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Original Article

# Exploring the Protective Effect of Ascorbic Acid and Aqueous Extract of *Spirulina platensis* on Methotrexate-Induced Lipid Peroxidation

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## Abstract
This study was designed to explore the protective effects of ascorbic acid and water extract of *Spirulina platensis* (blue green algae) on methotrexate-induced lipid peroxidation using goat liver as the 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 study reveals the lipid peroxidation induction capacity of methotrexate, the anti-peroxidative potential of ascorbic acid and aqueous extract of *S. platensis* on methotrexate-induced lipid peroxidation.

*Keywords*: Ascorbic acid; Lipid peroxidation; Methotrexate; Nitric oxide; Reduced glutathione; *Spirulina platensis*.
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## 1. Introduction
Lipid peroxidation is oxidative deterioration of polyunsaturated lipid that occurs through free radical mediated chain reaction [1]. Reactive oxygen free radicals are responsible for damage of tissues through lipid peroxidation [2]. Free radicals are constantly formed in the human body, but protection of cellular structures from damage by free radicals can be accomplished through enzymatic and non-enzymatic defense mechanisms [3]. Large production of some free radicals, particularly reactive oxygen species (ROS) and their high activity lead to oxidative stress, a condition in which endogenous antioxidant mechanisms are insufficient for scavenging ROS. So the balance between pro-oxidants and antioxidants is very important for survival. All antioxidants generally influence the redox status, thereby protecting cells against ROS under certain circumstances, while promoting ROS generation in others [4]. 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. [5]. Oxidative stress in lipid peroxidation is responsible for initiating and developing many

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condition and diseases of modern time like diabetes, liver cirrhosis, nephrotoxicity, ageing etc. [6, 7]. In case of reduced or impaired defense mechanism and excessive generation of free radicals that are not counter balanced by endogenous antioxidant defense, exogenously administered antioxidants have been proven useful to overcome oxidative damage [8].

Vitamin C (ascorbic acid) is an essential water-soluble antioxidant in cells and plasma. Besides metabolic functions, vitamin C is also known to contribute to immune homeostasis. It has also potential antioxidant property. Ascorbic acid has been reported to have protective role against cadmium-induced thyroid dysfunction due to its antioxidant action [9]. Recently, it has been demonstrated that vitamin C has an inhibitory effect on the expression of pro-inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor alpha (TNF-alpha) in adult whole blood cells *in vitro* [10]. *Spirulina platensis*, planktonic blue green algae, is gaining increasing attention because of its nutritional and medicinal properties [11]. Spirulina has 60-70% protein by weight and contains a rich source of vitamins especially vitamin $B_{12}$, β-carotene (provitamin A), and minerals, especially iron [12]. It was found that spirulina potentiate the immune system to suppress cancer development and viral infections [13]. 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/g of dry mass) that acts indirectly by slowing down the rate of oxygen radical generating reactions [12]. In view of the above findings and widespread use of ascorbic acid and spirulina, it would be of interest if further studies were carried out to obtain more information regarding antiperoxidative potential of ascorbic acid and spirulina.

Methotrexate, a folic acid antagonist, is one of the chemotherapeutic agents widely used in the treatment of some types of cancer. But nephrotoxicity is one of the complications of methotrexate treatment [14]. It has been reported that methotrexate leads to reduction

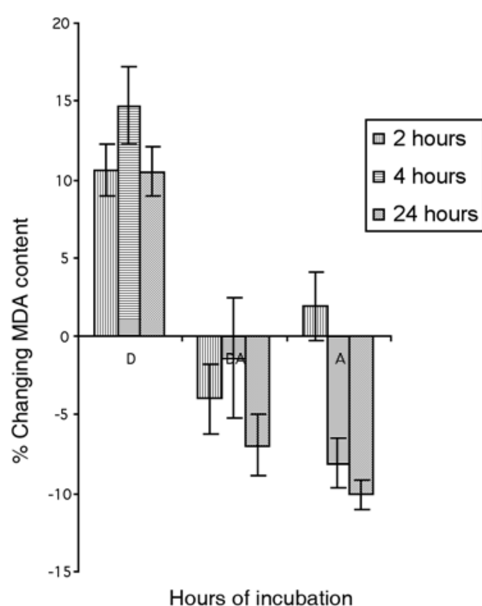


**Figure 1.** Effects of ascorbic acid on methotrexate-induced lipid peroxidation: Changes in MDA profile (n=5). D: drug; A: ascorbic acid.

