Chapter 3

*IN VIVO* ANTIOXIDANT ACTIVITY OF *ACTINIOPTERIS RADIATA*
3.0. INTRODUCTION:

Oxidative stress has been implicated in the pathogenesis of many diseases and conditions, including ageing (Ames and Shigenaga, 1992), atherosclerosis (Esterbauer et al., 1997) cancer (Cheeseman, 1993) smoking (et al., 1995) and respiratory disease (Wood et al., 2005). Oxidative stress occurs when excessive quantities of reactive oxygen species (ROS), such as the superoxide (O$_2^-$) and hydroxyl (OH) radicals, overwhelm host antioxidant defenses, resulting in cellular damage. ROS generation is essential to many important biological processes. For example, ROS are generated by all aerobic organisms as an unwanted by-product of normal oxygen metabolism (Davies, 1995). Thus, the body carries antioxidants, which inhibit the damaging reactions of ROS in tissues.

An antioxidant is a substance that when present at low concentrations, compared to those of the oxidisable substrate, significantly delays, or inhibits, oxidation of that substrate (Halliwell, 1997). Some of the mechanisms by which antioxidants act include - removing O$_2$ or decreasing local O$_2$ concentrations, removing catalytic metal ions, removing key ROS, e.g. O$_2^-$ and H$_2$O$_2$ scavenging initiating radicals, e.g. 'OH, RO', RO$_2$', breaking the chain of an initiated sequence, quenching or scavenging single oxygen, enhancing endogenous antioxidant defenses by up-regulation the expression of genes encoding the antioxidant enzymes, repairing oxidative damage caused by radicals, increasing elimination of damaged molecules and not repairing excessively damaged molecules in order to minimize introduction of mutations (Gutteridge, 1994).

The most suitable antioxidants are those that perform one or more of the above functions, without generating any toxic or reactive end products. As long as adequate amounts of antioxidants are present to provide sufficient protection, the oxidant-antioxidant balance is maintained. However, if antioxidant protection is overwhelmed, due to disease, diet, lifestyle or other environmental factors, oxidative damage will occur (Gutteridge, 1994; Halliwell, 1996; Sen, 1995; Rock et al., 1996).

The recognition of oxidative stress and impaired antioxidant defenses as a feature underpinning many chronic diseases and conditions has resulted in many different antioxidant supplementation trials. These trials have been aimed at correction the
oxidant-antioxidant imbalance, thereby preventing and improving adverse clinical outcomes. However, to date, interpretation of many of these trials has been limited because an appropriate antioxidant marker has not been included as an outcome measure. Consequently, if a treatment effect is not observed, it is not possible to determine if this is due to inadequate antioxidant supplementation or lack of clinical efficacy of adequate supplement. When monitoring antioxidant supplementation, it is important to firstly confirm that the supplement has been absorbed, by monitoring host antioxidant levels. Secondly, it is important to examine the effect of the antioxidant supplement on markers of oxidative stress. This combined data will help elucidate that mechanism by which various antioxidants act.

Animal models, which mimic specific characteristics of human oxidative stress, are useful in evaluating biomarkers as surrogate endpoints for oxidative stress incidence. Particularly, the correlation of surrogate end point modulation, to effects on oxidative stress incidence, in such models can provide strong evidence for validating the surrogate endpoint. This correlation can strengthen efficacy claims prior to definitive clinical validation (Kelloff et al., 1999). The animal models especially rat and mice have been used extensively to study different deleterious effects of oxidative stress (Van Zwieten, 1984). As the architecture, anatomy and physiology of the internal organs of rodents resembles the human anatomy (Tamayo, 1983), Albino rats of Wistar strain are used in the present study to evaluate the therapeutic effect of EEAR.

Oxidative stress and disturbed antioxidant status are well described characteristics of many disease and conditions. This highlights the potential for antioxidant supplementation to correct the oxidant-antioxidant balance and potentially improve clinical outcomes. When supplementing the antioxidants, it is important that appropriate biomarkers are used to assess the suitability of the supplement.

**Carbon tetrachloride as oxidative stress inducer:**
Chemical-induced oxidative stress has been extensively studied in animal models, and the changes in biochemical pathways in association with pathological progress in various diseases under toxic insults have been well documented. Many compounds including clinically useful drugs can cause cellular damage through metabolic activation of the
compound to highly reactive substances such as free radicals. One such xenobiotic which causes oxidative damage to cells is carbon tetrachloride (Jeon et al., 2003).

Exposure to various organic compounds including a number of environmental pollutants and drugs can cause cellular damages through metabolic activation of those compounds to highly reactive substances such as reactive oxygen species (ROS). Free radical induced lipid peroxidation is believed to be one of the major causes of cell membrane damage leading to a number of pathological situations (Halliwell, 1993; Oberley, 1988; Slater, 1984). Reports from several investigators have established that the industrial solvent, carbon tetrachloride (CCl₄) is a potent environmental hepatotoxin (Sarkar et al., 2006; Abraham et al., 1999; Szymonik-Lesiuk et al., 2003; Guven et al., 2003). A number of recent reports clearly demonstrated that in addition to hepatic problems, CCl₄ also causes disorders in kidneys, lungs, testis and brain as well as in blood by generating free radicals (Charbonneau et al., 1986; Ahmad et al., 1987; Ohta et al., 1997; Ozturk et al., 2003). Perez et al., (1987), Ogeturk et al., (2005) and Churchill et al., (1983) suggested that exposure to this solvent causes acute and chronic renal injuries. In addition, reports on various documented case studies established that CCl₄ produces renal diseases in humans (Ruprah et al., 1985; Gosselin et al., 1984). Extensive evidence demonstrated that 'CCl₃ and 'Cl are formed as a result of the metabolic activation of CCl₄, which in turn, initiate lipid peroxidation process. A known potent antioxidant, vitamin E, could protect CCl₄ induced liver injury indicating that oxidative stress is responsible for CCl₄ induced hepatic disorder in this particular model (Yoshikawa et al., 1982; Weber et al., 2003).

Generation of free radicals from carbon tetrachloride:
The first step in CCl₄ metabolism is a one-electron reduction and homolytic cleavage catalyzed by Cytochrome P₄₅₀ of the mixed function oxidase system to yield the trichloromethyl radical (Brattin et al., 1985; Recknagel et al., 1977). Cytochrome P₄₅₀ is maintained in the reduced form by NADPH. The CCl₃ radical rapidly reacts with molecular oxygen to form the trichloromethyl peroxyl radical (Slater, 1982). The CCl₃OO' radical is more electrophilic than the CCl₃ radical and may be more responsible for attacks on unsaturated fatty acids, leading to lipid peroxidation (Slater, 1982). CCl₃'
may be more involved in covalent binding reactions of CC14 (Slater, 1982). The CC13 radical can bind to cellular molecules (nucleic acid, protein, lipid), impairing crucial cellular processes such as lipid metabolism with the potential outcome of fatty degeneration (steatosis). Adduct formation between CC13 and DNA is thought to function as initiator of hepatic cancer. This radical can also react with oxygen to form the trichloromethylperoxy radical (CC13OO'), a highly reactive species. CC13OO' initiates the chain reaction of lipid peroxidation which attacks and destroys PUFA’s (Fig. 3.1), in particular those associated with phospholipids. This affects the permeabilities of mitochondrial, endoplasmic reticulum and plasma membranes resulting in the loss of cellular calcium sequestration and homeostasis, which can contribute heavily to subsequent cell damage. Among the degradation products of fatty acids are reactive aldehydes, especially 4-hydroxynonenal which bind easily to functional groups of proteins and inhibit important enzyme activities.

![Fig. 3.1. Biotransformation of CC14 (Source: McGregor and Lang, 1996).](image-url)
CCl₄ intoxication also leads to hypomethylation of cellular components; in the case of RNA the outcome is thought to be inhibition of protein synthesis, in the case of phospholipids it plays a role in the inhibition of lipoprotein secretion. Oxidative stress plays an important role in the pathogenesis of CCl₄ induced tissue injury. Various studies demonstrated that liver is not the only target organ of CCl₄ and it causes free radical generation in other tissues also such as kidneys, heart, lung, testis, brain and blood (Ozturk et al., 2003; Ohta et al., 1997; Ahmad et al., 1987). It has also been reported that exposure to CCl₄ induces acute and chronic renal injuries (Naveen Tirkey et al., 2005; Perez et al., 1987; Churchill et al., 1983). Suja et al., (2004) reported that hepatitis caused by viral infection is similar to the hepatitis due to the CCl₄ intoxication. Abraham et al., (1999) reported that alcoholic liver cirrhosis is similar to the liver cirrhosis caused by CCl₄ toxicity. Oxidative stress caused by CCl₄ toxicity is similar to the oxidative stress caused due to the radiation and aging (American family physician, 1992).

Antioxidative action plays an important role in protection against CCl₄ induced tissue injury. Protective effects of various natural products with antioxidant and hepatoprotective efficacy like red ginseng, Salvia miltiorrhiza, Antrodia camphorata, silymarin, vitamin E, vitamin C etc., against CCl₄ hepatotoxicity have been reported (Hsiao et al., 2003; Lee et al., 2003; Halim et al., 1997; Jeong et al., 1996). Studies also showed that various herbal extracts could protect organs against CCl₄ induced oxidative stress by altering the levels of increased lipid peroxidation, and enhancing the decreased activities of antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) as well as enhanced the decreased level of the hepatic reduced glutathione (GSH) (Ko et al., 1995; Rajesh and Latha, 2004). Knowledge on the protective mechanisms against toxin and drug induced organ-toxicities leads scientists to look for biologically active relevant compounds from herbal plants, which can possess intrinsic antioxidant activity and protect those organs from unwanted oxidative stress. In the modern medicine, plants occupy a significant birth as raw materials for some important drug preparations (de Mejia et al., 2002; Iwu et al., 1994; Chopra et al., 1986). However use of these medicines is mainly in raw and semi-standardized form and often based on empirical evidence. The lack of pharmacological and clinical data on the majority of herbal medicinal products is a major impediment to the integration of herbal
medicines into conventional medical practice. In view of the evergrowing importance of herbal drugs, the present study has been undertaken with an objective to investigate the possible antioxidant activity of the ethanol extract of *Actiniopteris radiata* against CCl₄ induced acute and chronic oxidative stress in albino rats.

### 3.1. EXPERIMENTAL DESIGN:

The experimental design was divided into two phases namely short term (10 days) and long term (8 weeks) with CCl₄ as oxidative stress inducer.

**Acute treatment - 10 days**

Healthy male albino rats were divided into four groups each containing six animals. Group I vehicle control (olive oil 1 ml/kg body weight, i.p.), Group II (toxin control) received 30% CCl₄ in olive oil (1 ml/kg body weight, i.p.). Group III and Group IV received EEAR (250 and 500 mg/kg body weight, p.o. respectively) once in a day and CCl₄ as mentioned above. Treatment duration was 10 days and the dose of CCl₄ was administered after every 72 hr (Shahjahan *et al.*, 2004).

**Chronic treatment - 8 weeks**

Healthy male albino rats were divided into four groups each containing six animals. Group I vehicle control (olive oil 1 ml/kg body weight, i.p.), Group II (toxin control) received 30% CCl₄ in olive oil (1 ml/kg body weight, i.p.). Group III and Group IV received EEAR (250 and 500 mg/kg body weight, p.o. respectively) once in a day and CCl₄ as mentioned above. Treatment duration was 8 weeks and the dose of CCl₄ was administered after every 72 hr (Yang-Jun Luo *et al.*, 2004).

The division of groups is as represented below:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle (olive oil) control rats</td>
</tr>
<tr>
<td>II</td>
<td>30% CCl₄ in olive oil (toxin control) treated rats</td>
</tr>
<tr>
<td>III</td>
<td>CCl₄ along with ethanol extract of <em>A. radiata</em> (250mg/kg b.wt)</td>
</tr>
<tr>
<td>IV</td>
<td>CCl₄ along with ethanol extract of <em>A. radiata</em> (500mg/kg b.wt)</td>
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</tbody>
</table>
In the present study, standard antioxidant treated group treated group was not maintained as the activity of a crude plant extract which contain a variety of compounds can not be compared with a single antioxidant compound. Plant extract alone treated group also was not maintained as we have already proved in toxicological studies (Chapter-2) that the plant extract is safe.

