ORIGINAL ARTICLE

# Fate of biomolecules during carbon tetrachloride induced oxidative stress and protective nature of *Ammoniac baccifera* Linn.: A natural antioxidant

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Ethanol extract of *Ammannia baccifera* was studied for its protective nature against the oxidative damage of lipids, proteins and DNA in carbon tetrachloride ($CCl_4$)-induced toxicity in rats. $CCl_4$ administration to albino Wistar rats increased the levels of lipid peroxidation, protein carbonyls and decreased the levels of total sulfhydryls. $CCl_4$ also induced the elevation of DNA damage measured by the comet assay. The study revealed that the administration of the ethanol extract of *A. baccifera* to $CCl_4$ intoxicated rats could significantly ($P < 0.01$) decrease the levels of lipid peroxidation, protein carbonyls and increased the levels of total sulfhydryls in a dose-dependent manner. It was also found that the ethanol extract of *A. baccifera* prevent the $CCl_4$-induced elevation of DNA damage in hepatocytes. These results suggest that treatment with the ethanol extract of *A. baccifera* can minimize the deleterious effects caused by $CCl_4$ through its strong antioxidative and free radical scavenging properties.

**Key words:** Antioxidants, comet assay, DNA, protein carbonyls, total sulfhydryls

## INTRODUCTION

Oxidative stress occurs when antioxidant defence mechanisms are overwhelmed by free radicals. This imbalance can be caused by either increased free radical formation or decreased antioxidant capacity.[1] Oxidative stress is associated with DNA damage, lipid peroxidation and protein cross-linking and, via oxidative changes to key biomolecules, is believed to increase the risk of cancer, coronary heart disease, cataract and dementia and to be deeply involved in the ageing process.[2,3] Epidemiological studies have shown that high intake of antioxidants is associated with lower risk of chronic diseases.[4,5] Great efforts have been made in an attempt to find safe and potent natural antioxidants from plant resources, as the synthetic antioxidants, such as propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ), have been suspected of being responsible for liver damage and carcinogenesis.[6] Consequently, over recent years many different supplementation trials have been implemented, aimed at improving clinical outcomes by boosting antioxidant levels. These trials have included supplementation with individual antioxidants, antioxidant combinations and antioxidant-rich foods, such as fruit and vegetable juices and other plant extracts. To ensure that data from these trials are interpreted correctly, it is essential that suitable biomakers of oxidative damage to lipids (thiobarbituric acid (TBA) reactive substances/ malondialdehyde (MDA), proteins (inzy for tuberculosis and typhoid fever. This herb is used as laxative, rubefacient and external remedy for ring worm.[8] It is also reported to possess antiurolithiasis, antibacterial, CNS depressant[9-17] and antisteroidogenic activities.[12] However, no work has been reported on the protective effect, if any, of ethanol extract of *A. baccifera* (EEAB) during $CCl_4$-induced oxidative damage. The objective of the present work was to examine if external supplementation with the EEAB affords protection against $CCl_4$ toxicity. Rats were exposed to a toxic dose of $CCl_4$ and administered with two doses of EEAB (250 and 500 mg/kg body wt.) and compared with those receiving $CC1_4$ alone. The protective action was monitored by measuring the levels of lipid peroxidation, protein carbonyl and total sulfhydryl groups. The protective effect of EEAB against DNA damage caused by $CCl_4$ was also studied using the comet assay.

## MATERIALS AND METHODS

### Plant Material and Extraction

*A. baccifera* whole herb was collected from the surroundings of Tirumala Tirupathi hills, Chittoor dist., Andhra Pradesh, India, and identified by comparison with a voucher specimen deposited in the herbarium of the Department of Botany, Sri Venkateswara University, Tirupati, Chittoor dist., Andhra Pradesh, India. A 100 g of dried powder of

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whole herb of *A. baccifera* was soxhlet extracted with 500 mL of ethanol for more than 6 h. The extract was concentrated in a rotary evaporator and was stored at −20°C for further usage.

**Animals**

Male Wistar rats (200 ± 50 g) were selected for the study and maintained at controlled temperature of 25-28°C with a 12 h light/dark cycle and fed a standard diet and water *ad libitum*. Animal studies were conducted according to the Institute Animal Ethics Committee regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The animals were acclimatized to laboratory condition for 10 days before commencement of experiment.