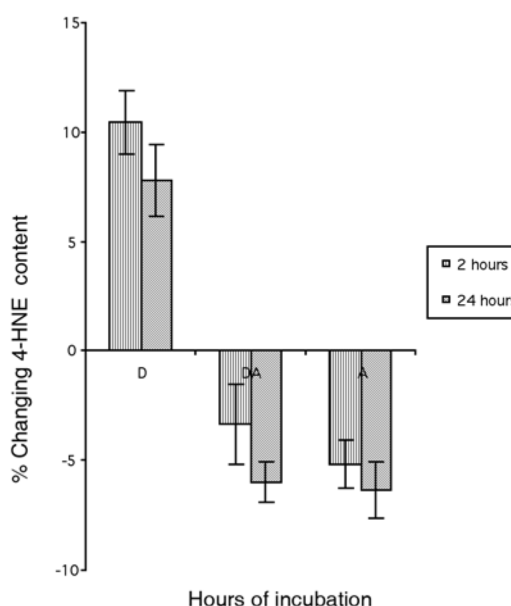


**Figure 2.** Effects of ascorbic acid on methotrexate-induced lipid peroxidation. Changes in 4-HNE profile (n=5). D: drug; A: ascorbic acid.



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in antioxidant enzymatic defense capacity and causes lipid peroxidation in renal tissue [15]. It has been observed that oxidative stress is occurred in CNS membrane phospholipids during chemotherapy with methotrexate [16]. Methotrexate along with cyclophosphamide and 5-fluorouracin produce lipid peroxidative damages of the intestinal basolateral membrane of rats [17]. To control and reduce lipid peroxidation antioxidants have been proven helpful to a significant extent. Thus, evaluation of antioxidants as suppressor of drug-induced lipid peroxidation provides a scope to select free radical scavengers, which on co-administration with drug for therapeutic purpose may reduce the toxic effects of drugs. In the ongoing search of the present authors for antioxidants that may reduce drug induced lipid peroxidation [18-28], the present work has been carried out *in vitro* to evaluate the antioxidant effect of ascorbic acid (AA) and water extract of *S. platensis* (SP) on methotrexate-induced lipid peroxidation.

## 2. Material and methods

This study was performed on goat (*Capra capra*) liver using some common laboratory markers 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 profile [29].

### *2.1. Preparation of aqueous 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 aqueous extract. For this purpose, first 2.5 g of spirulina powder was weighed and added to 200 ml of water. The mixture was heated cautiously until the volume was reduced to 50 ml. The hot solution was filtered at a suction pump using one filter paper. After that the filtrate was again filtered at 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

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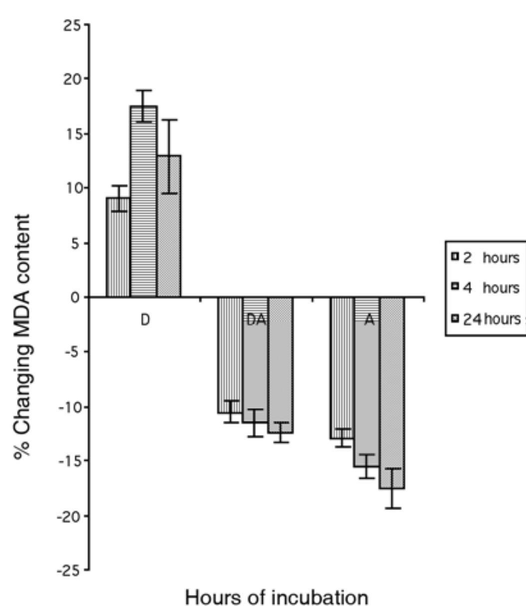


**Figure 3.** Effects of aqueous extract of *Spirulina platensis* on methotrexate-induced lipid peroxidation: Changes in MDA profile (n=5). D: drug; A: ascorbic acid.

