

Evaluation of Protective Effects of Water Extract of *Spirulina platensis* (blue green algae) on Cisplatin-Induced Lipid Peroxidation

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Attempt has been made to evaluate free radical scavenging activity of water extract of *Spirulina platensis* on cisplatin-induced lipid peroxidation using some common laboratory markers. In this present study goat liver has been used as lipid source. This *in vitro* evaluation was done by measuring the malondialdehyde, 4-hydroxy-2-nonenal, reduced glutathione and nitric oxide content of tissue homogenates. The results suggest that cisplatin could induce lipid peroxidation to a significant extent and it was also found that water extract of the algae has the ability to suppress the cisplatin-induced toxicity.

Key words: Lipid peroxidation, *Spirulina platensis*, cisplatin, malondialdehyde, reduced glutathione, 4-hydroxy-2-nonenal, nitric oxide.

Spirulina also called arthospira is a microscopic and filamentous cyanobacterium (blue green algae) that has a long history of use as food¹⁻³. *Spirulina* is 50-70% protein by weight and contain a rich source of vitamins especially vitamin B₁₂, β-carotene (provitamin A), vitamin E. It also contains carbohydrates like rhamnose, fructose, ribose, mannose and some minerals like copper, magnesium, zinc, potassium and iron. Beside γ-linolenic acid (GLA), it also contains a host of other phytochemicals that have potential health benefits⁴⁻⁵. *Spirulina* contains phycocyanin (7% dry weight basis) and polysaccharides, both of them have antioxidant properties. They have a direct effect on reactive oxygen species. It also contains an important enzyme superoxide dismutase (1700 units/g) that acts indirectly by slowing down the rate of oxygen radical generating reactions⁵. It has been reported that spirulina has a protective role in cisplatin-induced nephrotoxicity in rats⁶. It was found that besides antioxidant effects spirulina had properties like immunomodulation effects⁷, anticancer effects⁸.

Cisplatin [cis-diaminedichloroplatinum(II)] is one of the widely used antineoplastic drugs. However it has

major side effects such as acute tubular necrosis⁹. It has also strong side effects on gastrointestinal tract¹⁰. It has been found that cisplatin impairs the respiratory function and DNA of mitochondria in renal proximal tubules and small intestinal mucosal cells, thereby inducing apoptosis of epithelial cell¹⁰. It has been suggested that cisplatin induced toxicity is closely associated with an increase in lipid peroxidation¹¹. Lipid peroxidation is oxidative deterioration of polyunsaturated lipid that occurs through free radical chain reaction¹². Free radicals are generated inside the body and cause several damages to vital cellular organs. To control and reduce lipid peroxidation antioxidants have been proven helpful to a significant extent.

In the ongoing search of the present authors for antioxidant that may reduce drug-induced lipid peroxidation¹³⁻²⁴, the present work has been carried out *in vitro* to evaluate the antioxidant effect of water extract of *Spirulina platensis* on cisplatin-induced lipid peroxidation.

MATERIALS AND METHODS

The study had been performed on the goat (*capra capra*) liver using some common laboratory markers

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of lipid peroxidation like measurement of the malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), reduced glutathione (GSH) and nitric oxide (NO) content of the tissue. The goat liver was selected because of its easy availability and close similarity to the human liver in its lipid profile²⁵.

Preparation of water extract of *Spirulina platensis*:

Spirulina was obtained from Indo Leena Biotech Private Ltd., Spirulina Farm, Namakkal, Tamil Nadu. Attempt was made to determine the maximum concentration of the algae in water extract. For this purpose, first 2.5 g of spirulina powder was weighed accurately and taken in a beaker. Then 200 ml of water was added to it. The mixture was heated cautiously in a steam bath until the volume was reduced to 50 ml. The hot solution was filtered at a suction pump using single filter paper. After that the filtrate was again filtered at a suction pump using double filter paper. Then the filtrate was transferred in a 50 ml volumetric flask and the volume was made up to the mark with double distilled water. The concentration of the solution was determined as follows: At first a clean petridish was weighed accurately. Then 1 ml of the extracted solution was placed on it. Then the solution was heated on a steam bath to remove water and last traces of water were removed by drying in hot air oven. It was then kept in a desiccator to cool to room temperature. The weight of the petridish along with the solid material was weighed. Then further 1 ml of the extract was added and same procedure was done. In this way a total of 5 ml of extract was added to petridish and water was evaporated. Finally the weight of the petridish and solid material was taken. The amount of solid present in 5 ml extract was calculated by difference from the empty weight of petridish. The concentration of the water extract determined in this way was 0.92% w/v. The same procedure was followed with 4, 5, 6 and 7 g of spirulina powder and the concentrations were 1.4, 1.7, 1.7 and 1.7% w/v respectively. It was found that the maximum extractable concentration of the algae using 200 ml of water would be 1.7% w/v. The λ_{\max} of the water-extracted solution was found at 259 nm.