At the end of the experimental period the animals were sacrificed by decapitation under anesthesia. Blood and tissues were collected and used for further biochemical and molecular analysis. Also a section of the tissues were kept aside for histopathological studies.

Biochemical constituents analyzed in the study:

- Liver marker enzymes—SGOT/AST, SGPT/ALT, ALP, LDH, GGT and bilirubin were assayed.
- Serum urea, uric acid and creatinine levels were estimated to assess kidney damage.
- Alteration in lipid profiles (cholesterol, triglycerides and phospholipids) were estimated in serum and liver, kidney, brain and heart tissues.
- Oxidative stress markers—lipid peroxidation, protein oxidation (protein carbonyls and protein thiols) and xanthine oxidase were estimated in liver, kidney, brain and heart tissues.
- Antioxidant enzymes such as SOD, CAT, GPx, GR, G-6-PDH, GST and non enzymic antioxidants such as reduced glutathione (GSH), vitamin C and E were estimated in liver, kidney, brain and heart tissues.
- Isozyme profiles of antioxidant enzymes – SOD, CAT and GPx were performed in liver by native-PAGE.
- mRNA expressions of antioxidant enzymes (SOD, CAT and GPx) in liver were determined using RT-PCR.
- DNA damage was analyzed in blood by comet assay.
Histopathology:

- Histopathological studies were performed to assess the tissue damage due to \( \text{CCl}_4 \) toxicity and protection due to the administration of EEAR.

Statistical analysis:

The experimental data has been analyzed using one-way Analysis of Variance for comparing the difference in the means across the groups. Duncan's Multiple Range Test (DMRT) is used to identify significantly differing group means. The calculations were performed using SPSS 15.0. The results are presented as Tables showing mean and standard error and the F values obtained in ANOVA are reported in each Table. Results with \( p < 0.01 \) are considered as significant. Graphs are plotted for selected parameters.

3.2. MATERIAL AND METHODS

Chemicals

Coomassie Brilliant Blue G-250, Thiobarbituric acid (TBA), Sodium dodecyl sulphate (SDS), epinephrine (Adrenaline), Glutathione reductase, reduced glutathione (GSH), Nicotinamide adenine dinucleotide phosphate reduced NADPH, cumene hydroperoxide, glutathione oxidized (GSSG), 1-chloro-2,4-dinitro benzene (CDNB), 5, 5'-dithiobis-2-nitro benzoic acid (DTNB), Normal melting agarose, Low melting agarose, Bromophenol blue, Acrylamide, Bis-acrylamide, \( \text{N}_2\text{N}_2\text{N}'\text{N}' \) tetramethylene diamine (TEMED), 2, 6-dichloro phenol indophenol (DCPIP), Diethyl pyrocarbonate (DEPC), ethidium bromide were purchased from Sigma chemical company. Total RNA extraction kit was obtained from Genei, Bangalore, India. Primers were obtained from MWG-Biotech Pvt. Ltd. Bangalore. One step RT-PCR kit was procured from Qiagen chemical company, Germany. All other acids, bases, salts and solvents used were of analytical grade.

Animals

Experiments were carried out with male albino rats of Wister strain, weighing 250 ± 50 g. They were obtained from Sri Raghavendra suppliers, Bangalore and maintained in standard laboratory conditions, with a 12 hr light/dark cycle and fed with commercial rat feed supplied by Hindustan Lever Ltd., Mumbai under the trade name Gold Mohur rat feed and water \textit{ad libitum}. Experimental animals were handled according to the
University and Institutional Legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of social justice and empowerment, Government of India.

**Estimation of serum glutamate oxaloacetate transaminase/Aspartate amino transferase (SGOT/AST) activity** (Methodology is similar as in Chapter-2).

**Estimation of serum glutamate pyruvate transaminase/Alanine amino transferase (SGPT/ALT) activity** (Methodology is similar as in Chapter-2).

**Estimation of Alkaline phosphatase (ALP) activity** (Methodology is similar as in Chapter-2).

**Estimation of γ-glutamyl transpeptidase activity, (E.C.2.3.2.2)**

The activity of γ-glutamyl transpeptidase was estimated according to the method modified by Rosalki and Rao (1972).

γ-glutamyl transpeptidase catalyses the transfer of the γ-glutamyl group from γ-glutamyl peptides to another peptide or the L-amino acid or the removal of the glutamyl group from some compounds which contain it.

**Reagents:**

1. Tris-HCl buffer, 0.1 M, pH 8.5
2. Glycylglycine: 13.2 mg/10 mL.
3. Substrate: 30.37 mg of L-γ-glutamyl-p-nitroanilide was dissolved in 10 mL of water by heating at 50-60°C.
4. Standard: 13.8 mg of p-nitroaniline was dissolved in 100 mL of distilled water. This contained 1 μmol/mL.

**Procedure:**

The incubation mixture contained 0.5 mL of substrate, 1 mL of Tris-HCl buffer, 2.2 mL of glycylglycine, 0.2 mL of enzyme was added to the above mixture and the total volume was made up to 4 mL with water. After incubation for 30 min at 37°C, the samples were heated at 100°C for 5 min and centrifuged. The amount of p-nitroaniline in the supernatant was measured at 410 nm. The activity of γ-glutamyl transpeptidase was expressed as units/L.
Estimation of lactate dehydrogenase activity, (EC 1.1.1.27)

Lactate dehydrogenase activity was determined by the method of King (1965).

Reagents:
1. 0.1 M glycine buffer (pH 7.6): 7.5 g of glycine and 5.85 g of sodium chloride was dissolved in one liter of distilled water.
2. Buffered substrate: 2.76 g of lithium lactate was dissolved in 1.25 mL of glycine buffer containing 75 mL of 0.1 N NaOH solutions. This was prepared just before use.
3. 0.4 N NaOH solution
4. 5.0 mg of NAD⁺ was dissolved in 10 mL of distilled water (Prepared freshly every time).
5. 2, 4-dinitrophenylhydrazine (DNPH): 200 mg of DNPH was dissolved in one liter of 1.0 N HCl.
6. Standard pyruvate solution: 12.5 g of sodium pyruvate was dissolved in 100 mL of buffered substrate solution.

Procedure:
To 1.0 mL of the buffered substrate, 0.1 mL of the sample was added and the tubes were incubated at 37°C for 15 min. After adding 0.2 mL of NAD⁺ solution, the incubation was continued for another 15 minutes. The reaction was arrested by adding 1.0 mL of DNPH reagent. The tubes were then incubated for a further period of 15 min after which 7.0 mL of 0.4 N NaOH was added and color developed was measured at 420 nm in a Shimadzu UV spectrophotometer. The calibration curve was constructed simultaneously with the test sample. LDH activity was expressed as units/L (one unit corresponds to 1 micro mole of pyruvate formed / min).

Estimation of bilirubin (Methodology is similar as in Chapter-2)

Estimation of urea (Methodology is similar as in Chapter-2)

Estimation of uric acid (Methodology is similar as in Chapter-2)

Estimation of creatinine (Methodology is similar as in Chapter-2)
Assay of lipid profiles

Preparation of tissue lipid extract
The tissues were washed with saline and dried between filter paper and a weighed amount of tissue (500 mg) was homogenized with 7 mL of methanol. The homogenate was then filtered through a Whatman No. 1 filter paper into a conical flask. The residue after filtration was scraped carefully and homogenized in 14 mL of chloroform. The residue after filtration was once again scraped from filter paper and ground with 10 mL of chloroform-methanol mixture (2:1 v/v) and the resulting filtrate was evaporated to dryness.

Purification of lipids was done using Folch’s wash procedure (Folch et al., 1957). The dried lipid residue obtained after evaporation was dissolved in 5 mL of chloroform-methanol mixture. The contents were then mixed with 1 mL of 0.1 N KCl and shaken well. The upper aqueous phase containing gangliosides and other water-soluble compounds were separated. The lower chloroform phase containing neutral and phospholipids was again washed thrice with 2 mL of Folch’s reagent (0.1 N KCl: methanol: chloroform mixed in the ratio of 10:10:1) and the upper aqueous phase was aspirated. The lower chloroform phase was made up to a known volume and aliquots were used for the analysis of total cholesterol, triglycerides and phospholipids.

Estimation of triglycerides (Methodology is similar as in Chapter-2)

Estimation of cholesterol (Methodology is similar as in Chapter-2)

Estimation of phospholipids
Phospholipids were estimated by the method of Rouser et al., (1970) after digesting the lipid extract with perchloric acid.

Reagents:
1. Ammonium molybdate, 3%.
2. Ascorbic acid, 3%
3. Perchloric acid, 70%.
4. Standard phosphate: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 mL of distilled water to give a concentration of 80 µg of phosphorous/mL.
5. Working standard: A concentration of 8 μg/mL was prepared by diluting 1 mL of the stock solution to 10 mL with distilled water.

**Procedure:**

0.1 mL of serum/Folch-wash tissue extract was dissolved in 1 mL of perchloric acid and digested on a sand bath till the solution become colorless. After cooling, the solution was made up to 5 mL with double distilled water. To this, 0.5 mL of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 min. The blue colour developed was read at 710 nm. Serum and tissue phospholipid content are expressed as mg/dL and mg/g wet tissue, respectively.

**Preparation of the tissue homogenate:**

Samples of liver, kidney, brain and heart (100 mg/mL) were homogenized in 50 mM phosphate buffer (pH 7.0), and then centrifuged at 10,000 rpm for 15 min; the supernatant thus obtained was used for assaying antioxidant enzymes and other biochemical parameters.

For lipid peroxidation, 10% tissue homogenate was prepared in 1.15% KCl and for estimating total sulfhydryl content the tissue was homogenized in 0.2 M EDTA.

**Estimation of lipid peroxidation in serum**

Lipid peroxide concentration was determined by thiobarbituric acid reaction as described by Okhawa *et al.*, (1979).

**Reagents:**

1. TCA, 10%.

2. Thiobarbituric acid (TBA), 0.33% dissolved in acetic acid: water in the ratio of 1:1.

3. n-butanol.

**Procedure:**

To 0.5 mL of plasma, 0.5 mL distilled water and 1 mL of 10% TCA was added. From this mixture, 1 mL of protein free supernatant was taken after centrifugation at 2000 rpm for 20 min. To this supernatant 0.25 mL of TBA was added and boiled for 1 hr at 95°C.
The tubes were cooled and the malondialdehyde formed were extracted in 1 mL of n-butanol and the intensity of the colour developed is read at 532 nm. 1, 1, 2, 2-tetraethoxy propane (TEP) was taken as standard.

Estimation of lipid peroxidation in tissues

Lipid peroxidation in tissues was carried out by the method of Okhawa et al., (1979). Estimation of lipid peroxides was based on the reduction of thiobarbituric acid to give a pink coloured complex, MDA which is measured at 532 nm. MDA formed as an end product of the peroxidation of lipids served as an index of the intensity of oxidative stress.

Reagents:
1. 1.15% KCl.
2. 8.1% Sodium dodecyl sulphate (SDS)
3. 0.8% Thiobarbituric acid.
4. n-butanol: pyridine mixture (15:1 v/v).
5. Standard: 1, 1, 2, 2-tetraethoxy propane (TEP)

Procedure:
The assay mixture contained 0.1 mL of 10% tissue homogenate (prepared in 1.15% KCl), 0.2 mL of SDS and 1.5 mL of TBA. The mixture was finally made up to 4 mL with distilled water and boiled at 95°C for 1 hr. After cooling, 1 mL of distilled water and 5 mL of n-butanol: pyridine mixture were added and shaken vigorously and then centrifuged at 4000 rpm for 10 min. Then the absorbance of the organic layer was measured at 532 nm. Amount of lipid peroxidation is expressed as nano moles of MDA produced/mg protein. A standard curve of TEP was prepared in a similar condition and used for calculation.