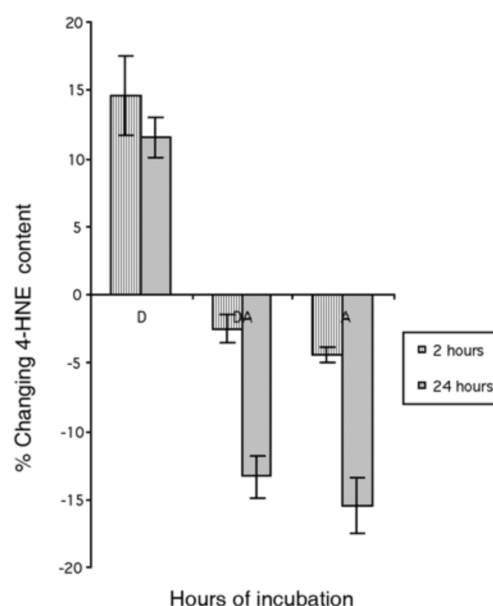


**Figure 4.** Effects of aqueous extract of *Spirulina platensis* on methotrexate-induced lipid peroxidation. Changes in 4-HNE profile (n=5). D: drug; A: ascorbic acid.



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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 solution was heated on steam bath to remove the water and last traces of water were removed by drying in hot air oven. It was then kept in desecrator to cool to room temperature. The weight of the Petri dish 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 Petri dish and water was evaporated. Finally the weight of the Petri dish 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 aqueous extract determined in this way was 0.92% w/v. The same procedure was followed with 4, 5, 6 and 7 gm of spirulina powder and the concentrations were 1.4%, 1.7%, 1.7%, 1.7% w/v, respectively. It was found that the maximum concentration of the algae in the aqueous extract was 1.7% w/v. The $\lambda_{max}$ of the water-extracted solution was found at 259 nm.

*2.2. 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 mg/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.

*2.3. 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 methotrexate (D) at a concentration of 0.0288 mg/g tissue homogenate. The third portion was treated with methotrexate at a concentration of 0.0288 mg/g tissue homogenate and antioxidant (AA/SP) at a concentration of 0.1667 mg/g tissue homogenate (DA) and the fourth one was treated with antioxidant (AA/SP) alone at a concentration of 0.1666 mg/g tissue homogenate (A). After treatment with methotrexate and/or antioxidant (AA/SP), the liver homogenates were shaken for 2 h and incubated at 18±2 °C for a period of maximum

**Table 1.** ANOVA and Multiple comparison for changes of MDA content.

| Antioxidant | Incubation (h) | Analysis of variance and multiple comparison |
|---|---|---|
| Ascorbic acid | 2 | F1=13.61 [df=(2, 8)], F2=1.16 [df=(4,8)], Pooled variance $(S^2)^*$ =19.57, Critical difference (p=0.05)# LSD =8.33, Ranked means** (D) (DA, A) |
| | 4 | F1=23.43 [df=(2, 8)], F2=1.98 [df=(4,8)], Pooled variance $(S^2)^*$ =29.30, Critical difference (p=0.05)# LSD =10.19, Ranked means** (D) (DA, A) |
| | 24 | F1=55.57 [df=(2, 8)], F2=1.24 [df=(4,8)], Pooled variance $(S^2)^*$ =11.09, Critical difference (p=0.05)# LSD =6.27, Ranked means** (D) (DA, A) |
| Aqueous extract of *S. platensis* | 2 | F1=140.96 [df=(2, 8)], F2=0.846 [df=(4,8)], Pooled variance $(S^2)^*$ =5.16, Critical difference(p=0.05)# LSD =4.28, Ranked means** (D) (DA, A) |
| | 4 | F1=165.78 [df=(2, 8)], F2=0.543 [df=(4,8)], Pooled variance $(S^2)^*$ =9.80, Critical difference (p=0.05)# LSD =5.89, Ranked means** (D) (DA, A) |
| | 24 | F1=35.21 [df=(2, 8)], F2=0.094 [df=(4,8)], Pooled variance $(S^2)^*$ =37.59, Critical difference (p=0.05)# LSD =11.54, 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.

*Error mean square, # Critical difference according to least significant procedure (34-35), ** Two means not included within same parenthesis are statistically significantly different at $p$=0.05 level.



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24 h for further work.

