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Flutamide-induced Lipid Peroxidation: Protective Role of Water extract of *Spirulina platensis*

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Abstract: The present study deals with exploration of lipid peroxidation induction capacity of flutamide, an anticancer drug, and *in vitro* evaluation of water extract of *Spirulina platensis* as a suppressor of flutamide-induced lipid peroxidation. Goat liver homogenate has been used as the lipid source. This evaluation was done by measuring the malondialdehyde and reduced glutathione content of the tissue as markers of lipid peroxidation. The study revealed that water extract of the *Spirulina platensis* could suppress the flutamide-induced lipid peroxidation to a significant extent.
Keywords: lipid peroxidation, flutamide, water extract of the *Spirulina platensis*, malondialdehyde, reduced glutathione.

Introduction

Lipid peroxidation leads to generation of peroxides and hydroperoxide that can decompose to yield a wide range of cytotoxic end products most of which are aldehydes as exemplified by molondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) etc (1). It is a free radical related process that may occur in the biological system under enzymatic control or nonenzymatically (2-4). The latter form is associated mostly with cellular damage as a result of oxidative stress (5). Free radicals are constantly being generated in the body through various mechanisms and also being removed by endogenous antioxidant defense mechanism that acts by scavenging free radicals, decomposing peroxides and / or binding with pro-oxidant metal ion. Free radical mediated oxidative stress results usually from deficient natural antioxidant defense. 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 (6). So the evaluation of antioxidants as suppressor of drug induced lipid peroxidation provides a scope of further investigation for their co-administration with drugs to reduce drug-induced toxicities that are possibly mediated by free radical mechanism.

Flutamide, a phototoxic anticancer drug acts as androgen receptor antagonist and used mainly as an anticancer drugs in certain type of prostrate cancer. It specifically inhibits androgen uptake and / or nuclear binding of androgen in target tissues. When used as monotherapy, it causes a gradual increase in plasma testosterone due to blockage of feedback inhibition of the hypothalamus and pituitary by testosterone. But it

shows a photo hemolytic effect on human erythrocytes and photo induces lipid peroxidation (7).
Spirulina platensis, planktonic blue green algae, is gaining increasing attention because of its nutritional and medicinal properties (8). Spirulina is 60-70% protein by weight and contain a rich source of vitamins especially vitamin $B_{12,}$ β-carotene (provitamin A), and minerals, especially iron (9). It was found that spirulina potentiate the immune system leading to suppression of cancer development and viral infection (10). It also contains phycocyamin (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 (9).

The protective effect of various antioxidants on anticancer drug-induced lipid peroxidation had been reported earlier by us (10-14). In continuation of ongoing search for antioxidants, the present work has been carried out *in vitro* to evaluate the antiperoxidative potential of water extract of *Spirulina platensis* on flutamide-induced lipid peroxidation.

Experimental

Materials

Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi. 5, 5' dithiobis (2-nitrobenzoic acid) was from SRL Pvt. LTd., Mumbai. 1,1,3,3, tetraethoxypropane, reduced glutathione were from Sigma chemicals Co. St. Louis, MO, USA. All other reagents were of analytical grade. The drug sample (flutamide) was provided by Cipla Ltd. Mumbai, India, Mumbai. Spirulina was obtained from INDO LEENA, Biotech private ltd., Spirulina Farm, Namakkal, Tamil Nadu Goat liver was used as the lipid source.

Methods

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.

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 (15). 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 flutamide (D) at a concentration of 0.0125 mg/g tissue homogenate. The third portion was treated with both flutamide at a concentration 0.0125 mg/g tissue homogenate and water extract of *Spirulina platensis* at a concentration of 0.1666 mg / g 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 five hours.

Estimation of malondialdehyde (MDA) level from tissue homogenate

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thio - barbuturic acid (TBA) method (16). The estimation was done at 5 hours 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 room temperature at 3000 rpm for 30 minutes to precipitate protein. Then 2.5 ml of the supernatant was treated with 5 ml of 0.002 (M) TBA solutions and then volume was made up to 10 ml with distilled water. The mixture was heated on a boiling water bath for 30 minutes. Then tubes were cooled to a 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 Shimadju UV-1700 double beam spectrophotometer. 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 minutes. 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=0.007086M, 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 with Ellman's method (17). The estimation was done at 5 hours 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% (w/v) TCA in 1 mM EDTA centrifuged at 2000 g for 10 minutes. 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 (0.01% in phosphate buffer pH=8.0) (DTNB) 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, which was constructed as follows. Different aliquots of standard reduced glutathione stock solution were taken in 10 ml volumetric flasks. To each solution 0.4 ml of DTNB solution was added and volume was adjusted up to the mark with phosphate buffer (pH=8.0). The absorbance of each solution was measured at 412 nm against a blank containing 9.6 ml of phosphate buffer (pH=8.0) and 0.4 ml DTNB solution. By plotting absorbances against concentration a straight line passing through the origin of grid was obtained. The best-fit equation was A= 0.00151C, where C= nanomoles of reduced glutathione, A= absorbance, r =0.997, SEE= 0.008.

