



EVALUATION OF ANTIPEROXIDATIVE POTENTIAL OF WATER EXTRACT OF *SPIRULINA PLATENSIS* ON BUSULFAN-INDUCED LIPID PEROXIDATION USING 4-HYDROXY NONENAL AND NITRIC OXIDE AS MODEL MARKERS

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ABSTRACT

This *in vitro* study was designed to evaluate free radical scavenging activity of water extract of *Spirulina platensis* on busulfan-induced lipid peroxidation using 4-hydroxy nonenal and nitric oxide as model markers. In this study goat liver has been used as liver source. The results suggest that busulfan could induce lipid peroxidation to a significant extent and it was also found that water extract of the *Spirulina platensis* has the ability to suppress the busulfan-induced toxicity.

Keywords: Busulfan, *Spirulina platensis*, lipid peroxidation, 4-hydroxy-2-nonenal, nitric oxide

INTRODUCTION

Reactive oxygen free radicals are responsible for damage of tissues through lipid peroxidation (Guio Q et al. 1996). Free radicals are constantly formed in the human body, but the protection of cellular structures from damage by free radicals can be accomplished through enzymatic and non-enzymatic defense mechanisms (Durak I et al. 1994). Lipid peroxidation leads to generation of peroxides and hydroperoxides that can decompose to yield a wide range of cytotoxic products, most of which are aldehydes, as exemplified by malondialdehyde, 4-hydroxynonenal etc. (Esterbauer H et al. 1998). Oxidative stress in lipid peroxidation is responsible for initiating and developing many condition and diseases of modern time like diabetes, liver cirrhosis, nephrotoxicity, ageing etc. (Marx JL, 1987; Halliwell B 1981). In case of reduced or impaired defense mechanism and excess generation of free radicals that are not counter balanced by endogenous antioxidant defense, exogenously administered antioxidants

have been proven useful to overcome oxidative damage (Halliwell B 1991).

Busulfan, an alkylating agent used extensively in bone marrow transplantation. But there are evidences that organ toxicity in bone marrow transplantation may in part be due to free radical damage (Dürken M et al. 1995).

Spirulina platensis, planktonic blue green algae, is gaining increasing attention because of its nutritional and medicinal properties (Pinero EJE et al. 2001). *Spirulina* is 60-70% protein by weight and contain a rich source of vitamins especially vitamin B₁₂, β -carotene (provitamin A), and minerals, especially iron (Belay A 2002). It was found that spirulina potentiate the immune system leading to suppression of cancer development and viral infection (Hirahashi T et al. 2002). It also contains phycocyanin (7% dry weight basis) and polysaccharides, both of them have antioxidant

properties. Spirulina has direct effect on reactive oxygen species. It also contains an important enzyme superoxide dismutase (SOD) (1700 units / gm of dry mass) that acts indirectly by slowing down the rate of oxygen radical generating reactions (Belay A 2002).

In view of the above findings and the ongoing search of the present author for antioxidant that may reduce drug induced lipid peroxidation (Chakraborty S et al. 2005; Ray S et al. 2005; Ray S et al. 2006; Ray S et al. 2010) the present work has been carried out *in vitro* to evaluate the antiperoxidative potential of water extract of *Spirulina platensis* on busulfan-induced lipid peroxidation.

EXPERIMENTAL

1.1 Materials

The drug sample (busulfan) was provided by Elder Pharmaceuticals, Mumbai. Goat liver was used as the lipid source. Chemicals of analytical grade were used for the present study. 2, 4-Dinitrophenylhydrazine (DNPH) and trichloroacetic acid (TCA) were procured from SD Fine Chem. Ltd., Mumbai and Merck, Mumbai, respectively. The standard sample of 4-HNE was purchased from ICN Biomedicals Inc., Aurora, Ohio. Sulfanilamide was from SD Fine Chem. Ltd., Mumbai; N-naphthylethylenediamine dihydrochloride was from Loba Chemie Pvt. Ltd., Mumbai; Spirulina was obtained from INDO LEENA, Biotech private ltd., Spirulina Farm, Namakkal, Tamil Nadu.

1.2 Methods

1.2.1.Preparation of water extract of *Spirulina platensis*

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 the 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 4g, 5g, 6g, 7g of spirulina powder and the concentrations were 1.4%, 1.7%, 1.7%, 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.

1.2.2.Preparation of tissue homogenate

Goat liver was collected from Drugapur Municipal Corporation (DMC) approved outlet. Goat liver was selected because of its easy availability and close similarity with human liver in its lipid profile (Hilditch TP and Williams PN, 1964). 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

One portion of the homogenate was kept as control (C) while a second portion was treated with the busulfan (D) at a concentration of 0.0013 mg/g tissue homogenate. The third portion was treated with both busulfan at a concentration 0.0013 mg/g

tissue homogenate and water extract of *Spirulina platensis* at a concentration of (0.1666 mg / g tissue homogenate (DA) and the fourth portion was treated only with water extract of *Spirulina platensis* at a concentration of 0.1666 mg / g tissue homogenate (A). After flutamide and /or water extract of *Spirulina platensis* treatment, the liver tissue homogenate samples were shaken for two hours.