*2.4. Estimation of malondialdehyde (MDA) level from tissue homogenate*

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thiobarbuturic acid (TBA) method [30]. The estimation was done at 2, 4 and 24 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 room temperature at 3000 rpm for 30 min. 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 min. 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 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 min. The solutions were cooled to 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

*2.5. Estimation of reduced glutathione (GSH) level from tissue homogenate*

Reduced glutathione (GSH) was measured in accordance with Ellman's method [31]. The estimation was done at 2, 4 and 24 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% (w/v) 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 0.1 M 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

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**Table 2:** ANOVA and multiple comparison for changes of 4-HNE content.

| Antioxidant | Incubation (h) | Analysis of variance and multiple comparison |
|---|---|---|
| Ascorbic acid | 2 | F1=34.37 [df=(2,8)], F2=1.16 [df=(4,8)], Pooled variance (S2)* =10.56, Critical difference (p=0.05)# LSD =6.12, Ranked means** (D) (DA, A) |
| | 24 | F1=27.36 [df=(2,8)], F2=0.178 [df=(4,8)], Pooled variance (S2)* =11.86, Critical difference (p=0.05)# LSD =6.48, Ranked means** (D) (DA, A) |
| Aqueous extract of *S. platensis* | 2 | F1=36.95 [df=(2,8)], F2=1.26 [df=(4,8)], Pooled variance (S2)* =14.80, Critical difference (p=0.05)# LSD =7.24, Ranked means** (D) (DA, A) |
| | 24 | F1=74.74 [df=(2,8)], F2=0.924 [df=(4,8)], Pooled variance (S2)* =15.12, Critical difference (p=0.05)# LSD =7.09, 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.
*Error mean square, # Critical difference according to least significant procedure (34-35), ** Two means not included within same parenthesis are statistically significantly different at $p$=0.05 level.



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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

*2.6. Estimation of 4-hydroxy-2-nonenal (4-HNE) level from tissue homogenate*

The estimation was done at 2 and 24 h of incubation and it was repeated in five animal sets. In each case three samples of 2 ml of incubation mixture were treated with 1.5 ml of 10% (w/v) TCA solution and centrifuged at 3000 rpm for 30 min. Then 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 h at room temperature. After that, the samples were extracted with hexane, and the extract was evaporated to dryness under argon at 40 °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 [32]. 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 was pipetted out and transferred into stoppered glass tube. One ml of DNPH solution was added to all the samples and kept at room temperature for 1 h. 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 40 °C and the residue was reconstituted in 1 ml of methanol. The absorbance was measured at 350 nm using the 0 µM 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, SEE=0.007

*2.7. Estimation of nitric oxide (NO) level from tissue homogenate*

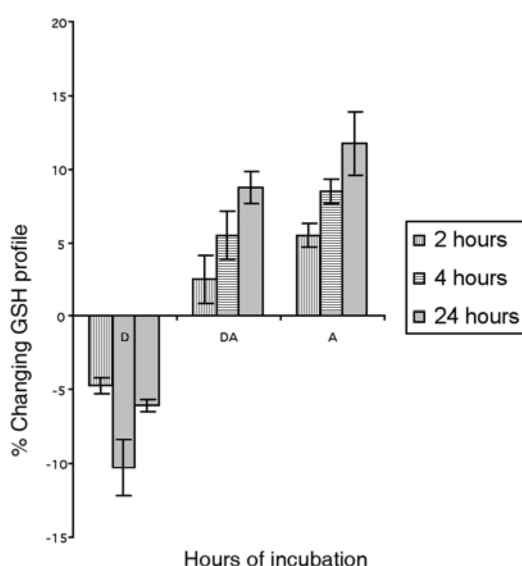


**Figure 5.** Effects of ascorbic acid on methotrexate-induced lipid peroxidation: Changes in GSH profile (n=5). D: drug; A: ascorbic acid.

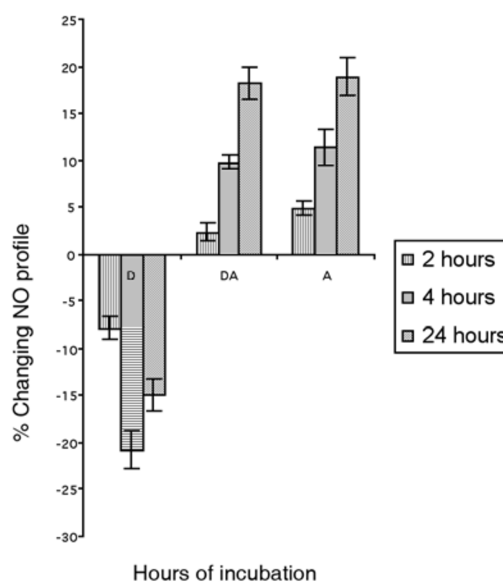


**Figure 6.** Effects of ascorbic acid on methotrexate-induced lipid peroxidation: Changes in NO profile (n=5). D: drug; A: ascorbic acid.