Estimation of xanthine oxidase activity, (E.C.1.2.3.2):

Xanthine oxidase activity was estimated by the dye reduction method of Srikanthan and Krishnamurthy (1955). The assay mixture contained 100 μmoles of sodium phosphate buffer (pH 7.4), 50 μmoles of xanthine, 0.1 μmole of NAD, 0.4 μmoles of INT and the
enzyme source. The reaction was initiated by the addition of enzyme source and incubated at 37°C for 30 min. The reaction was stopped by the addition of 5 mL of glacial acetic acid and the formazan formed was extracted into toluene and read at 495 nm against toluene blank. The activity was expressed as µmoles of formazan formed/hr/mg protein.

Estimation of protein carbonyl content:
The carbonyl content of liver, kidney, brain and heart homogenates of control and treated rats was evaluated by the method of Levine et al., (1990). 100 µL of homogenate (10%) were incubated with 100 µL of DNPH (100 mM/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. 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 sulphydryl (thiol) content:
Total sulphydryl group was estimated by the method of Sedlack and Lindsay (1968).

The method is based on the reaction of tissue homogenate with DTNB, where in DTNB is reduced by the thiol group to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole-SH. The absorbance of the supernatant was read at 412 nm.

Reagents:
1. Tris-HCl buffer: 0.2 M, pH 8.2 containing 0.2 M EDTA.
2. 5, 5'-dithiobis-2-nitro benzoic acid (DTNB): 99 mg of DTNB was dissolved in 25 mL of absolute ethanol.
3. Standard: 10 mg of reduced glutathione was dissolved in 100 mL of distilled water.

Procedure:
200 mg of tissue was homogenized in 0.8 mL of 0.2 M EDTA. 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 sample blank without DTNB were prepared in the same manner. The test tubes were stoppered and 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 sulphhydryl group is expressed as μg of GSH/mg protein.

Estimation of glucose-6-phosphate dehydrogenase activity, (E.C.1.1.1.49)
Glucose-6-phosphate dehydrogenase was assayed by the method of Ellis and Kirkman (1961).

Reagents:
1. Tris-HCl buffer, 0.05 M (pH 7.5).
2. Magnesium chloride, 1M.
3. NADP, 1M.
4. Phenazine methosulphate (PMS), 0.005%.
5. 2, 6-dichloro phenol indophenol (DCPIP), 0.01%.
6. Glucose-6-phosphate, 0.02 M.

Procedure:
The incubation mixture in a total volume of 5.5 mL contained the following: 1 mL of Tris-HCl, 0.5 mL of PMS, 0.4 mL of DCPIP solution, 0.1 mL of magnesium chloride, 0.1 mL of NADP and a required amount of enzyme preparation. The mixture was allowed to stand at room temperature for 10 min to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 mL of glucose-6-phosphate. The change in optical density at 640 nm was monitored for 3 min at 30 second interval in
a spectrophotometer against water blank. The activity of glucose-6-phosphate dehydrogenase activity was expressed as units/min/mg protein.

**Antioxidants**

**Enzymic antioxidants**

**Estimation of superoxide dismutase activity, (E.C.1.15.1.1)**

Superoxide dismutase was measured according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine transition by the enzyme.

**Reagents:**

1. 50 mM Sodium carbonate-bicarbonate buffer, pH 10.2 containing 0.1 mM of ethylene diamine tetra acetic acid (EDTA).
2. 0.6 mM epinephrine (Adrenaline).

**Procedure:**

To 0.5 mL of the homogenate 2 mL of carbonate buffer and 0.5 mL of 0.6 mM epinephrine was added. Epinephrine was the last component to be added and the adrenochrome formed in the next 4 min was recorded at 470 nm in spectrophotometer.

SOD activity is expressed in units/min/mg protein (One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of epinephrine auto-oxidation).

**Estimation of catalase activity, (E.C.1.11.1.6):**

Catalase assay was carried out by the method of Aebi (1984). The decomposition of H$_2$O$_2$ was followed directly by measuring the decrease in absorbance at 240 nm.

**Reagents:**

1. 50 mM Phosphate buffer, pH 7.0
2. 30 mM H$_2$O$_2$: 340 µL of 30% (v/v) H$_2$O$_2$ was dissolved in 100 mL of phosphate buffer (pH 7.0).

**Procedure:**

0.5 mL of the tissue homogenate was mixed with 1.5 mL of phosphate buffer. Then 1 mL of H$_2$O$_2$ was added and change in absorbance was recorded after every 15 seconds up to 1 min. The activity of catalase was expressed as µmoles of H$_2$O$_2$ utilized/min/mg protein.
Estimation of Glutathione peroxidase activity, (E.C.1.11.1.9)

Assay of glutathione peroxidase was carried out by continuous monitoring of NADPH oxidation in a recycling assay as described by Wendel (1981). Total GPx was measured by using cumene hydroperoxide as a substrate.

Reagents:

1. 0.25 M phosphate buffer, pH 7.0 containing 2.5 mM disodium ethylene diamine tetra acetic acid and 2.5 mM sodium azide.
2. Glutathione reductase: 0.3 U/mL.
3. 10 mM reduced glutathione (GSH).
4. 2.5 mM NADPH in 0.1% sodium carbonate.
5. 12.5 mM cumene hydroperoxide.

Procedure:

The reaction mixture contained 0.1 mL of phosphate buffer, 0.1 mL glutathione reductase, 0.1 mL reduced glutathione and 0.1 mL of NADPH. To this 0.5 mL of homogenate was added and incubated at 37°C for 10 min. The reaction was started by the addition of 100 μL of cumene hydroperoxide. The linear decrease in absorption was recorded at 340 nm. The spontaneous reaction was assayed without enzyme and was subtracted from the samples. Activity of GPx was expressed as μmoles of GSH oxidized/min/mg protein.

Estimation of glutathione reductase activity, (E.C.1.6.4.2)

Glutathione reductase was assayed by the method of Staal et al., (1969).

Reagents:

1. 0.3 M sodium phosphate buffer (pH 6.8).
2. 250 mM EDTA
3. 12.5 mM glutathione oxidized (GSSG).
4. 3 mM Nicotinamide adenine dinucleotide phosphate reduced (NADPH).
Procedure:
The reaction mixture containing 1 mL of phosphate buffer, 0.5 mL EDTA, 0.5 mL of glutathione oxidized and 0.2 mL of NADPH was made up to 3 mL with distilled water. After the addition of 0.1 mL of tissue homogenate, the change in optical density at 340 nm was monitored every 30 seconds for 2 min. The enzyme activity is expressed as μmoles of GSH utilized/min/mg protein.

Estimation of glutathione-s-transferase activity, (E.C.2.5.1.18)
Glutathione-s-transferase activity was determined by measuring the increase in absorbance at 340 nm using 1-chloro-54-dinitro benzene (CDNB) as a substrate by the method of Habig et al., (1974).

This enzyme catalyzes the reaction of CDNB with the sulfhydryl group of GSH to form CDNB-GSH conjugate that absorbs light at 340 nm.

Reagents:
1. 0.5 mM phosphate buffer, pH 6.5
2. 30 mM CDNB.
3. 30 mM reduced glutathione (GSH).

Procedure:
The reaction mixture contained 2.7 mL phosphate buffer, 0.1 mL CDNB and 0.1 mL of the tissue homogenate which was incubated at 37°C for 10 min. The reaction was initiated by adding 0.1 mL of glutathione. The reaction was monitored spectrophotometrically for increase in absorbance at 340 nm. Measuring and subtracting the rate in the absence of enzyme made correction for the spontaneous reaction. The enzyme activity is expressed as units/min/mg protein.

Non-enzymic antioxidants
Estimation of reduced glutathione
Reduced glutathione was determined by the method of Moron et al., (1979).

Reagents:
1. Phosphate buffer, 0.2 M (pH 8.0).
2. Trichloroacetic acid, 10%.
3. 5, 5' dithiobis-2-nitro benzoic acid (DTNB), 0.6 mM in 1% sodium citrate.

4. Standard glutathione: 10 mg of reduced glutathione was dissolved in 100 mL of distilled water.

Procedure:
An aliquot of 1 mL of the homogenate was precipitated with 10% TCA. The precipitate was removed by centrifugation. To an aliquot of the supernatant was added 2 mL of phosphate buffer, 0.5 mL of DTNB reagent and the final volume was made up to 3 mL with distilled water. Standard was also treated in a similar manner. The colour developed was read at 412 nm. The amount of reduced glutathione was expressed as µg/mg protein.

Estimation of Ascorbic acid / Vitamin C
Vitamin C was estimated by the method of Omaye et al., (1979). Ascorbic acid is oxidized by copper to form dehydroascorbic acid which reacts with 2, 4-dinitrophenyl hydrazine; this under goes further rearrangement to form a product with an absorption maximum at 520 nm.

Reagents:
1. Trichloroacetic acid, 5%.
2. 2, 4-Dinitrophenyl hydrazine-Thiourea-Copper sulphate (DTC) reagent: 0.4 g thiourea, 0.05g copper sulphate and 3 g of 2, 4-dinitrophenyl hydrazine were dissolved in 100 mL of 9 N sulphuric acid.
3. Sulphuric acid, 65% (v/v).
4. Standard ascorbic acid: 50 mg of ascorbic acid was dissolved in 100 mL of 5% trichloroacetic acid.

Procedure:
To 0.5 mL tissue homogenate, 0.5 mL of distilled water and 1 mL of 5% trichloroacetic acid were added, mixed thoroughly and centrifuged for 20 min. To 1 mL of the supernatant, 0.2 mL of DTC reagent was added and incubated at 37°C for 3 hr. Then 1.5 mL for 65% sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 min. The colour developed was read at 520 nm. Graded amount of standards was also treated similarly. The level of ascorbic acid was expressed as µg/mg protein.
**Estimation of α-tocopherol /Vitamin E:**

Vitamin E was determined by the method of Quaife and Dju (1948).

**Reagents:**

1. Absolute ethanol
2. Xylene
3. Dipyridyl reagent: 120 mg of 2, 2'-dipyridyl in 100 mL of n-propanol.
4. Ferric chloride reagent: 120 mg of ferric chloride was dissolved in 100 mL of ethanol.
5. Standard: 10 mg of α-tocopherol was dissolved in 100 mL of ethanol.

**Procedure:**

To 3 stoppered centrifuge tubes, 1 mL of homogenate, 1.5 mL of standard and 1.5 mL of distilled water were added respectively. To test and standard, 1.5 mL of ethanol and to blank 1.5 mL of distilled water were added. Then 1.5 mL of xylene was added to all the tubes and shaken well. After centrifugation, 1 mL of xylene layer was taken and 1 mL of dipyridyl reagent was added and mixed well. From this, 1.5 mL was pipetted out and read at 460 nm against the blank. Then 0.33 mL of ferric chloride reagent was added to all the tubes and exactly after 1.5 min the test and standards were again read at 520 nm against the blank. The level of vitamin E is expressed as µg/mg protein.

**Detection of isozymes by electrophoresis:**

**Native - Polyacrylamide gel electrophoresis (Native-PAGE)**

Non-denaturing polyacrylamide gel electrophoresis was performed as described by Laemmli (1970), except that SDS was omitted from all buffers and the samples were not boiled before electrophoresis. The enzymes were run on the basis of equal amounts of protein (70 µg) in a 10% gel for SOD and GPx and 8% gel for CAT. Electrophoretic separation was performed at 4°C with a constant power supply of 50 V for stacking gel and 100 V for separating gel.