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 (18, 19) were also performed on the percent changes data of various groups such as flutamide-treated (D), flutamide 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 MDA and GSH content of different samples at five 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 flutamide-induced lipid peroxidation and its inhibition with water extract of *Spirulina platensis* were shown in Tables 1-2.

From Table 1 it was evident that tissue homogenates treated with flutamide showed an increase in MDA (9.17 %) content in samples with respect to control to a significant extent. The observations suggest that flutamide could significantly induce the lipid peroxidation process. MDA is a highly reactive three-carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism (20). But the MDA (-5.48%) content were significantly reduced in comparison to control as well as flutamide-treated group when tissue homogenates were treated with flutamide in combination with water extract of *Spirulina platensis* . Again the tissue homogenates were treated only with the water extract of *Spirulina platensis* then the MDA (-7.61 %) level were reduced in comparison to control and flutamide treated group. So the decrease in MDA content of tissue homogenate, when treated with flutamide 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*.

Table 1: Effects of water extract of *Spirulina platensis* on flutamide-induced lipid peroxidation: changes in MDA profile

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in MDA 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 *Spirulina platensis*	Flutamide	5	An 1	8.87[a]	-7.21[a]	-11.79[d]	F1=89.34 [df=(2,8)]
			An 2	7.54[a]	-2.39[b]	-4.17[b]	F2=1.49 [df=(4,8)]
			An 3	9.54[a]	-4.68[a]	-5.64[b]	Pooled variance
			An 4	11.67[a]	-7.52[b]	-6.84[c]	(S^2)*=4.67
			An 5	8.24[b]	-5.62[a]	-9.63[a]	Critical difference
							(p=0.05)[#]
			Av.	**9.17**	**-5.48**	**-7.61**	LSD =4.07
			(±SEM)	(±0.71)	(±0.93)	(±1.37)	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 flutamide or water extract of *Spirulina platensis*), only flutamide -treated, flutamide 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 MDA 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 (18, 19) **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

It was evident from Table 2 that tissue homogenates treated with flutamide caused a decrease in GSH (-10.47%) content with respect control to a significant extent. The decrease in GSH content was associated with an increase in lipid peroxidation. When tissue homogenates were treated both with flutamide and water extract of *Spirulina platensis* then the GSH (1.77%) levels increased in comparison to control and flutamide treated group. Tissue homogenates treated only with water extract of *Spirulina platensis* also increase the GSH (4.37%) contents in comparison to the control samples. The increase in GSH level suggests the antiperoxidative potential of water extract of *Spirulina platensis*. Glutathione is an important antioxidant and plays a very important role in the defense mechanism for tissue against the reactive oxygen species (21). The depletion of GSH is associated with increase in lipid peroxidation. The decrease in GSH level may be a consequence of enhanced utilization of this compound by the antioxidant enzymes glutathione peroxidase and glutathione-S-transferase.

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 flutamide-treated, flutamide 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 MDA / GSH in flutamide -treated group is only statistically significantly different from the flutamide and water extract of *Spirulina platensis* -treated group as well as only water extract of *Spirulina platensis*-treated group. But there is no statistically significantly difference among the flutamide and water extract of *Spirulina platensis* -treated group and only water extract of *Spirulina platensis* -treated group.

Table 2: Effects of water extract of *Spirulina platensis* on flutamide-induced lipid peroxidation: changes in GSH profile

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in GSH 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 *Spirulina platensis*	Flutamide	5	An 1	-12.52[a]	-1.24[a]	5.42[d]	F1=100.50 [df=(2,8)]
			An 2	-10.26[b]	2.54[a]	3.26[b]	F2=1.53 [df=(4,8)]
			An 3	-13.52[b]	2.88[a]	3.54[b]	Pooled variance
			An 4	-8.52[a]	1.24[b]	4.22[a]	(S^2)*=3.12
			An 5	-7.52[b]	3.42[a]	5.42[a]	Critical difference
							(p=0.05)[#]
			Av.	**-10.47**	**1.77**	**4.37**	LSD =4.32
			(±SEM)	(±1.14)	(±0.83)	(±0.455)	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 flutamide or water extract of *Spirulina platensis*), only flutamide -treated, flutamide 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 GSH 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 (18, 19) **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Conclusion

The results also suggest the antiperoxidative effects of water extract of *Spirulina platensis* and demonstrate its potential to reduce flutamide-induced lipid peroxidation and thus to increase therapeutic index of the drug by way of reducing toxicity that may be mediated through free radical mechanisms. However a detailed study is required to conclude such hypothesis.

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