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The estimation was done at 2, 4 and 24 h of incubation and it was 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-naphthylethylenediamine dihydrochloride) [33]. In each case three samples of 4.0 ml of tissue homogenate were treated with 2.5 ml of 10% (w/v) TCA solution and centrifuged at 3000 rpm 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 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 540 nm 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:

$$A=0.0108\ M$$

where M=nanomoles of NO, A=absorbance, r=0.99581, SEE=0.0064

### 2.8. Statistical analysis

Analysis of variance (ANOVA) and multiple comparisons [34, 35] were done based on the percent changes data with respect to control of corresponding h to check statistical significance of the results. In multiple comparisons there are two possible sources of error: The random error associated with the replicate measurements and the other due to animal variations. The variations may be calculated and their effects estimated by a statistical method known as analysis of variance (ANOVA), where the square of the standard deviation $s^2$ is called the variance V. Thus $F= s_1^2/s_2^2$ where $s_1^2/s_2^2$, and may be written as $F=V_1/V_2$ where $V_1>V_2$. ANOVA is done to compare the means of more than two treatment groups. In our study the degree of freedom (df) between samples is (2, 8) and that between animals is (4, 8).

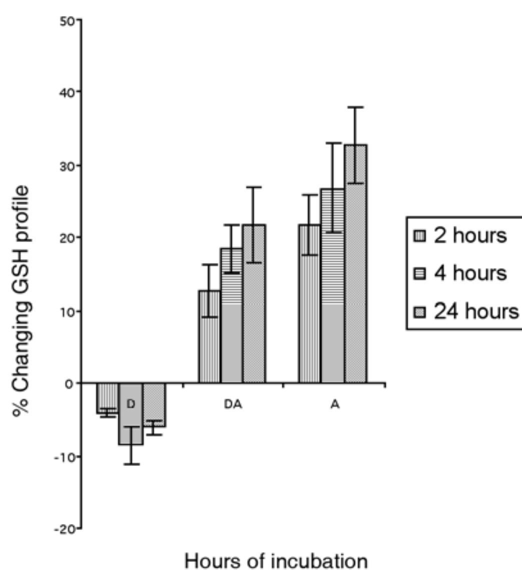


**Figure 7.** Effects of aqueous extract of *Spirulina platensis* on methotrexate-induced lipid peroxidation: Changes in GSH profile (n=5). D: drug; A: ascorbic acid.

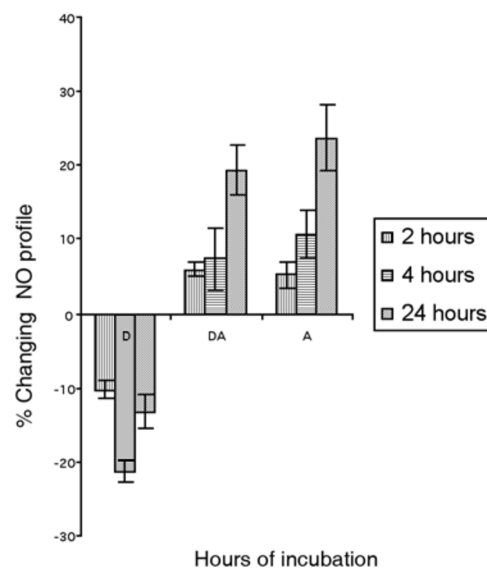


**Figure 8.** Effects of aqueous extract of *Spirulina platensis* on methotrexate-induced lipid peroxidation: Changes in NO profile (n=5). D: drug; A: ascorbic acid.



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If the F test (F=mean square between regimens / mean square within regimens) is significant and more than two treatments are included in the experiment, it may not be obvious immediately which treatments are different. To solve the problem, multiple comparisons in ANOVA have been done. Various multiple comparison procedures have been proposed to solve this problem. The general procedure of multiple comparisons is to list the ranked means from lowest to highest (or the reverse) and the means that are not statistically significantly different from each other are placed in the same parenthesis. The procedure is carried out by calculating a 5% allowance, which is defined as the critical difference between means which allows one to reject the null hypothesis and accept the alternative hypothesis for any two sample means at $p$=0.05.