**Reagents:**

- **Acrylamide:** Bis (29.2:0.8%)
  - Acrylamide - 29.2 g
  - Bis-acrylamide - 0.8 g
  - Made to 100 mL with deionized water

- 1.5M Tris pH 8.8 (Separating gel)
  - Tris-18.15 g
  - Adjust the pH to 8.8 with HCl and make the volume to 100 mL with deionized water.
**Gel Casting:**

10 % separating gel (10 mL)
- Acrylamide: Bis (29.2:0.8) - 3.3 mL
- Tris-HCl pH 8.8 (1.5 M) - 2.5 mL
- Deionized water - 4.0 mL
- APS (10%) - 0.1 mL
- TEMED - 0.004 mL

5% stacking gel (5 mL)
- Acrylamide: Bis (29.2:0.8) - 0.83 mL
- Tris-HCl pH 6.8 (1 M) - 0.63 mL
- Deionized water - 3.4 mL
- APS (10%) - 0.05 mL
- TEMED - 0.005 mL

**Gel loading dye**

- 1 M Tris-HCl (pH 6.8) : 1.25 mL
- Glycerol (100%) : 1 mL
- Bromophenol blue : a pinch
- Deionized water : 7.25 mL

**Staining:**

(i) CAT activity was detected by the method of Woodbury et al., (1971). The gel soaked in 5 mM H₂O₂ solution for 10 min was washed with water and stained with a reaction mixture containing 1% potassium ferricyanide (w/v) and 1% ferric chloride. The enzyme appeared as a yellow band superimposed on a dark green background. The reaction was terminated by adding water, and the gel was photographed at once.

(ii) SOD activity was identified by the method of Beauchamp and Fridovich (1971). The gel was soaked in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mg nitroblue tetrazolium (NBT), 1 mg ethylene diamine tetra acetic acid (EDTA), 2 mg riboflavin (50 mL final volume) and kept in the dark for 30 min. The gel was then placed on an illuminated light box to locate the area of SOD activity, which appeared as a clear zone on a bluish-violet background.
GPx isozymes were separated by the method of Lin et al., (2002). The gel was soaked in 50 mL of 50 mM Tris–HCl buffer (pH 8.0) containing 200 mg reduced glutathione, and 8 μL of 30% H₂O₂ for 20 min. The gel was then transferred to 50 mL of 50 mM Tris–HCl buffer (pH 8.0) containing 25 mg NBT and 25 mg phenazine methosulphate. The appearance of white bands in the gel was taken to indicate the presence of GPx isozymes.

Quantification of the isozyme bands for each enzyme studied was performed in a densitometer.

Isolation of RNA:

Total RNA was isolated from liver using manufacturer's instruction (Genei Total RNA extraction kit, Genei, Bangalore). Total RNA extraction kit is based on widely used guanidine thiocyanate phenol: chloroform procedure of Chomoczynsky and Sacchi (1987). Samples are lysed in highly denaturing condition inactivating RNases, followed by Phenol: chloroform extraction where DNA and proteins are removed and then precipitated with isopropanol. An additional step of suspending and reprecipitating ensures further purity of the RNA. All the precautions were taken to isolate RNA without any contamination.

Procedure:

About 100 mg of tissue is homogenized in 1 mL of denaturing solution. Add 1 mL of water saturated phenol followed by 200 μL of chloroform-isoamyl alcohol mix (freshly prepared in the ratio of 49:1), mix thoroughly and incubate in ice for 15 minutes. Then centrifuge at 10,000 rpm for 20 min at 4°C. Transfer upper aqueous phase carefully to another tube. To this add 1 mL of 100% isopropanol to precipitate RNA and incubate at -20°C for 30 minutes. Now centrifuge at 10,000 rpm for 20 min at 4°C and discard the supernatant. Resuspend the pellet in 0.3 mL of denaturation solution, precipitate RNA by adding 0.3 mL of 100% isopropanol, incubate at -20°C for 30 minutes, centrifuge at 10,000 rpm for 20 min at 4°C and discard the supernatant. Resuspend the RNA pellet in 75% ethanol. Incubate at room temperature for 10-15 minutes to dissolve residual amounts of guanidine. Centrifuge at 10,000 rpm for 20 min at 4°C, discard the supernatant and dry the pellet. Do not over dry the pellet. Dissolve in 100-200 μL of DEPC water. Incubate for 10-15 min at 55°C to ensure complete solubilisation of RNA.
Store the total RNA at -70°C. Subsequently, the purity of RNA was done by measuring the absorbance of RNA solution at 260 nm and 280 nm (Absorbance ratio of 260/280 nm ranges from 1.6-1.8 was taken for further reaction).

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

RT-PCR for SOD, CAT and GPx mRNA expression was carried out according to manufacturer's guidelines (Qiagen One step RT-PCR mix). The reaction mixture contained 10 µL of 5X Qiagen One step RT-PCR buffer (2.5 mM MgCl₂ as final concentration), 2 µL of dNTP mix (10 mM of each dNTP as final concentration), 3 µL of each sense and anti-sense primers of SOD, CAT and GPx, so as to give a final concentration of 0.6 µM, 1 µg of template RNA, 1 µL of RNase inhibitor and 2 µL of Qiagen one step RT-PCR enzyme mix and the volume was finally made up to 50 µL with RNase free water.

The RT-PCR reaction was performed in the thermal cycler (Eppendorf) using the following conditions (Pallavi et al., 2003):

1. Reverse transcription, 30 min, and 50°C
2. Initial PCR activation step, 15 min, 95°C,
3. 3-step cycling for 30 cycles, each cycle consisting of denaturation for 30 seconds at 94°C followed by annealing for 30 seconds at 58°C and extension for 1 min at 72°C.

The template concentration and the cycle number were optimized to ensure linearity of response and to avoid saturation of the reaction. To compare the amount of steady state mRNA, 10 µL of each PCR product was resolved onto 2% agarose gel using TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). After electrophoresis, the gels were viewed under UV light, and the digital images were captured on Alpha Innotech gel documentation system. The expression of each target gene was standardized with internal control gene (β-actin) and represented as a ratio.
Table 3.1: Primer sequences and expected product sizes for genes amplified.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Genbank Accession No.</th>
<th>Primers</th>
<th>RT-PCR product size</th>
</tr>
</thead>
</table>
| β-actin    | V01217                | Forward primer: 5'-CTGCTTGCTGATCCACA  
Reverse primer: 5'-CTGACCCGAGCTGGCTAC  | 505bp                |
| CuZnSOD    | X05634                | Forward primer 5'-GCAGAAGGCAAGCGGTGAAC  
Reverse primer 5'-TAGCAGGACAGCAGATGAGT  | 387bp                |
| GPx        | M21210                | Forward primer 5'-CTCTCCGGCGTGCCACAGT  
Reverse primer 5'-CCACCACCGGCGTGGACATAC  | 290bp                |
| CAT        | AH004967              | Forward primer 5'-GCAGAATTGAGAGGGACGTGTAC  
Reverse primer 5'-GAGTGAACGTGGTCATAGCCTG  | 670bp                |

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

Possible DNA damage induced by CCl₄ at different doses was detected using the alkaline single cell gel electrophoresis (Comet) assay following a simplified protocol with slight modifications (Endoh et al., 2002; Singh et al., 1988).

Reagents:

1. 1% Normal melting agarose.
2. 1% Low melting agarose.
3. 0.5% Low melting agarose.
4. Lysing solution: 36 mL of lysing buffer (73.5 g of NaCl, 18.612 g of EDTA, 5 g of Sodium lauryl sarcosinate, 0.6 g of Tris dissolved in 500 mL of distilled water and adjust the pH to 10.0), 4 mL DMSO and 400 μL TritonX-100.
5. Electrophoresis buffer: 30 mL of 10 N NaOH and 5 mL of 0.5 M EDTA made up to 1000 mL with distilled water and adjust the pH to 10.0
6. Neutralizing buffer: 24.228 g of Tris dissolved in 500 mL of distilled water and pH was adjusted to 7.5.
Procedure:
At the end of the treatment (short term and long term) with CCl₄, blood was collected freshly from the retro-orbital plexus and used for the assay. 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 fresh blood 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. 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 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 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⁻¹; 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. 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 occlulometer 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 (Sebastien et al., 2003). 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.

Histopathological studies (Raphael, 1976)
The tissues (liver, kidney, brain and heart) preserved in neutral buffered formalin were used for the study of histopathological changes. Tissues were processed, which involves dehydration, clearing and infiltration of the tissue with paraffin and then the tissues were sectioned. The sections are mounted on glass slides and smeared with a drop of Mayer’s egg albumin. The slides are dried on a hot plate at about 50°C for 30 min. The sections are then stained with Mayer’s Hematoxylin-Eosin stain and observed under light microscope.

3.3. RESULTS AND DISCUSSION:
Marker enzymes:
Serum transaminases (SGOT/AST and SGPT/ALT):
SGOT and SGPT are hepatospecific serum markers. The changes in the marker levels will reflect in hepatic structural integrity. The markers are cytoplasmic in origin and released into the circulation after cellular damage (Mohan Rao et al., 1989; Lin et al., 2000). Therefore, their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage (Mitra et al., 1998). The rise in SGOT is usually accompanied by an elevation in the levels of SGPT, which play a vital role in the conversion of aminoacids to keto acids (Sallie et al., 1999).

In the assessment of liver damage by CCl₄, the determination of enzyme levels such as SGOT and SGPT is largely used. Necrosis or membrane damage releases the enzyme into circulation; therefore, it can be measured in serum. High levels of SGOT indicate liver damage, such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and
is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury (Williamson, 1996).

In the present study, administering CCl4 to rats markedly increases serum transaminase (SGOT and SGPT) levels. This increase commonly reflects the severity of liver injury (Lin et al., 1996). The leakage of large quantities of enzymes into the bloodstream was associated with massive centrilobular necrosis, ballooning degeneration and cellular infiltration of the liver. There was a significant increase in the activities of SGOT and SGPT ($p<0.01$; $p<0.001$) in CCl4 treated (Group II) rats when compared to the control rats during both acute and chronic treatments and are depicted in Table 3.2. However, the increased levels of SGOT and SGPT were significantly reduced ($p<0.01$; $p<0.01$) by treatment with EEAR extract (Group III and IV) in serum, dose dependently when compared to the Group II (CCl4 treated) rats. The reversal of the levels of serum transaminases activities in CCl4-induced hepatic damage by the administration of EEAR extract could be explained by the prevention of a leakage of intracellular enzymes through its membrane-stabilizing effects (Thabrew et al., 1987). Raju et al. (2003); Shanmugasundaram and Venkataraman (2006) reported a decrease in SGOT and SGPT levels with the administration of antioxidants like dried fruits of Solanum nigrum Linn. and Hygrophila auriculata during CCl4 toxicity. The results in the present study are in consistent with these reports.

**Alkaline phosphatase (ALP):**

Serum ALP is also related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure (Moss and Butterworth, 1974). Effective control of alkaline phosphatase activity points towards an early improvement in the secretory mechanism of the hepatic cell. The changes in ALP will reflect in hepatic structural integrity. Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells (Zimmerman and Seef, 1970). CCl4 damage to liver raises the serum level of ALP by releasing into the bloodstream (Asha, 2001). In the present study, a significantly elevated activity ($p<0.01$; $p<0.01$) of ALP was noticed in the serum of CCl4 treated (Group II) rats when compared to the control (Group I) rats during acute and chronic CCl4 toxicity. The
raise in the activity of ALP in CCl₄ treated rats may be due to the disturbance in the secretory activity or in transport of metabolites or may be due to altered synthesis of certain enzymes in these conditions. Treatment with the EEAR extract at two doses in group III (250 mg/kg b.wt) and group IV (500 mg/kg b.wt) rats significantly lowered (p<0.001; p<0.001) the enzyme activity, dose dependently, when compared to CCl₄ treated (Group II) rats (Table 3.2). Thus lowering of enzyme content in serum is a definite indication of antioxidant action of the drug which stabilizes the membrane integrity of hepatic cells by scavenging the free radicals generated due to CCl₄ toxicity. The results obtained in this study are consistent with the previous literature of Chandan et al., (2007) and Vanitha et al., (2007) who reported that natural antioxidants like Aloe barbadensis Mill. and Dunaliella bardawil reduce the levels of ALP elevated due to CCl₄ toxicity.