1.2.3 Determinations of 4-hydroxy-2-nonenal (4-HNE) level in tissue homogenate

The estimation was done only at 2 hours of incubation and repeated in five animal sets. In each case three samples of 2 ml of incubation mixture was treated with 1.5 ml of 10% (w/v) TCA solution then centrifuged at 3000 rpm for 30 min. 2 ml of the filtrate was treated with 1 ml of 2, 4-dinitrophenyl hydrazine (DNPH) (100 mg / 100 ml of 0.5 M HCl) and kept for 1 hour at room temperature. After that, the samples were extracted with hexane, and the extract was evaporated to dryness under argon at 40⁰ C. 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 blank (Kinter M 1996) using Shimadju UV-1700 double beam spectrophotometer. The values were determined from the standard curve. The standard calibration curve was drawn based on the following procedure. A series of dilutions of 4-HNE in different concentrations of solvent (phosphate buffer) were prepared. From each solution 2 ml of sample pipette out and transferred into stoppered glass tube. 1 ml of DNPH solution was added to all the samples and kept at room temperature for 1 hour. Each sample was extracted with 2 ml of hexane for three times. All extracts were collected in stoppered test tubes. After that extract was evaporated to dryness under argon at 40⁰C and the residue was reconstituted in 1 ml of methanol. The absorbance was measured at 350 nm using the 0 μ M standard as blank. The best-fit equation is: Nanomoles of 4-HNE = $(A_{350} - 0.005603185) / 0.003262215$, where A_{350} = absorbance at 350nm, $r = 0.999$, SEM = 0.007.

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

The estimation was done at 2 hours of incubation and repeated in five animal sets. NO content was determined by reaction with Griess reagent. Griess reagent was prepared by mixing equal volumes of sulphanilamide (1% w/v in 3N HCl) and (0.1%w/v N-naphthyl ethylenediamine dihydrochloride) (Sastri KVH et al. 2002). 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 3000 rpm for 30 minutes. Then 5 ml of the filtrate were treated with 0.5 ml Griess reagent. After 10 minutes 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) using Shimadju UV-1700 double beam spectrophotometer. The values were calculated from standard curve, which was constructed as follows. Different aliquots from standard sodium nitrite solution were taken in 5 ml volumetric flasks. To each solution 0.5 ml of Griess reagent was added and volume was adjusted up to the mark with phosphate buffer. The absorbances of each solution were noted at 540nm against a blank containing the buffer and Griess reagent. By plotting absorbance against concentration a straight line passing through the origin was obtained. The best-fit equation is $A = 0.0108M$, where M = nanomoles of NO, A = absorbance, $r = 0.99581$, SEE = 0.0064.

1.3 Statistical analysis

Interpretation of the result is supported by student “t” test. Analysis of variance (ANOVA) and multiple comparison analysis using least significant different procedure (Snedecor GW and Cochran WG 1967; Bolton S 2000) were also performed on the percent changes data of various groups such as busulfan-treated (D), busulfan and water extract of *Spirulina platensis* (DA) and only water extract of *Spirulina platensis* -treated (A) with respect to control group of corresponding time.

RESULTS & DISCUSSION

The percent changes in 4-HNE and NO content of different samples at two hours of incubation were calculated with respect to the control of the

corresponding time of incubation and was considered as indicator of the extent of lipid peroxidation. The results of the studies on busulfan-

induced lipid peroxidation and its inhibition with water extract of *Spirulina platensis* were shown in Tables 1-2.

Table 1: Effects of water extract of *Spirulina platensis* on Busulfan-induced lipid peroxidation: changes in 4-HNE profile

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in 4-HNE content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Water extract of <i>Spirulina platensis</i>	Busulfan	2	An 1	10.50 ^a	-5.67 ^b	-5.52 ^a	F1=92.25 [df=(2,8)]
			An 2	9.85 ^a	-4.55 ^b	-5.25 ^a	F2=0.746 [df=(4,8)]
			An 3	12.50 ^a	-7.71 ^a	-8.02 ^b	Pooled variance
			An 4	11.25 ^a	-3.25 ^b	-4.36 ^c	(S ²)*=5.44
			An 5	11.52 ^a	-4.52 ^c	-12.6 ^a	Critical difference
			Av.	11.12	-5.14	-7.15	(p=0.05) [#]
			(±SEM)	(±0.45)	(±0.75)	(±1.49)	LSD =4.39
						Ranked means ^{**}	
						(D) (DA, A)	

Percent changes with respect to controls of corresponding hours are shown. C, D, DA & A indicate control (not treated with busulfan or water extract of *Spirulina platensis*), only busulfan -treated, busulfan and water extract of *Spirulina platensis* -treated and only water extract of *Spirulina platensis*-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of estimate (df=4); Significant of 't' values of the changes of 4-HNE content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; d=90-95%; e=80-90%; f=70-80%; g=60-70%; h<60%; 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; * Error mean square, # Critical difference according to least significant procedure (Snedecor GW and Cochran WG 1967; Bolton S 2000) **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Table 2: Effects of water extract of *Spirulina platensis* on Busulfan-induced lipid peroxidation: changes in NO profile