We have used least significant difference procedure in multiple comparison analysis. Least significant different procedure is the least conservative procedure, and this assures the probability that any one comparison is judged to be significant would be greater than 5%. Any two means not included in the same parenthesis do not differ significantly at $p$=0.05. Any two means not included in the same parenthesis are statistically significantly different at $p \leqq 0.05$.

## 3. Results

The results of the studies on methotrexate-induced lipid peroxidation and its inhibition with ascorbic acid and water extract of *S. platensis* are shown in bar diagram along with standard error of estimate (Figures 1-8). Interpretation of the result is supported by ANOVA analysis and also by multiple comparison analysis using least significant different procedure.

From Figures 1-4, it is evident that incubation of the liver homogenates with methotrexate caused an increase in MDA and 4-HNE content with respect to control to a significant extent after incubation for varying period of time. But the MDA and 4-HNE contents were significantly reduced with respect to drug treated group when the tissue homogenates were treated with methotrexate in combination with antioxidants (AA/SP). The observations suggest that methotrexate could significantly induce the lipid peroxidation process. So the lipid peroxidation induction capacity of the drug may be related to its toxic potential. It was also found that the antioxidants (AA/SP) could suppress the methotrexate-induced lipid peroxidation to a significant extent. It was also noted that when the tissue homogenates were treated with ascorbic acid alone, it showed some increase in MDA content with respect to control (2 h). But when the tissue

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**Table 3**: Anova and multiple comparison for changes of GSH content.

| Antioxidant | Incubation (h) | Analysis of variance and multiple comparison |
|---|---|---|
| Ascorbic acid | 2 | F1=29.31 [df=(2,8)], F2=1.95 [df=(4,8)], Pooled variance (S2)* =4.64, Critical difference (p=0.05)# LSD =4.05, Ranked means** (D) (DA, A) |
| | 4 | F1=33.57 [df=(2,8)], F2=0.29 [df=(4,8)], Pooled variance (S2)* =15.17, Critical difference (p=0.05)# LSD =7.33, Ranked means** (D) (DA, A) |
| | 24 | F1=44.22 [df=(2,8)], F2=1.05 [df=(4,8)], Pooled variance (S2)* =10.19, Critical difference (p=0.05)# LSD =6.01, Ranked means** (D) (DA, A) |
| Aqueous extract of *S. platensis* | 2 | F1=32.78 [df=(2,8)], F2=3.79 [df=(4,8)], Pooled variance (S2)* =26.35, Critical difference (p=0.05)# LSD =9.66, Ranked means** (D) (DA, A) |
| | 4 | F1=23.31 [df=(2,8)], F2=1.78 [df=(4,8)], Pooled variance (S2)* =73.10, Critical difference (p=0.05)# LSD =16.09, Ranked means** (D) (DA, A) |
| | 24 | F1=30.32 [df=(2,8)], F2=2.17 [df=(4,8)], Pooled variance (S2)* =66.14, Critical difference (p=0.05)# LSD =15.31, 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.

* Error mean square, # Critical difference according to least significant procedure (34-35), ** Two means not included within same parenthesis are statistically significantly different at $p$=0.05 level.



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homogenates were treated with spirulina alone then there is decrease in MDA content with respect to corresponding control.

From Figures 5-8 it is evident that incubation of the liver homogenates with methotrexate caused decrease in GSH and NO content with respect to control to a significant extent. The decrease in GSH and NO content was associated with an increase in lipid peroxidation. When the tissue homogenates were treated with drug and antioxidants (AA/SP) then the GSH and NO levels were increased in comparison to drug treated group of corresponding hours. Again, when the tissue homogenates were treated with antioxidants (AA/SP) alone the GSH and NO contents were also increased in comparison to the control samples. The increase in GSH and NO level suggest the antiperoxidative potential of antioxidants (AA/SP). From Tables 1-4, it is seen that there is significant differences among various groups (F1), but within a particular group, differences (F2) are insignificant. The Tables also indicate that the content of MDA/ GSH/ 4-HNE/NO in drug-treated group is statistically significantly different from the drug and antioxidant treated group and only antioxidant treated group.