**Gamma glutamyl transferase (γ-GT):**

The estimation of γ-GT level is valuable screening test with high negative, predictive nature for liver disease (Nemesanszky, 1996). In present study γ-GT activity was significantly (p<0.001 and p<0.01) elevated in CCl₄ treated rats for a period of 10 days and 8 weeks. Increased activity of γ-GT indicate a severe damage to tissue membrane during CCl₄ toxicity, because of γ-GT which is a membrane bound enzyme (Chander et al., 1994). Various reports show that serum γ-GT activity was significantly increased when CCl₄ administered at different dosages via different routes in rats (Ogeturk et al., 2004 and Fahim et al., 1999). Administration of EEAR in two doses along with CCl₄ in Group III and IV rats showed significant (p<0.001 and p<0.01; p<0.001 and p<0.01) reduction in γ-GT activity thus, it could indicate and reflect the membrane stabilizing activity of EEAR (Table 3.2). This indicated that EEAR resulted in improving in liver function. Zerin et al., (2004); Sengottuvelu et al., (2007); Bodakhe and Ram, (2007) reported that CCl₄ treatment caused an increase in the activity of γ-glutamyl transferase in serum and it was showed that treatment with medicinal herb extracts like Nigella sativa oil, Cleome viscosa seed extract and Bauhinia variegata bark extract reversed the elevated levels of the enzymes in serum. The results of present study are in accord with these reports.
Lactate dehydrogenase (LDH):
Lactate dehydrogenase (LDH) is a key enzyme of anaerobic glycolysis and catalyses the reversible oxidation of lactate to pyruvate in the terminal step of glycolysis. The reaction catalysed by LDH interlinks anaerobic and aerobic oxidation of glucose (Robert et al., 2000). LDH increase in serum may occur in any injury that causes loss of cell cytoplasm. Elevation of serum LDH is observed due to in vivo effects of toxic agents like clofibrate, dicumarol, ethanol, carbon tetrachloride etc. In view of its role in glucose oxidation the NAD dependent LDH activity levels were assayed in serum to assess the metabolic significance of this enzyme in compensatory mechanism operating in the tissues of rat during CCl₄ and plant treatment. In the present study, LDH levels were significantly (p<0.001 and p<0.001) increased in CCl₄ treated rats (Group II) for 10 days and 8 weeks. In groups treated with two different dose of plant extract, EEAR (Group III and IV) there was a significant (p<0.001 and p<0.001; p<0.01 and p<0.001) decrease in the LDH levels towards the respective normal levels, dose dependently. Reduction in the levels of LDH towards the respective normal values by plant extract of two different doses (250 and 500 mg/kg b.wt) (Group III and IV) is an indication of the stabilization of plasma membranes as well as repair of hepatic tissue damage caused by CCl₄ (Table 3.2). The possible reason for the decrease in the serum LDH activity when EEAR administered to the rats intoxicated with CCl₄ is due to the antioxidant activity of EEAR, by which it scavenges or neutralizes free radicals inhibits the peroxidation of membrane lipids and maintains cell membrane integrity and their function.

The results obtained in this study are corroborates with the earlier findings of Malathi and Patrick Gomez, (2007); Mitcheva et al., (2006); Raghavendran et al., (2004) reported similar decrease in serum LDH activity levels with the administration of Tylophora asthmatica, Hypericum annulatum and a sea weed against toxic agents induced liver damage respectively. Similar results are reported by El-Khatib et al., (2002) with the administration of aqueous Propolis extract against CCl₄ induced oxidative stress in rats and these reports are in correlation with the findings of present investigation.
Bilirubin:

Bilirubin, an endogenous organic anion binds reversibly to albumin and it is transported to the liver, and then conjugated with glucuronic acid and excreted in the bile. Serum bilirubin levels on the other hand, are related to the function of hepatic cell (Moss and Butterworth, 1974). When bilirubin in the blood exceeds, hyperbilirubinemia exists. Hyperbilirubinemia may be due to the production of more bilirubin than the normal liver can excrete, or it may result from the failure of a damaged liver to excrete bilirubin (Dangerfield and Finlayson, 1953). As a guide in the prognosis of liver condition, estimation of serum bilirubin level has assumed greater importance. The elevation in the levels of bilirubin has been reported (Table 3.2) in CCl₄ induced hepatotoxicity (Sallie et al., 1999). In the present study, significant (p<0.01 and p<0.001) elevated level of serum bilirubin has been observed in the rats with acute and chronic CCl₄ toxicity when compared to control group (Group I). Administration of two doses of EEAR (Group III and Group IV) to the rats induced with CCl₄ significantly (p<0.001 and p<0.01; p<0.01 and p<0.001) decreased the level of bilirubin when compared to CCl₄ alone treated rats (Group II). This suggests that EEAR has protective effect on liver damage by scavenging free radicals.

The results in the present study are in accordance with the findings of Sethuraman et al., 2003 and Jain et al., (2008) who reported the antioxidant and hepatoprotective activities of Sarcostemma brevistigma and Momordica dioica against CCl₄ toxicity in albino rats.

Non-protein nitrogenous substances

Urea and Creatinine:

Urea is the major end product of nitrogen excretion and creatinine is a waste product produced during the formation of creatine, should be excreted. Kidney, the principle excretory system maintains the levels of these molecules. Damage to the kidney results in
Table 3.2: Effect of ethanol extract of *A. radiata* on liver markers in serum of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>72.96 ± 1.64²</td>
<td>328.45 ± 2.959ᵇ</td>
<td>165.48 ± 4.937ᶜ</td>
<td>116.82 ± 3.138ᵈ</td>
<td>1090.816</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>46.61 ± 3.85ᵈ</td>
<td>326.46 ± 3.68³ᶜ</td>
<td>145.71 ± 3.88ᵇ</td>
<td>75.69 ± 3.98ᵃ</td>
<td>1063.371</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>184.68 ± 3.64ᵈ</td>
<td>335.29 ± 4.14³ᵃ</td>
<td>235.33 ± 3.64ᶜ</td>
<td>220.25 ± 5.36ᵇ</td>
<td>229.441</td>
</tr>
<tr>
<td>γGT (IU/L)</td>
<td>12.67 ± 0.33ᵃ</td>
<td>24.58 ± 0.37ᵇ</td>
<td>20.37 ± 0.40ᶜ</td>
<td>18.47 ± 0.38ᵈ</td>
<td>173.322</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>930.95 ± 2.70ᵃ</td>
<td>2784.25 ± 3.60ᵇ</td>
<td>1474.86 ± 3.96ᶜ</td>
<td>1134.5 ± 3.64ᵈ</td>
<td>56195.29</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.34 ± 0.03ᵃ</td>
<td>1.85 ± 0.04ᵇ</td>
<td>1.64 ± 0.04ᶜ</td>
<td>1.35 ± 0.04ᵃ</td>
<td>37.6</td>
</tr>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>132.74 ± 3.30ᵃ</td>
<td>581.57 ± 5.64ᵇ</td>
<td>335.71 ± 2.76ᶜ</td>
<td>162.99 ± 3.96ᵈ</td>
<td>2564.507</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>56.26 ± 4.5³ᵃ</td>
<td>660.86 ± 6.8ᵇ</td>
<td>321.06 ± 4.66ᶜ</td>
<td>134.86 ± 3.8ᵈ</td>
<td>2780.07</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>195.78 ± 4.2³ᵇ</td>
<td>582.88 ± 4.64ᵇ</td>
<td>385.60 ± 3.58ᶜ</td>
<td>232.20 ± 8.35ᵈ</td>
<td>1016.955</td>
</tr>
<tr>
<td>γGT (IU/L)</td>
<td>15.45 ± 0.39ᵃ</td>
<td>54.02 ± 4.09ᵇ</td>
<td>44.77 ± 3.25ᵇ</td>
<td>34.65 ± 3.58ᶜ</td>
<td>27.096</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>879.83 ± 4.8³ʳᵃ</td>
<td>3206.23 ± 4.69ᵇ</td>
<td>2182.12 ± 5.4¹ᶜ</td>
<td>1109.18 ± 5.3³ᵈ</td>
<td>44431.75</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.24 ± 0.03ᵃ</td>
<td>2.35 ± 0.04ᵇ</td>
<td>1.84 ± 0.04ᶜ</td>
<td>1.35 ± 0.04ᵃ</td>
<td>158.011</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).
impaired excretion of these molecules. Increases in levels of urea and creatinine are indications of damage to renal tissues (Kaplan et al., 1988). Creatinine has been reported to be a chemical marker of renal function with elevated concentration often taken as an indication of muscular dystrophy or atrophy (Baron, 1982; Sodeman and Sodeman, 1979). In the present study, the levels of urea and creatinine are increased in CCl₄ treated rats (Group II) during acute (10 days) and chronic (8 weeks) studies. There is a controversy about the effect of CCl₄ on serum urea and creatinine. No clear reasons are observed about this controversy. Some investigators found a decrease in serum urea and creatinine (Cruz et al., 1993) in CCl₄ toxicity and others have shown that the urea and creatinine levels increase due to CCl₄ toxicity (Stephen Adewole et al., 2007). The results in the present study are in accord with these reports. There was a significant increase (p<0.01; p<0.001) in the levels of urea and creatinine when compared to the control (Group I) rats during both acute and chronic studies. CCl₄-induced free radicals and an imbalanced antioxidant system may damage the kidney functions and probably contributed to the increased serum urea and creatinine concentrations seen in this study. Administration of EEAR extract at two different doses, 250 mg/kg b.wt (Group III) and 500 mg/kg b.wt (Group IV) significantly reduced (p<0.001; p<0.001) the urea and creatinine levels and brought back to near normal levels (Table 3.3). This may be due to the protective effect of the plant extract against the renal damage by CCl₄ toxicity. The results in this study are in accordance with Konishi et al., (2005) who reported that the urea and creatinine levels increase due to CCl₄ toxicity and administration of micafungin could revert the urea and creatinine levels to near normal values. Yang Yan et al., (2006) also reported an increase in creatinine during CCl₄ toxicity and administration of chitosan oligosaccharide caused normalization of creatinine levels.

Uric acid:
Uric acid, the metabolic end product of purine metabolism, has proven to be a selective antioxidant, capable especially of reacting with free radicals and hypochlorous acid. Its antioxidant action was first reported by Howell and Wyngarden (1960). The reducing properties of uric acid might explain its physiological and evolutionary role as substitute for ascorbate, another antioxidant (Shamsi and Hadi, 1996). Uric acid is known to act as a potent hydrophilic antioxidant (Esterbauer et al., 1989; Niki, 1991). Sevanian et al.,
imported that under physiological conditions urate prevents the prooxidant action of ascorbic acid. Uric acid also inhibits the oxidation of low density lipoproteins (Esterbauer et al., 1989; Frie et al., 1991). The main site of uric acid formation is liver, from where it is transported to kidneys through blood. Plasma contains high concentrations of uric acid compared to other body compartments. Uric acid is synthesized and lost from the body. Further it is excreted as urea and ammonia by intestinal cells (Chatterjee and Shinde, 1993). Kidneys, the main site of filtration, absorption and secretion of biomolecules will affect the excretion of uric acid. Analysis of uric acid gives the clear picture of the functional state of kidneys.

In the present study, the levels of uric acid decreased significantly (p<0.01; p<0.01) in CCl₄ treated rats (Group II) for 10 days and 8 weeks. The reduced level of uric acid during CCl₄ toxicity may be due to the increased utilization of uric acid against increased production of the free radicals in kidney. Administration of EEAR extract to Group III (250 mg/kg b.wt) and IV (500 mg/kg b.wt) could alter uric acid level significantly ((p<0.001; p<0.001) to near normal levels in a dose dependant manner (Table 3.3). Similar results were reported by Pombya Bose et al., (2007) by the administration of Eupatorium ayapana extract in CCl₄ induced liver damage in rats.