**Chemicals**

For the present study, the following chemicals were used: Sodium dodecyl sulphate (SDS), 1, 1, 2, 2-tetraethoxy propane (TEP), TBA, di-nitrophenyl hydrazine (DNPH), guanidine hydrochloride, 5, $5^1$ dithiobis-2-nitro benzoic acid (DTNB), normal melting agarose, low melting agarose, lodium lauryl sarcosinate (SLS) and Triton X-100 that were obtained from Sigma-Aldrich chemicals Pvt. Ltd, USA. All other acids, bases, salts and solvents used were of analytical grade.

**Carbon Tetrachloride-induced Liver Damage in Rats**

Healthy male albino rats were divided into four groups each containing six animals. Group I (control) received olive oil (1 mL/kg body weight, i.p.), Group II ($CCl_4$ induced) received 30% $CCl_4$ in olive oil (1 mL/kg body weight, i.p.), Group III, and IV received 250 and 500 mg/kg body weight of EEAB p.o, respectively, once in a day and $CCl_4$ as mentioned above. Treatment duration was 10 days and the dose of $CCl_4$ was administered after every 72 h.[13] Animals were sacrificed, 24 h after the last injection. After killing, livers were collected from all groups and washed in PBS and soaked on a blotting paper to remove the blood and to be used for biochemical studies.

**Determination of Biochemical Parameters in Liver**

**Preparation of liver homogenate**

Prior to biochemical analysis, each liver sample (100 mg/mL buffer) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10,000 rpm for 15 min and the supernatant obtained was used for biochemical analysis. All liver parameters were expressed as activity per mg protein. The protein concentration in each fraction was determined by Lowry's method[14] using crystalline bovine serum albumin as a standard.

**Determination of lipid peroxidation**

The mean MDA content (l mol/mg protein), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reacting substances (TABRS) by the method of Ohkawa *et al*.[15]

**Estimation of protein carbonyl content**

The carbonyl content of liver homogenates of control and treated rats was evaluated by the method of Levine *et al*.[16] A 100 μL of homogenates (10%) were incubated with 100 μL of DNPH (100 mmol/L) for 60 min with vigorous intermittent shaking with a time gap of 10 min. Subsequently, the protein was precipitated from the solution with the use of 20% TCA. The pellet was washed after centrifugation (3400 g) with ethyl acetate: Ethanol (1: 1 v/v) mixture three times to remove excess of DNPH (Each washing step was followed by centrifugation). After the final centrifugation, the protein pellet was dissolved in 1.5 mL of 6 μM guanidine hydrochloride, pH 6.5 and should be incubated at 50°C with continuous shaking till the pellet dissolves. The carbonyl content was evaluated in a spectrophotometer at wavelength of 370 nm. The control sample was made of the equivalent amount of the homogenate and 100 μL of 2 M HCl and the same procedure was followed. A standard curve of bovine serum albumin was included in each assay to determine linearity and measure the extent of derivatization. The results were presented in nmol/mg protein.

**Estimation of total sulfhydryl (thiol) content**

The total sulphydryl group was estimated by the method of Sedlack and Lindsay.[17] The method is based on the reaction of tissue homogenate with 5, $5^1$ dithiobis-2-nitro benzoic acid (DTNB), where in DTNB is reduced by the thiol group to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole-SH. The aliquot of tissue homogenate was mixed with 1.5 mL of buffer and 0.1 mL of DTNB. The mixture was made up to 10 mL with absolute ethanol. A reagent blank without the sample and the blank sample without DTNB were prepared in the same manner. The test tubes had stopper and were allowed to stand with occasional shaking for 15 min at room temperature. The absorbance of the clear supernatant was read at 412 nm. Calibration curves were obtained with reduced glutathione as standard. Total sulfhydryl group is expressed as μg of GSH/mg protein.