## 4. Discussion

It has been understood that lipid peroxidation induction capacity of drugs may be related to their toxic potential. This is an analogy to insulin deficiency diabetes induced by alloxan [36] that are mediated through free radical mechanism. Increase in MDA and 4-HNE levels or decrease of GSH and NO levels of the drug treated group suggest the occurrence of lipid peroxidation. Lipid peroxidation leads to the generation of variety of cytotoxic products. More over it causes disruption of membrane structure and change in fluidity. MDA is a highly reactive three-carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism [37]. 4-HNE is a major product formed due to lipid peroxidation through the oxidative degradation of arachidonic acid bound to phospholipid [38, 39]. So the decrease in MDA and 4-HNE content of tissue homogenates when treated with drug and antioxidants (AA/SP) implies the free radical scavenging property of antioxidants. The increase in MDA content (2 h) with respect to control when the tissue homogenates were treated with ascorbic acid alone indicates its pro-oxidants effect. It was postulated that ascorbic acid could reduce $Fe^{3+}$ to $Fe^{2+}$, which promotes generation of hydroxyl radicals and other reactive oxygen species through Fenton's reaction [40]. Many known antioxidants like vitamins [41], estrogen [42, 43], superoxide

**Table 4**: Anova and multiple comparison for changes of no content.

| Antioxidant | Incubation (h) | Analysis of variance and multiple comparison |
|---|---|---|
| Ascorbic acid | 2 | F1=38.01 [df=(2,8)], F2=0.175 [df=(4,8)], Pooled variance (S2)* =6.06, Critical difference (p=0.05)# LSD =4.63, Ranked means** (D) (DA, A) |
| | 4 | F1=95.53 [df=(2,8)], F2=0.334 [df=(4,8)], Pooled variance (S2)* =17.28, Critical difference (p=0.05)# LSD =7.83, Ranked means** (D) (DA, A) |
| | 24 | F1=91.83 [df=(2,8)], F2=0.334 [df=(4,8)], Pooled variance (S2)* =20.40, Critical difference (p=0.05)# LSD =8.50, Ranked means** (D) (DA, A) |
| Aqueous extract of *S. platensis* | 2 | F1=46.04 [df=(2,8)], F2=0.974 [df=(4,8)], Pooled variance (S2)* =9.06, Critical difference (p=0.05)# LSD =5.67, Ranked means** (D) (DA, A) |
| | 4 | F1=43.37 [df=(2,8)], F2=2.16 [df=(4,8)], Pooled variance (S2)* =35.63, Critical difference (p=0.05)# LSD =11.24, Ranked means** (D) (DA, A) |
| | 24 | F1=37.43 [df=(2,8)], F2=1.39 [df=(4,8)], Pooled variance (S2)* =54.38, Critical difference (p=0.05)# LSD =13.88, 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.
* Error mean square, # Critical difference according to least significant procedure (34-35), ** Two means not included within same parenthesis are statistically significantly different at $p$=0.05 level.



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dismutase [44] and flavonoids have been reported to act as pro-oxidant in presence of transition metals [45, 46] or at high concentration [47]. Glutathione is an important antioxidant and plays a very important role in the defense mechanism for tissue against the reactive oxygen species [48]. The depletion of GSH is associated with increase in lipid peroxidation. The decrease in GSH level may be the consequence of enhanced utilization of this compound by the antioxidant enzymes glutathione peroxidase and glutathione-S-transferase. NO plays a very important role in host defense [49]. So the increase in GSH and NO content of tissue homogenates when treated with drug and antioxidants (AA/SP) implies the free radical scavenging activity of the antioxidants. It has been found that spirulina reduces the hepatic cytochrome $P_{450}$ content and increases the hepatic glutathione-S-transferase activity [50]. It has also been proposed that NO causes chain termination reactions during lipid peroxidation as observed in low-density lipoprotein oxidation as well as in chemical systems [51-54]. The data presented in this work demonstrate the lipid peroxidation induction potential of methotrexate, which may be related to its toxic potential. The results also suggest the antiperoxidative effects of ascorbic acid and water extract of *S. platensis* and demonstrate their potential to reduce methotrexate-induced lipid peroxidation and thus to increase therapeutic index of the drug by the 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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