Preparation of tissue homogenate:

Goat liver was collected from Kolkata Municipal Corporation (KMC) approved outlet. Goat liver perfused with normal saline through hepatic portal vein was harvested and its lobes were briefly dried

between filter papers to remove excess blood and thin cut with a heavy-duty blade. The small pieces were then transferred in a sterile vessel containing phosphate buffer (pH 7.4) solution. After draining the buffer solution as completely as possible, the liver was immediately grinded to make a tissue homogenate (1 g/ml) using freshly prepared phosphate buffer (pH 7.4). The homogenate was divided into four equal parts, which were then treated differently as mentioned below.

Incubation of tissue homogenate with drug and / or antioxidant:

The tissue homogenate was divided into four parts of 50 ml each. The first portion was kept as control (C), while the second portion was treated with cisplatin (D) at a concentration of 0.0244 mg/g tissue homogenate. The third portion was treated with cisplatin at a concentration of 0.0244 mg/g tissue homogenate and water extract of spirulina at a concentration of 0.1666 mg/g tissue homogenate (DA) and the fourth one was treated with water extract of spirulina alone at a concentration of 0.1666 mg/g-tissue homogenate (A). After treatment with cisplatin and/or water extract of spirulina, the liver homogenates were shaken for 1 h and incubated at $18^{\circ}\pm 2^{\circ}$ for a period of maximum 4 h for further work.

Estimation of malondialdehyde (MDA) level from tissue homogenate:

The extent of lipid peroxidation was measured in terms of MDA content using thiobarbuturic acid (TBA) method²⁶. The estimation was done at 2, 4 h of incubation and repeated in five animal sets. In each case three samples of 2.5 ml of incubation mixture were treated with 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 1200 g for 30 min to precipitate protein. Then 2.5 ml of the filtrate was treated with 5 ml of 0.002 (M) TBA solution and the volume was made up to 10 ml with distilled water. The mixture was heated on a boiling water bath for 30 min and then tubes were cooled to room temperature and the absorbance was measured at 530 nm against a TBA blank (prepared from 5 ml of TBA solution and 5 ml of distilled water) using Elico Mini Spec (SL 171). The concentrations of MDA were determined from standard curve, which was constructed as follows. Different aliquots from standard 1,1,3,3-tetrahydroxypropane (TEP) solution were taken in graduated stoppered test tubes and volume of each solution was made up to 5 ml. To

each solution, 5 ml of TBA solution was added and the mixture was heated in a steam bath for 30 min. The solutions were cooled to a room temperature and their absorbances were measured at 530 nm against TBA as blank. By plotting absorbances against concentrations a straight line passing through the origin of grid was obtained. The best-fit equation is $A=0.007086 M$, where M = nanomoles of MDA, A = absorbance, $r= 0.995$, $SEE= 0.006$.

Estimation of reduced glutathione (GSH) level from tissue homogenate:

Reduced glutathione (GSH) was measured in accordance to the Ellman's method²⁷. The estimation was done at 1, 2 h of incubation and repeated in five animal sets. In each case three samples of 1 ml of incubation mixture were treated with 1 ml of 5% TCA in 1 mM EDTA centrifuged at 2000 g for 10 min. After that, 1 ml of the filtrate was mixed with 5 ml of 0.1M phosphate buffer (pH 8.0) and 0.4 ml of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01% in phosphate buffer) was added to it. The absorbances of the solutions were measured at 412 nm against blank (prepared from 6.0 ml of phosphate buffer and 0.4 ml of DTNB). The concentrations of reduced glutathione were determined from standard curve.

Estimation of 4-hydroxy-2-nonenal (4-HNE) level from tissue homogenate:

The estimation was done only at 2 h of incubation and it was repeated in 5 animal sets. In each case three samples of 2 ml of incubation mixture were treated with 1.5 ml of 10% TCA solution and centrifuged at 1200 g for 30 min. Then 2 ml of the filtrate was treated with 1 ml of 2,4 dinitrophenyl hydrazine (100 mg/100 ml of 0.5 M HCl) and kept for 1 h at room temperature. After that the samples were extracted with hexane and the extract was evaporated to dryness under argon at 40°. After cooling to a room temperature, 2 ml of methanol was added to each sample and the absorbance was measured at 350 nm against methanol as blank²⁸. The concentrations of 4-HNE content were determined from the standard curve.