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in NO content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Water extract of <i>Spirulina platensis</i>	Busulfan	2	An 1	-9.2 ^b	5.2 ^c	5.42 ^c	F1=158.85 [df=(2,8)]
			An 2	-8.01 ^a	5.12 ^b	4.86 ^a	F2=0.76 [df=(4,8)]
			An 3	-7.13 ^b	6.48 ^c	6.52 ^d	Pooled variance (S ²)*=1.87
			An 4	-10.5 ^b	5.23 ^e	5.76 ^c	Critical difference
			An 5	-5.42 ^a	4.5 ^b	4.02 ^b	(p=0.05) [#]
			Av.	-8.05	5.31	5.32	LSD =2.57
			(±SEM)	(±0.868)	(±0.322)	(±0.42)	Ranked means **
						(D) (DA, A)	

Percent changes with respect to controls of corresponding hours are shown. C, D, DA & A indicate control (not treated with busulfan or water extract of *Spirulina platensis*), only busulfan -treated, busulfan and water extract of *Spirulina platensis* -treated and only water extract of *Spirulina platensis*-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of estimate (df=4); Significant of 't' values of the changes of NO content (df=2) are shown as: a>99%; b=97.5-

99%; c=95-97.5%; d=90-95%; e=80-90%; f=70-80%; g=60-70%; h<60%; 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; * Error mean square, # Critical difference according to least significant procedure (Snedecor GW and Cochran WG 1967; Bolton S 2000) **Two means not included within same parenthesis are statistically significantly different at $p=0.05$ level.

From Table 1 it was evident that tissue homogenates treated with busulfan showed an increase in 4-HNE (11.12 %) content in samples with respect to control to a significant extent. The observations suggest that busulfan could significantly induce the lipid peroxidation process. 4-Hydroxy-2-nonenal (4-HNE), a lipid aldehydes that form due to lipid peroxidation occurring during episodes of oxidant stress, readily forms adducts with cellular proteins; these adducts can be assessed as a marker of oxidant stress in the form of lipid peroxidation (Esterbauer H et al. 1991). But the 4-HNE (-5.14%) content were significantly reduced in comparison to busulfan-treated group when tissue homogenates were treated with busulfan in combination with water extract of *Spirulina platensis*. Again the tissue homogenates were treated only with the water extract of *Spirulina platensis* then the 4-HNE (-7.15%) level were reduced in comparison to the control and the busulfan treated group. This decrease may be due to the free radical scavenging property of the water extract of *Spirulina platensis*. So the decrease in 4-HNE content of samples, when treated with busulfan and water extract of *Spirulina platensis* as well as only with water extract of *Spirulina platensis* implies the free radical scavenging property of water extract of *Spirulina platensis*.

It was evident from Table 2 that tissue homogenates treated with busulfan caused a decrease in NO (-8.05%) content with respect control to a significant extent. The decrease in NO content was associated with an increase in lipid peroxidation. When tissue homogenates were treated both with busulfan and water extract of *Spirulina platensis* then the NO (5.31%) levels increased in comparison to busulfan treated group. Tissue homogenates treated only with the water extract of *Spirulina platensis* also increase the NO (5.32%) contents in comparison to the control samples. The increase in NO level suggests the antiperoxidative potential of water extract of *Spirulina platensis*. NO plays a very important role

in host defense (Hogg N et al. 1993; Rubho H et al. 1995). So the increase in NO content of tissue homogenate, when treated with busulfan and water extract of *Spirulina platensis* as well as only with water extract of *Spirulina platensis* implies the free radical scavenging activity of water extract of *Spirulina platensis*.

To compare means of more than two samples, multiple comparison analysis along with analysis of variance was performed on the percent changes data with respect to control of corresponding hours. It is seen that there is significant differences among various groups ($F1$) such as busulfan-treated, busulfan and water extract of *Spirulina platensis* -treated and only water extract of *Spirulina platensis* -treated. But within a particular group, differences ($F2$) are insignificant which shows that there is no statistical difference in animals in a particular group (Tables 1-2). The Tables also indicate that the level of 4-HNE / NO in busulfan -treated group is only statistically significantly different from the busulfan and water extract of *Spirulina platensis*-treated group as well as only water extract of *Spirulina platensis* -treated group. But there is no statistically significant difference among the busulfan and water extract of *Spirulina platensis* -treated group and only water extract of *Spirulina platensis* -treated group.

CONCLUSION

The data presented in this work demonstrate the lipid peroxidation induction potential of busulfan, which may be related to its toxic potential. The results also suggest the antiperoxidative effects of water extract of the algae and demonstrate its potential to reduce busulfan induced toxic effects. The antioxidant effect is attributed due to its various constituents working individually or in synergy. However, further extensive study is required to draw any final conclusion.

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