**Detection of DNA damage by alkaline single-cell gel electrophoresis (Comet assay)**

Possible DNA damage induced by $CCl_4$ at different doses was detected using the alkaline single-cell gel electrophoresis (Comet) assay following a simplified protocol with slight modifications.[18,19] At the end of the treatment with $CCl_4$, rats of all groups were killed and the livers were dissected out. To prepare the single-cell suspension, about 200 mg of liver tissue was mixed with 3 ml of hypotonic solution [75 mM NaCl, 24 nM EDTA (pH 7.5)] homogenized with a Potter homogenizer, centrifuged at 700 rpm for 5 min, suspended

in the hypotonic solution, centrifuged and resuspended in the hypotonic solution. Each single-cell suspension of liver was prepared freshly.[20]

Half frosted microscope slides were coated with 1% normal melting agarose in physiological buffer saline (PBS). The slides were then allowed to dry at room temperature protected from dust and other particles. An aliquot of 10 μL of liver homogenate was mixed with 140 μL of 1% low melting point agarose in Milli-Q water. This mixture was then layered on the top of the pre-coated slide and covered with a 24 × 50 mm cover slip and kept on ice to allow the agarose to solidify. After the agarose had solidified on ice for at least 10-15 min, the cover slip was gently removed and a third layer of 0.5% low melting point agarose was layered on the top of the second layer and covered with a cover slip and kept on ice for 5-10 min. After the agarose had solidified, the cover slip was gently removed and the slides were carefully immersed in a freshly prepared ice-cold lysing solution (2.5 ml NaCl, 100 mM $Na_2EDTA$, 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% triton X-100 and 10% DMSO. pH was adjusted to 10 with NaOH). After lysis overnight at 4°C the slides were placed in an electrophoresis unit and the buffer reservoirs were gently filled with fresh electrophoresis buffer [1 mM $Na_2EDTA$ and 0.3 M NaOH (pH > 13.1)] to a level of 0.25 cm above the microscope slides and incubated for 20 min at 4°C to allow the unwinding of DNA. Keeping the same temperature, the slides were subjected to electrophoresis (25 V, 400 mA) for another 25 min. After electrophoresis, the slides were placed on a tray to remove alkali and detergents and neutralized with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 10 min. Excess liquid was carefully removed from each slide using a paper towel. The microscope slides were carefully dried at room temperature avoiding dust and other particles and then stored in a sealed container until the day of image analysis. The dried microscope slides were stained with ethidium bromide in water (20 μg/mL; 50 μL/slide). The slides with a cover slip were examined at 400x magnification under a fluorescence microscope,and the photomicrographs of cells were taken. A 150-200 randomly selected cells (5-7 zones/slide) in each slide were counted (4 slides/animals in each group) to determine the number of damaged cells and then the percentage of damage cells were calculated using the formula:

% Damage = (Number of damaged cells/Total number of cells counted) × 100.

The length of the comet tail was determined by using an oculometre affixed in the eye piece of the microscope. The comet tail length was measured between the edge of the comet head and the end of the comet tail, calculated in micrometers.[21] The results were expressed as

1. percentage of cells with tail (tailed cells) in each group was scored and
2. average tail length due to DNA migration in each group.

**Statistical Analysis**

The data obtained from experiments were analysed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT). The calculations were performed using SPSS 15.0 to identify the differences between the mean values of different groups. A *P*-value < 0.01 was considered significant.

## RESULT AND DISCUSSION

The $CCl_4$ is one of the most commonly used hepatotoxins in the experimental study of liver diseases.[22] Lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of $CCl_4$.[23] Lipid peroxidation is caused by the carbon trichloromethyl radical, $CCl_3$•. $CCl_4$ is biotransformed by cytochrome $P_{450}$ to the trichloromethyl free radical that induces membrane lipid peroxidation and disturbs $Ca_2$ + homeostasis to produce hepatocellular injury.[24] MDA, an end product of lipid peroxidation, is widely used as a marker of lipid peroxidation. $CCl_4$ administration resulted in a significant increase in MDA.[25] In the present study, a marked increase in the mean MDA level was found in the liver of Group II ($CCl_4$-exposed) rats relative to control (Group I) rats [Figure 1]; this increase was statistically significant ($P < 0.01$). Treatment with the EEAB in Group III and Group IV rats was found to result in a significant ($P < 0.01$) lowering of the mean MDA concentration, presumably by limiting lipid peroxidation in the hepatic tissue. Prasenjit *et al*.[26] have demonstrated that the administration of natural antioxidants reduces the accumulation of lipid peroxides