Estimation of nitric oxide (NO) level from tissue homogenate:

The estimation was done at 1, 2 h of incubation and it was repeated in five animal sets. NO content was determined by reaction with Griess reagent²⁹. In each case three samples of 4.0 ml of tissue homogenate

were treated with 2.5 ml of 10% TCA solution and centrifuged at 1200 g for 30 min. Then 5 ml of the filtrate were treated with 0.5 ml Griess reagent. After 10 min the absorbances of the solutions were measured at 540 nm against blank (prepared from 5.0 ml of distilled water and 0.5 ml of Griess reagent). The concentrations of NO content were calculated from standard curve.

RESULTS AND DISCUSSION

The percent changes in MDA, GSH, 4-HNE and NO level at different h of incubation were calculated with respect to the control of the corresponding h of incubation and the changes in MDA/GSH/4-HNE/NO level was considered as indicator of the extent of lipid peroxidation. The averages of percent changes in MDA/GSH/4-HNE/NO content of five animal sets along with standard error are listed in Table 1. Interpretation of the results is supported by analysis of variance and also by statistical multiple comparison analysis using least significant different procedure³⁰⁻³¹.

Incubation of tissue homogenates with cisplatin resulted an increase in MDA content with respect to corresponding control (Table 1). This observation suggests lipid peroxidation induction potential of the cisplatin. MDA is a highly reactive three-carbon dialdehyde produced as a biproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. Increase in the accumulation of MDA in cells can result into cellular degradation, some biochemical and functional changes and even cell death³². It was further found that MDA content was significantly reduced when tissue homogenates were treated both with cisplatin and water extract of *Spirulina platensis*. This implies the free radical scavenging property of the water extract of the algae. When tissue homogenates were treated only with the water extract of algae then there is also depletion of MDA level in comparison to control.

The average percent changes in reduced glutathione (GSH) level of five animal sets are shown along with statistical analysis in Table 1. Incubation of tissue homogenates with cisplatin decreased the GSH level with respect to corresponding controls. Glutathione is a small protein composed of three amino acid, such as cysteine, glutamic acid and glycine³³. It is an important antioxidant and plays a very important role in the defence mechanism for tissue against the reactive

TABLE 1: EFFECTS OF WATER EXTRACT OF *SPIRULINA PLATENSIS* ON CISPLATIN-INDUCED LIPID PEROXIDATION: CHANGES IN MDA/GSH/4-HNE/NO PROFILE

Parameters	Incubation (h)	% Changes due to treatment samples (Average±SE)			Analysis of variance and multiple comparison
		D	DA	A	
MDA	2	44.81 (±9.84)	14.38 (±6.12)	-5.04 (±5.49)	F1=13.24 [df=(2,8)], F2=1.45[df=(4,8)] Pooled variance (S ²)*=238.45, Critical difference, (p=0.05)# LSD=29.07, Ranked means** (D) (DA, A) F1=8.77[df=(2,8)], F2=2.22[df=(4,8)]
	4	23.70 (±10.24)	-11.07 (±5.17)	-18.89 (±10.70)	Pooled variance (S ²)*=287.30, Critical difference, (p=0.05)# LSD=31.91, Ranked means** (D) (DA, A)
GSH	1	-11.33 (±3.95)	02.69 (±4.05)	03.50 (±3.27)	F1=4.13 [df=(2,8)], F2=0.538[df=(4,8)] Pooled variance (S ²)*=84.19, Critical difference, (p=0.05)# LSD=17.27, Ranked means** (D, DA, A)
	2	-20.39 (±2.62)	06.67 (±8.06)	10.42 (±4.24)	F1=21.27[df=(2,8)], F2=4.76[df=(4,8)] Pooled variance (S ²)*=66.43, Critical difference, (p=0.05)# LSD=6.65, Ranked means** (D) (DA, A)
4-HNE	2	12.45 (±2.86)	-03.61 (±1.75)	-10.39 (±4.62)	F1=10.89[df=(2,8)], F2=0.586[df=(4,8)] Pooled variance (S ²)*=63.17, Critical difference, (p=0.05)# LSD=14.96, Ranked means** (D) (DA, A)
NO	1	-09.79 (±1.66)	05.83 (±4.57)	12.73 (±4.02)	F1=16.72 [df=(2,8)], F2=3.01 [df=(4,8)] Pooled variance (S ²)*=39.78, Critical difference (p=0.05)# LSD=11.87, Ranked means** (D) (DA, A)
	2	-06.35 (±1.35)	02.49 (±0.525)	06.82 (±2.22)	F1=15.10 [df=(2,8)], F2=0.375 [df=(4,8)] Pooled variance (S ²)*=14.90, Critical difference (p=0.05)# LSD=7.27, Ranked means** (D) (DA, A)

Theoretical values of F: P=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)], P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)]. F1 and F2 corresponding to variance ratio between groups and within groups respectively. D, DA, A indicate drug treated, drug and antioxidant treated, antioxidant treated respectively. SE= Standard Error (df=4); df= degree of freedom, *Error mean square, #Critical difference according to least significant procedure (31). **Two means not included within same parenthesis are statistically significantly different at P=0.05 level.

oxygen species³⁴. When the tissue homogenates were treated both with cisplatin and water extract of the algae then GSH level was increased in comparison to drug treated group. Incubation of tissue homogenates only with water extract of algae also enhances the GSH level. These observations suggest that increase in GSH level may be due to antioxidant property of the extract.