Lipid profiles:

Triglycerides, cholesterol and phospholipids:

Lipids of biological importance are triglycerides, cholesterol and phospholipids. Lipids are involved in cellular function, rendering substantial contribution to the surface properties of the cell. Triglycerides are esters of glycerol with the fatty acid molecules. Most triacylglycerol molecules contain fatty acids, of varying lengths, which may be unsaturated, saturated, or a combination. In animals, triacylglycerols have several roles. First, they are the major storage and transport form of fatty acids. Triacylglycerol molecules store energy more efficiently than glycogen for several reasons. Second important function of fat is to provide insulation in low temperatures (Christopher Mathews et al., 1996). Adipose tissue is a specialized connective tissue for synthesis, storage and hydrolysis of triacylglycerols (Thomas Devlin, 1997). During CCl₄ toxicity,
Table 3.3: Effect of ethanol extract of *A. radiata* on non-protein nitrogenous substances in serum of control and experimental rats against *CCL*4 induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCL4 treated)</th>
<th>Group III (CCL4 + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCL4 + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>12.608 ± 0.365a</td>
<td>37.605 ± 0.387b</td>
<td>30.197 ± 0.343c</td>
<td>19.442 ± 0.348d</td>
<td>945.222</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>2.550 ± 0.04a</td>
<td>1.557 ± 0.036b</td>
<td>1.856 ± 0.039c</td>
<td>2.265 ± 0.038d</td>
<td>128.715</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.050 ± 0.037a</td>
<td>1.938 ± 0.034b</td>
<td>1.540 ± 0.0347c</td>
<td>1.160 ± 0.031d</td>
<td>139.616</td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>16.323 ± 0.381a</td>
<td>45.312 ± 0.358 b</td>
<td>32.457 ± 0.328c</td>
<td>21.562 ± 0.373d</td>
<td>1267.11</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>2.853 ± 0.036a</td>
<td>1.452 ± 0.035 b</td>
<td>1.953 ± 0.039 c</td>
<td>2.647 ± 0.038d</td>
<td>302.632</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.933 ± 0.040a</td>
<td>2.558 ± 0.036 b</td>
<td>1.793 ± 0.029 c</td>
<td>0.867 ± 0.037a</td>
<td>494.609</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).
lipids are more easily attacked by the activated metabolites of CCl4 resulting in damage to intracellular membranes and the plasma membrane (Cheeseman et al., 1985).

Cholesterol, a weekly amphipathic lipid is the major sterol in human and virtually a component of intracellular membranes. It is the precursor for the synthesis of steroids such as corticosteroids, sex hormones, bile acids and vitamin-D in the body. Cholesterol indirectly facilitates absorption of dietary triacyl glycerols and fat soluble vitamins. The localization of cholesterol may also be attributed to the architecture and physiological functions of the tissues (Gurr and James, 1983). Serum cholesterol is one of the general indications of the synthetic and general metabolic capacity of the liver (Fregia and Jensen, 1994). The change in the levels of serum cholesterol is an indirect indicator of liver function (Hilaly et al., 2004).

Phospholipids are a class of structural lipids which are important constituent of the biomembranes. The most abundant phospholipids are phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine. Phospholipids are found in highest concentration in various cellular membranes where they serve as structural and functional components. Nearly one-half the mass of the erythrocyte membrane is comprised of various phospholipids. They play an important role in determining the membrane's physical characteristics such as membrane fluidity (Thomas Devlin, 1997). Significant increase of triglycerides, cholesterol and fatty acids in serum and in various tissues of experimental animals during stress conditions are well documented (Bopanna et al., 1997; Gueler et al., 1998). Extensive accumulation of lipids is regarded as a pathological condition, and when the accumulation becomes chronic, fibrotic changes occur in the cells that progress to cirrhosis and impaired function of the organ (Murray et al., 1993).

In the present investigation, there is a significant increase (p<0.001; p<0.001; p<0.005) in the levels of cholesterol and triglycerides were noted in serum, liver, kidney, brain and heart tissues of Group II rats (acute and chronic administration of CCl4) when compared to the Group I rats (Fig. 3.2-3.4 and 3.6-3.8) CCl4 increases the synthesis of fatty acids and triglycerides from acetate. This could be due to the transport of acetate into the liver cell, resulting in increased substrate (acetate) availability. In CCl4 toxicity, the synthesis of cholesterol is also increased (Boll et al., 2001a). On the other hand, CCl4 lowers β-oxidation of fatty acids and hydrolysis of triglycerides. This
increases the availability of fatty acids to esterification (Lieber, 2000). Reports have also shown that during CCl₄ toxicity, fat from the peripheral adipose tissue is translocated to the liver and kidney leading to its accumulation (Devarshi et al., 1986). Moreover, the synthesis of apolipoproteins is inhibited by CCl₄ (Honma and Suda, 1997) subsequently resulting in the decreased synthesis of lipoproteins. A decrease in the secretion of bile acids is also reported (Boll et al., 2001b). Administration of EEAR to CCl₄-treated rats (Group III and Group IV) for a period of 10 days (acute) and 8 weeks (chronic) significantly decreased (p<0.001; p<0.001; p<0.001; p<0.001) the levels of cholesterol and triglycerides in serum, liver, kidney, brain and heart tissues when compared to control (Group I) rats (Tables 3.4-3.8).

Phospholipid levels in serum, brain and heart tissues (Fig. 3.2, 3.5, 3.6 and 3.9) of rats treated with CCl₄ (Group II) rats were found to be significantly (p<0.001; p<0.001) increased whereas these levels are decreased significantly (p<0.001; p<0.01) in liver and kidney tissues, when compared the control (Group I) rats. The decrease in the levels of phospholipids in liver and kidney is probably due to an increase in phospholipase activity (Lamb et al., 1988). During normal lipoprotein metabolism, phospholipids are extensively converted into triglycerides (Wiggins and Gibbons, 1996). CCl₄-induced inhibition of lipoprotein-associated triglyceride export may also result in increased release of phospholipids from these tissues. An increase in phospholipid levels in brain and heart could be due to increased phospholipid content of their membranes. Administration of A. radiata extract to CCl₄-treated rats (Group III and Group IV) for a period of 10 days (acute) and 8 weeks (chronic) significantly decreased (p<0.001; p<0.001; p<0.01) the levels of phospholipids in serum, brain and heart tissues and significantly increased (p<0.01; p<0.01) in liver and kidney tissues when compared to control (Group I) rats. The decreasing effect of EEAR on lipids (Tables 3.4-3.8) may be due to the mobilization of excess cholesterol from extra hepatic tissues to liver where it is catabolized. It may also increase the activity of 7α-hydroxylase, which converts cholesterol to bile acids, facilitating the biliary cholesterol excretion. The decrease in the levels of triglycerides and phospholipids might be due to decreased free fatty acid (FFA) synthesis by EEAR, which may suppress the enzymes involved in FFA synthesis. The result obtained in the present study has correlation with the results of Kamalakkannan
et al., (2005) who reported that the administration of antioxidants such as curcumin and its analogue, bisdemethoxy curcumin analogue (BDMC-A) could alter the cholesterol, triglycerides and phospholipid content in serum, liver, kidney, brain and heart tissues and brought back to near normal values. Sureshkumar and Mishra (2007) reported the decrease in cholesterol levels due to the administration of extracts from *Pergularia daemia* Forsk. against CCl₄ induced toxicity in rats.

**Oxidative stress markers:**

**Lipid peroxidation:**

Lipid peroxidation has been shown to be of great importance in mammalian physiology and pathophysiology in the last three decades. Increased lipid peroxidation is generally believed to be an important underlying cause of the initiation of oxidative stress related various tissue injuries, and cell death, and the progression of many acute and chronic diseases (Halliwell, 1997). Lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl₄ (Srivastava et al., 1990). Lipid peroxidation is caused by the carbon trichloromethyl radical, CCl₃+. Peroxidation of lipids, particularly those containing PUFA, can dramatically change the properties of biological membranes, resulting in severe cell damage and play a significant role in pathogenesis of diseases. MDA, an end product of lipid peroxidation, is widely used as a marker of lipid peroxidation. CCl₄ administration resulted in significant increase in MDA (Mansour, 2000).

In the present study, significant elevations (*p*<0.01; *p*<0.001) in the levels of lipid peroxidation in liver, kidney, brain and heart tissues (Fig. 3.10 and 3.11) of rats treated with CCl₄ (Group II) were observed when compared to the control (Group I) rats during both acute and chronic treatments and are represented in Tables 3.4-3.8. The increase in MDA level in liver, kidney, brain and heart suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Administration of two doses of EEAR extract during both acute and chronic treatments in Group III and Group IV animals caused a significant (*p*<0.01 and *p*<0.001; *p*<0.01 and *p*<0.01 respectively) decrease in the levels of LPO, dose dependently, in serum, liver, kidney, brain and heart tissues when compared with CCl₄ induced animals (Group II). Treatment with EEAR clearly protected against the rise of
the MDA levels by CCl₄ probably due to its interference with cytochrome P450, thereby preventing the formation of hepatotoxic free radicals (Nadeem et al., 1997) or/and promotion of its glucuronidation (Gilman et al., 1992). The results in the present study are in accordance with the reports of Ko and Lim (2006) who found a decrease in LPO levels due to the administration of glycoprotein isolated from Ulmus davidiana NAKAI during CCl₄ induced liver injury in mice. Aneja et al., (2005) observed decreased LPO levels due to the administration of phytoestrogens on CCl₄-induced oxidative stress in the livers of male Wistar rats and the the result obtained in the present study are in accordance with these reports.

Protein carbonyls:
One assay that might have particular significance in assessing the extent of oxidative damage is the measure of protein oxidation. Damage to proteins may affect the function of such important molecules as receptors and enzymes, as well as contribute to the damage of other biomolecules, so the functional consequences of protein damage are significant (Wander and Du, 2000). Primary lipid oxidation products (hydroperoxides) and secondary lipid oxidation products (aldehydes and ketones) can react with proteins, and cause protein oxidation (Reytfmann et al., 1990; Kikugawa et al., 1991). Oxidation of proteins by lipid oxidation products can furthermore lead to the oxidation of amino acid residue side chains, cleavage of the peptide bonds, and formation of the covalent protein-protein cross-linked derivatives (Stadtman and Berlett, 1997). Oxidative cleavage of the peptide bond in the main chain leads to the formation of peptide fragments and the oxidation of the side chains of lysine, proline, tryptophan, arginine, and threonine, yielding to protein-carbonyl compounds. The reaction between amino acids and secondary lipid oxidation products leads to formation of carbonyl groups in the proteins (Zamora et al., 1999).

The carbonyl group content assay, according to Popadiuk et al., (2006) is a sensitive marker of free radical processes in vivo. So in the present study, the increase in protein carbonyl content was observed in liver, kidney, brain and heart tissues (Fig. 3.12 and 3.13) of control and experimental animals during both acute and chronic CCl₄ toxicity and is represented in Tables 3.9-3.12.
Table 3.4: Effect of ethanol extract of *A. radiata* on lipid peroxidation, lipid profiles and total protein in serum of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>2.333 ± 0.268ᵃ</td>
<td>3.498 ± 0.261ᵇ</td>
<td>3.463 ± 0.272ᶜ</td>
<td>2.828 ± 0.043ᵈ</td>
<td>5.769</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>138.517 ± 0.913ᵃ</td>
<td>171.628 ± 0.609ᵇ</td>
<td>169.405 ± 0.399ᵇ</td>
<td>145.358 ± 1.657ᶜ</td>
<td>273.427</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>86.347 ± 0.422ᵃ</td>
<td>132.403 ± 0.383ᵇ</td>
<td>116.363 ± 0.544ᶜ</td>
<td>91.507 ± 0.401ᵈ</td>
<td>2389.678</td>
</tr>
<tr>
<td>Phospholipids (mg/dl)</td>
<td>133.653 ± 0.449ᵃ</td>
<td>165.280 ± 0.444ᵇ</td>
<td>157.623 ± 0.832ᶜ</td>
<td>141.330 ± 0.405ᵈ</td>
<td>672.542</td>
</tr>
<tr>
<td>Total Protein (g/dl)</td>
<td>7.410 ± 0.259ᵃ</td>
<td>3.422 ± 0.372ᵇ</td>
<td>4.480 ± 0.242ᶜ</td>
<td>6.555 ± 0.353ᵃ</td>
<td>34.707</td>
</tr>
</tbody>
</table>

|                           |                   |                         |                                           |                                           |               |
|                           |                   |                         |                                           |                                           |               |
| Lipid peroxidation         | 3.058 ± 0.042ᵃ    | 5.165 ± 0.039ᵇ          | 4.752 ± 0.039ᶜ                           | 3.550 ± 0.035ᵈ                           | 646.302       |
| Triglycerides (mg/dl)      | 126.415 ± 0.346ᵃ  | 208.332 ± 0.788ᵇ        | 174.047 ± 0.963ᶜ                         | 145.580 ± 3.334ᵈ                         | 398.175       |
| Cholesterol (mg/dl)        | 75.317 ± 3.879ᵃ   | 184.433 ± 3.273ᵇ        | 164.898 ± 3.962ᶜ                         | 83.285 ± 0.307ᵃ                          | 299.011       |
| Total Protein (g/dl)       | 6.478 ± 1.657ᵃ    | 2.190 ± 0.030ᵇ          | 5.842 ± 0.039ᵃ                           | 7.348 ± 0.040ᵃ                           | 7.491         |