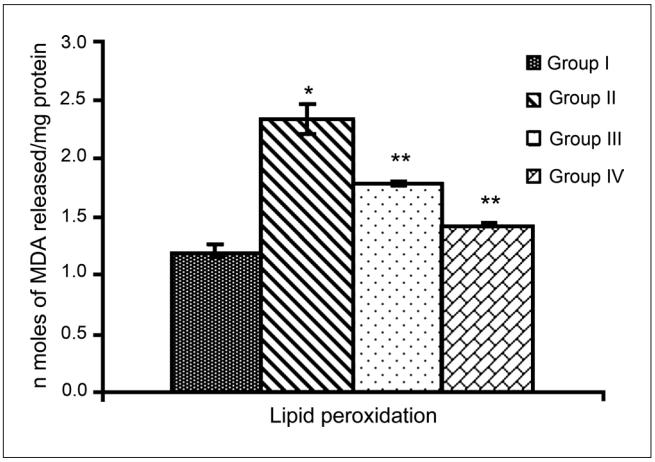


**Figure 1:** Effect of ethanol extract of *A. baccifera* on lipid peroxidation in the liver of control and experimental rats. Values are expressed as mean ± standard error of six rats in each group. *$P < 0.01$ when compared with group I; **$P < 0.01$ when compared with group II. MDA = malondialdehyde. group I, control; group II, $CCl_4$ treated; group III, $CCl_4$ + 250 mg/kg b. wt. of ethanol extract of *A. baccifera* and group IV, $CCl_4$ + 500 mg/kg b. wt. of ethanol extract of *A. baccifera*

in tissues; in the similar way, our results reveals that administration of the EEAB dose dependently reduced the accumulation of lipid peroxides in liver tissue.

Proteins constitute one of the major targets of ROS, and oxidation of proteins can lead to a loss of protein function as well as conversion of proteins to forms that are more susceptible to degradation by proteinases. ROS elicit a variety of modifications in amino acid residues, including cysteines, methionine, tryptophan, arginine, lysine, proline and histidine.[27] Among amino acid modifications by ROS is the formation of carbonyls as a result of oxidation of arginine, lysine, threonine or proline. Protein carbonylation is the result of secondary reactions of amino groups of lysine residues with reducing sugars or their oxidation production (glycation/glycoxidation reactions) and also by reactions of lysine, cysteines or histidine amino acids with α-and β-unsaturated aldehydes formed during the peroxidation of polyunsaturated fatty acids.[28] Thus, some studies, both *in vivo* and *in vitro*, have provided growing evidence that carbonyl proteins could be a useful biological marker of the oxidative stress.[29] In the present study, we have explored the effects of $CCl_4$ on the protein carbonyl formation and attenuation by two doses of EEAB in liver of rats [Figure 2]. The result demonstrated a significant increase ($P < 0.01$) in protein carbonyls in the liver tissue of rats administered with $CCl_4$ (Group II) when compared with the control animals (Group I). This might indicate an increased oxidatively modified protein in liver with the increase of hepatotoxicity. Administration of two doses of EEAB in Group III and Group IV animals caused a significant ($P < 0.01$ and $P < 0.001$, respectively) decrease in the levels of protein carbonyls in the liver tissue, when compared to $CCl_4$ intoxicated (Group II) rats [Figure 2]. Abira Sarkar *et al*.[30] reported that mangiferin, a natural antioxidant, protects proteins against benzo[a]pyrene-induced oxidative damage. Similar results have observed with EEAB administration to rats induced with $CCl_4$.

Sulfhydryl groups contribute significantly to the antioxidant capacity of tissues and their oxidation could be considered as oxidative damage.[31] These groups exist in two forms as protein bound sulfhydryls and free sulfhydryls. These sulfhydryl groups constitute the active site of major functional proteins, oxidation of − SH groups during oxidative damage leads to the inactivation of these proteins. The decreased levels of these − SH groups are good indicators of oxidative stress.[32] In the present study acute exposure of rats to $CCl_4$ (Group II) resulted in a significant ($P < 0.01$) decrease in total sulfhydryl groups when compared with control animals (Group I). The decrease of these − SH groups may be due to the oxidation of − SH groups to thiyl radicals by xenobiotics.[33] These changes were reversed to near normal values in EEAB-treated animals [Figure 3]. There was a significant ($P < 0.001$; $P < 0.01$) increase in the levels of total sulfhydryls in Group III and Group IV rats ($CCl_4$ and two doses of EEAB treated) when compared with the $CCl_4$ alone induced (Group II) rats [Figure 3]. This suggests that EEAB prevents oxidation of − SH groups by scavenging free radicals generated by $CCl_4$. Raja *et al*[34] reported that the decreased − SH content during oxidative stress induced by $CCl_4$ was brought back to normal levels by the administration of hydro-alcoholic extract of *Cytisus scoparius* and results obtained in the present study are inconsistent with this report.