The average percent changes in 4-HNE levels of five animal sets are shown along with statistical analysis in Table 1. Incubation of tissue homogenates with cisplatin caused significant increase in 4-HNE content with respect to control. 4-HNE is a specific and stable end product of lipid peroxidation. It can diffuse within or even escape from the cell and attack targets far from the site of the original free radical event³⁵⁻³⁶. 4-HNE can be produced from arachidonic acid, linolenic acid or their hydroperoxide in concentration of 1 μM to 5 nM in response to oxidative stress³⁵. When tissue homogenates were treated both with cisplatin and water extract of the algae, 4-HNE content was significantly reduced in comparison to the drug treated group. Incubation of tissue homogenates only with water extract of algae also reduces the 4-HNE levels. This implies that water extract of *Spirulina platensis*

inhibits cisplatin-induced lipid peroxidation to a significant extent.

The average percent changes in nitric oxide (NO) level of five animal sets are shown along with statistical analysis in Table 1. Incubation of tissue homogenates with cisplatin reduced the NO content with respect to corresponding controls. Nitric oxide has versatile role in biology because it can be a signaling molecule in vasodilation³⁷, a toxin³⁸, a prooxidant³⁹ and a potential antioxidant⁴⁰⁻⁴³. It was further found that incubation of tissue homogenates with cisplatin and water extract of algae resulted increase in NO content with respect to drug treated group. Incubation of tissue homogenates only with water extract of algae also enhances NO level with respect to corresponding controls. These results suggests that water extract of spirulina could inhibit lipid peroxidation to a significant extent. It has been proposed that NO causes chain termination reactions during lipid peroxidation as observed in low-density lipoprotein oxidation as well as in chemical systems⁴⁰⁻⁴³.

The data presented in this work demonstrate the lipid peroxidation induction potential of cisplatin. The results also suggest the antiperoxidative effects

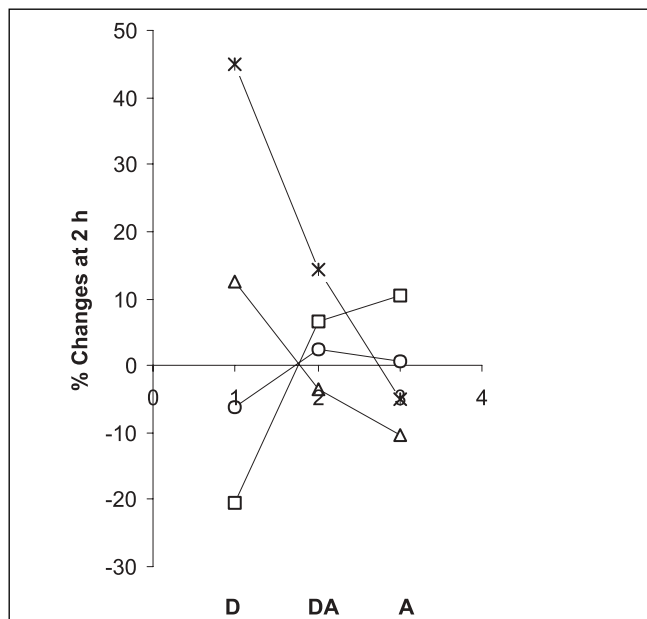


Fig. 1: Percent changes of MDA, GSH, 4-HNE and NO content at 2 h.

D, DA, A indicates drug-treated, drug- and antioxidant-treated, antioxidant-treated respectively, MDA is malondialdehyde (—X—), GSH is reduced glutathione (—□—), 4-HNE is 4-hydroxy-2-nonenal (—Δ—) and NO is nitric oxide (—◇—).

of water extract of spirulina and demonstrate its potential to reduce cisplatin-induced lipid peroxidation. The antioxidant effect is attributed due to its various constituents working individually or in synergy. From fig. 1 it is found that that the % changes of MDA and 4-HNE follow similar pattern while those of GSH and NO show similar trend for all three samples (D, DA and A). The results indicate that spirulina merits further extensive studies to explore potential to reduce cisplatin-induced toxicity.

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