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Lipid peroxidation – n moles of MDA/mg protein.
Table 3.5: Effect of ethanol extract of *A. radiata* on lipid peroxidation and lipid profiles in liver tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>1.44 ± 0.008ᵃ</td>
<td>2.343 ± 0.010ᵇ</td>
<td>1.956 ± 0.010ᶜ</td>
<td>1.546 ± 0.007ᵈ</td>
<td>2268.36</td>
</tr>
<tr>
<td>Triglycerides (mg/tissue)</td>
<td>3.005 ± 0.008ᵃ</td>
<td>4.665 ± 0.015ᵇ</td>
<td>4.041 ± 0.011ᶜ</td>
<td>3.3 ± 0.005ᵈ</td>
<td>5096.69</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>2.78 ± 0.012ᵃ</td>
<td>3.556 ± 0.018ᵇ</td>
<td>3.26 ± 0.010ᶜ</td>
<td>3.05 ± 0.017ᵈ</td>
<td>503.368</td>
</tr>
<tr>
<td>Phospholipids (mg/tissue)</td>
<td>13.766 ± 0.156ᵃ</td>
<td>11.933 ± 0.017ᵇ</td>
<td>12.653 ± 0.010ᶜ</td>
<td>13.15 ± 0.009ᵈ</td>
<td>97.573</td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>1.92 ± 0.015ᵃ</td>
<td>3.935 ± 0.011ᵇ</td>
<td>3.063 ± 0.013ᶜ</td>
<td>2.066 ± 0.014ᵈ</td>
<td>4918.781</td>
</tr>
<tr>
<td>Triglycerides (mg/tissue)</td>
<td>3.246 ± 0.011ᵃ</td>
<td>6.15 ± 0.018ᵇ</td>
<td>4.163 ± 0.012ᶜ</td>
<td>3.493 ± 0.025ᵈ</td>
<td>5938.311</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>3.068 ± 0.019ᵃ</td>
<td>4.356 ± 0.017ᵇ</td>
<td>3.925 ± 0.015ᶜ</td>
<td>3.343 ± 0.010ᵈ</td>
<td>1381.726</td>
</tr>
<tr>
<td>Phospholipids (mg/tissue)</td>
<td>15.04 ± 0.025ᵃ</td>
<td>10.881 ± 0.020ᵇ</td>
<td>12.38 ± 0.160ᶜ</td>
<td>14.793 ± 0.047ᵃ</td>
<td>556.562</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Lipid peroxidation– n moles of MDA/mg protein.
TG – Triglycerides; CH – Cholesterol; PL – Phospholipids.

Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.
TG – Triglycerides; CH – Cholesterol; PL – Phospholipids.

Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.
Table 3.6: Effect of ethanol extract of *A. radiata* on lipid peroxidation and lipid profiles in kidney tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>2.2 ± 0.012*</td>
<td>3.202 ± 0.012b</td>
<td>2.817 ± 0.018c</td>
<td>2.390 ± 0.017d</td>
<td>918.8</td>
</tr>
<tr>
<td>Triglycerides (mg/g tissue)</td>
<td>4.775 ± 0.017a</td>
<td>5.336 ± 0.016b</td>
<td>5.0467 ± 0.016c</td>
<td>4.941 ± 0.017d</td>
<td>200.661</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>3.51 ± 0.007a</td>
<td>4.657 ± 0.028b</td>
<td>4.177 ± 0.019c</td>
<td>3.843 ± 0.017d</td>
<td>656.032</td>
</tr>
<tr>
<td>Phospholipids (mg/g tissue)</td>
<td>13.863 ± 0.023a</td>
<td>11.34 ± 0.017b</td>
<td>11.883 ± 0.028c</td>
<td>12.76 ± 0.015d</td>
<td>2687.674</td>
</tr>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>2.316 ± 0.017a</td>
<td>4.51 ± 0.012b</td>
<td>3.046 ± 0.012c</td>
<td>2.79 ± 0.027d</td>
<td>2790.28</td>
</tr>
<tr>
<td>Triglycerides (mg/g tissue)</td>
<td>4.323 ± 0.019a</td>
<td>6.32 ± 0.027b</td>
<td>5.605 ± 0.017c</td>
<td>4.818 ± 0.027d</td>
<td>1475.217</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>3.906 ± 0.027a</td>
<td>6.031 ± 0.026b</td>
<td>4.723 ± 0.019c</td>
<td>4.286 ± 0.038d</td>
<td>1053.966</td>
</tr>
<tr>
<td>Phospholipids (mg/g tissue)</td>
<td>13.575 ± 0.055a</td>
<td>8.075 ± 0.017b</td>
<td>10.763 ± 0.028c</td>
<td>13.032 ± 0.030d</td>
<td>4962.701</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Lipid peroxidation - n moles of MDA/mg protein.
### Table 3.7: Effect of ethanol extract of *A. radiata* on lipid peroxidation and lipid profiles in brain tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>2.701±0.048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.465 ± 0.043&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.056 ± 0.070&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.895 ± 0.070&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.387</td>
</tr>
<tr>
<td>Triglycerides (mg/g tissue)</td>
<td>3.551±0.043&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.266 ± 0.050&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.026 ± 0.049&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.74 ± 0.036&lt;sup&gt;d&lt;/sup&gt;</td>
<td>49.162</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>12.051±0.055&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.261 ± 0.104&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.753 ± 0.040&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.138 ± 0.051&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.055</td>
</tr>
<tr>
<td>Phospholipids (mg/g tissue)</td>
<td>17.466±0.350&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.693 ± 0.328&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.663 ± 0.284&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.693 ± 0.328&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.304</td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>2.876 ± 0.058&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.765 ± 0.062&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.351 ± 0.054&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.095 ± 0.023&lt;sup&gt;d&lt;/sup&gt;</td>
<td>54.847</td>
</tr>
<tr>
<td>Triglycerides (mg/g tissue)</td>
<td>4.238 ± 0.057&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.091 ± 0.081&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.96 ± 0.126&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.735 ± 0.069&lt;sup&gt;c&lt;/sup&gt;</td>
<td>287.838</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>12.086±0.048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.745 ± 0.053&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.778 ± 0.112&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.226 ± 0.320&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50.318</td>
</tr>
<tr>
<td>Phospholipids (mg/g tissue)</td>
<td>21.438±0.355&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.826 ± 0.338&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.89 ± 0.075&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.6 ± 0.300&lt;sup&gt;d&lt;/sup&gt;</td>
<td>42.167</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Lipid peroxidation – n moles of MDA/mg protein.
Figure 3.10: Effect of ethanol extract of *A. radiata* on the level of LPO in liver, kidney, brain, heart and serum of control and experimental rats against CCl₄ acute toxicity.

Figure 3.11: Effect of ethanol extract of *A. radiata* on the level of LPO in liver, kidney, brain, heart and serum of control and experimental rats against CCl₄ chronic toxicity.
Table 3.8: Effect of ethanol extract of *A. radiata* on lipid peroxidation and lipid profiles in heart tissue of control and experimental rats against CCL₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCL₄ treated)</th>
<th>Group III (CCL₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCL₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation</td>
<td>2.85 ± 0.061ᵃ</td>
<td>3.653 ± 0.037ᵇ</td>
<td>3.258 ± 0.039ᶜ</td>
<td>3.058 ± 0.034ᵈ</td>
<td>60.453</td>
</tr>
<tr>
<td>Triglycerides (mg/g tissue)</td>
<td>3.051 ± 0.042ᵃ</td>
<td>4.646 ± 0.042ᵇ</td>
<td>3.83 ± 0.038ᶜ</td>
<td>3.136 ± 0.038ᵃ</td>
<td>342.335</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>1.653 ± 0.039ᵃ</td>
<td>2.745 ± 0.032ᵇ</td>
<td>2.248 ± 0.032ᶜ</td>
<td>1.743 ± 0.042ᵃ</td>
<td>188.938</td>
</tr>
<tr>
<td>Phospholipids (mg/g tissue)</td>
<td>7.933 ± 0.038ᵃ</td>
<td>12.605 ± 0.341ᵇ</td>
<td>11.331 ± 0.325ᶜ</td>
<td>8.463 ± 0.377ᵃ</td>
<td>55.325</td>
</tr>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid peroxidation (LPO)</td>
<td>3.157 ± 0.035ᵃ</td>
<td>3.828 ± 0.037ᵇ</td>
<td>3.533 ± 0.037ᶜ</td>
<td>3.253 ± 0.030ᵈ</td>
<td>74.578</td>
</tr>
<tr>
<td>Triglycerides (mg/g tissue)</td>
<td>3.157 ± 0.036ᵃ</td>
<td>6.357 ± 0.342ᵇ</td>
<td>4.455 ± 0.374ᵃ</td>
<td>3.752 ± 0.035ᵃ</td>
<td>29.789</td>
</tr>
<tr>
<td>Cholesterol (mg/g tissue)</td>
<td>1.738 ± 0.040ᵃ</td>
<td>4.443 ± 0.038ᵇ</td>
<td>3.345 ± 0.041ᶜ</td>
<td>2.057 ± 0.043ᵈ</td>
<td>930.429</td>
</tr>
<tr>
<td>Phospholipids (mg/g tissue)</td>
<td>10.420 ± 0.310ᵃ</td>
<td>16.525 ± 0.411ᵇ</td>
<td>13.510 ± 0.346ᶜ</td>
<td>11.493 ± 0.330ᵈ</td>
<td>58.461</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: LPO – n moles of MDA/mg protein.
A significant (p<0.001 and p < 0.01) increase in the levels of protein carbonyls was observed in the liver, kidney, brain and heart tissues of CCl₄ induced (Group II) animals during both acute and chronic toxicity when compared with the control animals (Group I). Administration of two doses of EEAR during both acute and chronic treatments in Group III (250 mg/kg b.wt) and Group IV (500 mg/kg b.wt) animals caused a significant (p<0.01 and p<0.001; p<0.001 and p<0.01 respectively) decrease in the levels of protein carbonyls in the liver, kidney, brain and heart tissues, when compared with CCl₄ induced (Group II) animals. Treatment with EEAR markedly and almost completely prevented the CCl₄ induced oxidative damage and prevented the increase in protein carbonyl levels and brought back to near normal values. Rudnicki et al., (2007) and Maksimchik et al., (2008) reported similar findings with Passiflora alata pretreatment during CCl₄ toxicity prevented oxidative damage and decreased the protein carbonyl content in liver, kidney, brain and heart tissues. Similarly, N-acetyl-L-cysteine prevented oxidative damage during CCl₄ toxicity and decreased the protein carbonyl content in liver (Maksimchik et al., 2008).