Oxidative damage to DNA seems to be an important factor in developing many human diseases. DNA strand breaks

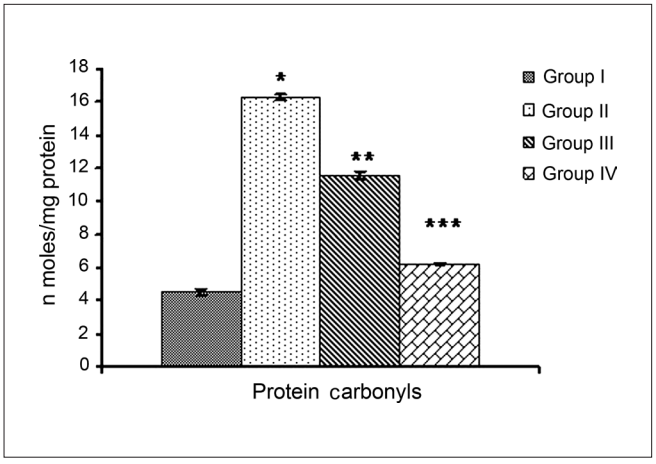


**Figure 2:** Effect of ethanol extract of *A. baccifera* on the level of protein carbonyls in the liver of control and experimental rats. Values are expressed as mean ± standard error of six rats in each group. *$P < 0.01$ when compared with group I; **$P < 0.01$ when compared with group II; ***$P < 0.001$ when compared with group II. group I, control; group II, $CCl_4$ treated; group III, $CCl_4$ + 250 mg/kg b. wt. of ethanol extract of *A. baccifera* and group IV, $CCl_4$ + 500 mg/kg b. wt. of ethanol extract of *A. baccifera*

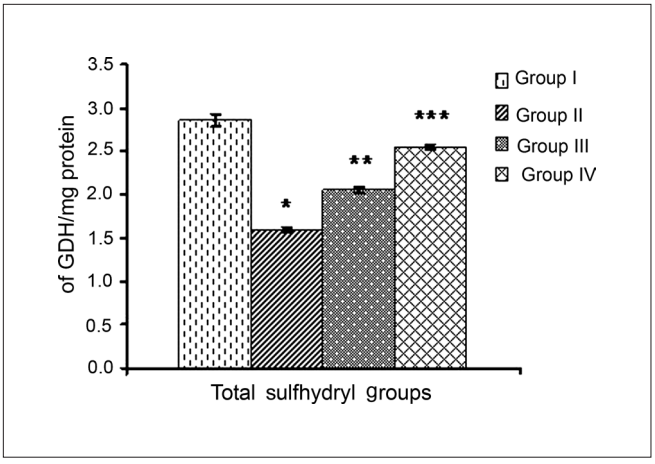


**Figure 3:** Effect of ethanol extract of *A. baccifera* on the level of total sulfhydryl groups in the liver of control and experimental rats. Values are expressed as mean ± standard error of six rats in each group. *$P < 0.01$ when compared with group I; **$P < 0.001$ when compared with group II; ***$P < 0.01$ when compared with group II. GSH = reduced glutathione. Group I, control; group II, $CCl_4$ treated; group III, $CCl_4$ + 250 mg/kg b. wt. of EEAB and group IV, $CCl_4$ + 500 mg/kg b. wt. of ethanol extract of *A. baccifera*

