic epoxide intermediate of BB produced by CYP mediated metabolism in rats and possessed anti per oxidative activity. However further evidences from molecular level studies are required to establish the exact drug mechanism. The levels of various cytokines and mRNAs of proteins involved in antioxidant mechanism need to be measured. A working hypothesis on the protective role of *Spirulina fusiformis* needs to be propounded. This will help in assessing the role of *Spirulina fusiformis* as a hepatoprotective food supplement against chemical inducing hepatotoxicity.

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