can originate from the direct modification of DNA by chemical agents or their metabolites; from the processes of DNA excision repair, replication and recombination; or from the process of apoptosis.[35] Direct breakage of the DNA strands occurs when reactive oxygen species interact with DNA.[36] $CCL_4$ is metabolized to reactive intermediates that then covalently bind to DNA and initiate oxidative stress and the formation of reactive oxygen species (ROS), resulting in the oxidation of DNA.[37] In the present investigation, possible DNA damage induced by $CCl_4$ in control and experimental rats was detected using the alkaline single cell gel electrophoresis (SCG) (comet) assay following a simplified protocol with slight modifications.[18] The use of this alkaline SCG assay as a method to detect genotoxicity and cytotoxicity *in vivo* is well documented, and DNA damage thus detected has been used to predict the presence of genotoxic metabolites in specific organs.[38] Percentage of damaged cells in each experimental group was represented in Table 1. Hepatocytes from the animals of Group I (control group) showed very few (4.32%) damaged cells at the time of experiment, whereas in Group II ($CCl_4$ induced) 59.13% cells showed a distinct comet tail which came down significantly to 34.61% in Group III ($CCl_4$ and 250 mg/kg b. wt. of EEAB treated) and 14.32% in Group IV ($CCl_4$ and 500 mg/kg b.wt. of EEAB treated) animals.

The microscopical image resulting from $CCl_4$-induced damaged hepatocytes (Groups II) are comets with small or non-existent head and large, diffused tails. The average tail length was increased to about 89.23% in Group II ($CCl_4$ injected) rats in comparison with Group I, whereas the tail lengths decreased by 41.6% in Group III (250 mg/kg b.wt. of EEAB treated) and 66.3% in Group IV (500 mg/kg b.wt. of EEAB treated), respectively, in comparison to the $CCl_4$-intoxicated rats (Group II) [Table 1]. These results demonstrated that EEAB provides a beneficial effect against $CCl_4$-induced oxidative DNA damage, although the mechanism is not entirely clear. The protective effect of EEAB on oxidative DNA damage may be due to its scavenging property of free radicals. Rajath *et al.*,[20] reported that supplementation with an antioxidant can reduce the DNA damage induced during oxidative stress and the results from the present investigation are in correlation with this report.

It can be concluded that EEAB exerts protective effect against the oxidative damage caused by $CCl_4$ on lipids, proteins and DNA. The protective effect of EEAB may be due to its strong antioxidant and free radical scavenging properties as evidenced by the significant dose-dependent increase in the level of sulfhydryl groups and decrease in the levels of lipid peroxidation and protein carbonyl groups. Comet assay results also indicate that EEAB can ameliorate DNA damage in $CCl_4$ intoxicated rats. This suggests that supplementation with EEAB is associated in lowering the deleterious effects caused during oxidative stress on biological molecules. However, the detailed mechanisms are not fully understood and remain to be further resolved.

**Table 1: Assessment of the protective activity of EEAB against $CCl_4$-induced DNA damage in hepatocytes of rats**

| Group | Damaged cells showing comet (%) | Average tail length (μM) |
|---|---|---|
| I | 4.32 ± 1.02[a] | 6.91 ± 0.23[a] |
| II | 59.13 ± 1.62[b] | 64.15 ± 4.01[b] |
| III | 34.61 ± 1.21[c] | 37.5 ± 2.04[c] |
| IV | 14.32 ± 0.89[d] | 21.6 ± 3.08[d] |

Values are expressed as mean ± SE. Means having same superscript in each column do not differ significantly at 0.01 level by Duncan's Multiple Range Test Group I: control; Group II: $CCl_4$ alone; Group III: $CCl_4$ + 250 mg/kg b. wt. of EEAB; Group IV: $CCl_4$ + 500 mg/kg b. wt. of EEAB

**Numerical data of the graphs**

| Group | Lipid peroxidation | Protein carbonyls | Total sulfhydryls |
|---|---|---|---|
| I | 1.201 ± 0.052[a] | 4.51 ± 0.159[d] | 2.855 ± 0.062[a] |
| II | 2.336 ± 0.119[c] | 16.311 ± 0.161[a] | 1.59 ± 0.022[b] |
| III | 1.781 ± 0.010[d] | 11.56 ± 0.243[b] | 2.05 ± 0.033[c] |
| IV | 1.426 ± 0.013[b] | 6.231 ± 0.071[c] | 2.548 ± 0.022[d] |

Values are expressed as mean ± SE; Means having same superscript in each column do not differ significantly at 0.01 level by Duncan's Multiple Range Test. Group I: control; Group II: $CCl_4$ alone; Group III: $CCl_4$ + 250 mg/kg b. wt. of EEAB; Group IV: $CCl_4$ + 500 mg/kg b. wt. of EEAB

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