Total sulfhydryl content:
Living systems contain large amounts of sulfhydryl compounds which must be maintained in the proper oxidation state to retain their biological activity. Serum -SH groups act as important cellular scavengers of peroxides and thus help to protect cells from damage by these molecules (Ahmet Isik et al., 2007). One of the mechanisms by which CCl₄ produce effects is through their interaction with cellular sulfhydryl groups in proteins. Sulfhydryl groups thus serve as a source of electrons for reduction and also mediate the methylation process. When the availability of free thiol group is low, enhanced expression of toxicity in the form of oxidative stress could occur. Thus reduction in total -SH group in our study indicated toxicity status of the tissue by these toxicants. Several physiological mechanisms (antioxidative defense mechanism) dispose of free radicals/ROS by directly scavenging them or by interrupting the already occurring lipid peroxidation chain reaction to limit their tissue damage (Venkateswara Rao et al., 2006). The decrease in -SH groups could be explained by the detection of high xanthine oxidase (XOD) activity (Miesel and Zuber, 1993). Snyder and Cornatzer (1958) reported
that histological examinations indicated that the decline of sulfhydryl compounds in the liver during CCl₄ toxicity is well correlated with the onset of necrosis and evidence has been presented indicating that sulfhydryl groups are important in nucleic acid metabolism and are thought to be involved in the transfer of formyl groups and it seems feasible that the degeneration of liver tissue may be a result of interference with the nucleic acid metabolism of the cell, mediated through the lack of -SH groups.

In the present study, the total sulfhydryl content was found to be significantly decreased (p<0.01 and p<0.001) in liver, kidney, brain and heart tissues of Group II (CCl₄ treated) rats when compared to the control (Group I) rats, during both acute and chronic CCl₄ induced oxidative stress (Fig. 3.14 and 3.15). Treatment with EEAR at two doses, Group III (250mg/kg b.wt) and Group IV (500mg/kg b.wt) during CCl₄ toxicity protected against the oxidative stress, dose dependently and restored the total sulfhydryl content of tissues (Tables 3.9-3.12). There was a significant (p<0.001; p<0.01; p<0.001 and p<0.001) increase in the levels of total sulfhydryls in Group III and Group IV rats when compared with the CCl₄ treated rats (Group II). Studies by Yan Yang et al., (2006) showed decreased levels of total -SH by CCl₄ intoxication and protective effects of chitosan oligosaccharide during toxicity which are in accord with our results. Cakatay and Kayali (2005) reported that the depletion of total sulfhydryl content was prevented by the administration of alpha-lipoic acid in aging rats.

Xanthine oxidase:
Xanthine oxidase is a flavoprotein, which catalyses the oxidation of hypoxanthine to xanthine and generates superoxide, hydroxyl radicals and uric acid (Cheng et al., 2003). Xanthine oxydoreductase is molybdenum-containing monooxygenase and exists as dehydrogenase (XDH) and oxidase (XOD). For Xanthine oxidase to be a source of oxygen free radicals, it must be converted from its normal dehydrogenase form to an oxidase, type (Della Corte and Stirpe, 1972; Weiss, 1986). Under physiological conditions, the enzyme is active as XDH, but several stimuli such as inflammation, ischemia reperfusion injury (Cuzzocrea et al., 2001) and hypoxia promote the conversion of XDH to the XOD form. During oxidative stress, Xanthine oxidase converts from dehydrogenase to oxidase form, along with increased purine catabolism, result in an
increased rate of $O_2^-$ and $H_2O_2$ production uncompensated for by normal cellular defense mechanisms. Xanthine oxidase-derived superoxide anion has been linked to post-ischaemic tissue injury and edema (Hearse et al., 1986; McCord and Fridovich, 1969). Hence, phytochemicals or extracts, which inhibit the superoxide anion regeneration by the enzymatic pathway, would be beneficial in preventing oxidative stress and tissue injury.

In the present study, xanthine oxidase was examined because it was found to be enhanced in rat liver damaged by $CCl_4$ poisoning (Villela and Mitidieri, 1955) and we have found that $CCl_4$ induced necrosis provokes XOD activity which produces oxidative stress by generating ROS, indicating its role in this type of tissue injury. The activity levels of XOD in liver, kidney, brain and heart tissues of control and experimental animals during both acute and chronic treatments were represented in Tables 3.9-3.12. A marked ($p<0.001$ and $p<0.01$) increase in the activity levels of XOD was observed in the liver, kidney, brain and heart tissues of $CCl_4$ treated (Group II) rats when compared with the control (Group I) rats. Administration of two doses of EEAR extract during both acute and chronic treatments in Group III (250 mg/kg b.wt) and Group IV (500 mg/kg b.wt) rats caused a significant ($p<0.01$ and $p<0.001$; $p<0.001$ and $p<0.01$ respectively) decrease in the levels of XOD in liver, kidney, brain and heart tissues (Fig. 3.16 and 3.17) when compared with $CCl_4$ induced (Group II) rats. The remarkable decrease of XOD activity observed in EEAR treated (Group III and IV) rats followed by $CCl_4$ treatment is in accordance with results of Ohta et al., (2004) and Pawa and Ali (2004) who reported the significant decrease in xanthine oxidase activity during liver necrosis due to the supplementation of melatonin and tungsten compounds respectively.

**Total protein**

Proteins are the macromolecular substances present in all living cells. They serve as major structural component in animal tissues. In the form of skin, hair, cartilage, tendons, and ligaments, proteins hold together, protect, and provide structure to the body. In the form of enzymes, hormones, antibodies, and globulins, they catalyse, regulate, and protect the body chemistry. In the form of haemoglobin, myoglobin, and various lipoproteins, they affect the transport of oxygen and other substances within the body.
(Thomas Devlin, 1997). In view of its importance it is assayed in serum (Table 3.4), liver, kidney, brain and heart of control and experimental rats during both acute and chronic treatments. In the present investigation, the level of protein was found to be significantly (p<0.001 and p<0.01) decreased in CCl₄ treated rats. In hepatotoxicity, a depression in total protein is observed due to the defect in protein biosynthesis (Dubey et al., 1994). This is due to the disruption and disassociation of polyribosomes from endoplasmic reticulum following CCl₄ administration (Clawson, 1989). Administration of EEAR at a dose of 250 and 500 mg/kg b.wt. (Group III and Group IV) prevented this change in a dose dependent manner (Tables 3.9-3.12). This may be due to the promotion of the assembly of ribosomes on endoplasmic reticulum to facilitate uninterrupted protein biosynthesis. The results reported in the present study are statistically significant between each group of rats at 0.01%. The results obtained are in accord with the reports of Rajesh and Latha, (2004).

**Glucose-6-phosphate dehydrogenase:**

For optimal glutathione reductase activity, NADPH must be supplied in adequate concentration. HMP shunt is the major path way in which a major portion of the cell’s NADPH is produced and Glucose-6-phosphate dehydrogenase is the important enzyme in this pathway which converts NADP to NADPH. The activity of G-6-PDH is an index for determining the efficiency of HMP shunt (Oh et al., 1998). Since, HMP shunt is an alternate pathway for the supply of energy and reduced co-enzymes (NADPH), which function in detoxification process of free radicals produced during oxidative stress. So, an attempt has been made in the present investigation to elucidate the G-6-PDH activity level in liver, kidney, brain and heart tissues (Fig. 3.18 and 3.19) of the rats under oxidative stress condition induced by CCl₄ and found that it is significantly (p<0.01 and p<0.001) decreased after acute and chronic CCl₄ administration. This decrease could be explained by the findings of Szweda et al. (1993), who described the inactivation of purified bacterial glucose-6-phosphate dehydrogenase by trans-4-hydroxy-2-nonenal, a toxic product of membrane lipid peroxidation. Administration of EEAR mitigated the reduced activity level of G-6-PDH in Group III and Group IV rats to normal level in dose dependent and in time dependent manner. Significant (p<0.01 and p<0.001; p<0.01 and p<0.001) increase in the activity of G6PDH was recorded (Tables 3.9-3.12) in rats.
Table 3.9: Effect of ethanol extract of *A. radiata* on protein carbonyl, total sulfhydryl, XOD and G6PDH in liver tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCL₄ treated)</th>
<th>Group III (CCL₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCL₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>125.85 ± 0.772ᵃ</td>
<td>68.017 ± 1.141ᵇ</td>
<td>115.55 ± 0.496ᶜ</td>
<td>122.95 ± 1.087ᵈ</td>
<td>881.101</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>3.966 ± 0.007ᵃ</td>
<td>15.036 ± 0.016ᵇ</td>
<td>10.856 ± 0.034ᶜ</td>
<td>5.841 ± 0.083ᵈ</td>
<td>11938.2</td>
</tr>
<tr>
<td>Total Sulfhydryl</td>
<td>2.536 ± 0.014ᵃ</td>
<td>1.453 ± 0.013ᵇ</td>
<td>1.955 ± 0.010ᶜ</td>
<td>2.17 ± 0.015ᵈ</td>
<td>1203.87</td>
</tr>
<tr>
<td>XOD</td>
<td>0.811 ± 0.012ᵃ</td>
<td>1.315 ± 0.012ᵇ</td>
<td>1.05 ± 0.010ᶜ</td>
<td>0.91 ± 0.018ᵈ</td>
<td>272.742</td>
</tr>
<tr>
<td>G6PDH</td>
<td>2.846 ± 0.008ᵃ</td>
<td>1.903 ± 0.032ᵇ</td>
<td>2.055 ± 0.015ᵇ</td>
<td>2.471 ± 0.099ᶜ</td>
<td>64.768</td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>153.867 ± 0.311ᵃ</td>
<td>129.627 ± 0.249ᵇ</td>
<td>131.497 ± 0.229ᶜ</td>
<td>146.153 ± 0.644ᵈ</td>
<td>873.562</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>4.291 ± 0.029ᵃ</td>
<td>22.635 ± 0.010ᵇ</td>
<td>17.776 ± 0.178ᶜ</td>
<td>9.011 ± 0.264ᵈ</td>
<td>2691.495</td>
</tr>
<tr>
<td>Total Sulfhydryl</td>
<td>3.048 ± 0.016ᵃ</td>
<td>0.868 ± 0.010ᵇ</td>
<td>1.633 ± 0.014ᶜ</td>
<td>2.763 ± 0.010ᵈ</td>
<td>6259.253</td>
</tr>
<tr>
<td>XOD</td>
<td>0.755 ± 0.008ᵃ</td>
<td>1.655 ± 0.020ᵇ</td>
<td>1.061 ± 0.014ᶜ</td>
<td>0.92 ± 0.017ᵈ</td>
<td>644.994</td>
</tr>
<tr>
<td>G6PDH</td>
<td>2.156 ± 0.013ᵃ</td>
<td>1.141 ± 0.018ᵇ</td>
<td>1.841 ± 0.018ᶜ</td>
<td>2.1 ± 0.026ᵃ</td>
<td>573.143</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Total protein—mg/dl; Protein carbonyls—μmol/mg protein; Total sulfhydryl—μg/mg protein; Xanthine oxidase (XOD)—μg of urate formed/h/mg protein; Glucose-6-phosphate dehydrogenase (G6PDH)—units/min/mg protein.
Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

Figure 3.12: Effect of ethanol extract of A. radiata on the level of protein carbonyls in liver, kidney, brain and heart of control and experimental rats against CCl₄ acute toxicity.

Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

Figure 3.13: Effect of ethanol extract of A. radiata on the level of protein carbonyls in liver, kidney, brain and heart of control and experimental rats against CCl₄ chronic toxicity.
Figure 3.14: Effect of ethanol extract of *A. radiata* on the level of total sulphydryl groups in liver, kidney, brain and heart of control and experimental rats against *CCl₄* acute toxicity.

Figure 3.15: Effect of ethanol extract of *A. radiata* on the level of total sulphydryl groups in liver, kidney, brain and heart of control and experimental rats against *CCl₄* chronic toxicity.
Table 3.10: Effect of ethanol extract of *A. radiata* on protein carbonyl, total sulphydryl, XOD and G6PDH in kidney tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>76.452 ± 0.354ᵃ</td>
<td>52.392 ± 0.262ᵇ</td>
<td>67.417 ± 0.352ᶜ</td>
<td>73.665 ± 0.264ᵈ</td>
<td>1191.133</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>-2.785 ± 0.016ᵃ</td>
<td>11.673 ± 0.017ᵇ</td>
<td>9.086 ± 0.041ᶜ</td>
<td>3.49 ± 0.015ᵈ</td>
<td>30207.63</td>
</tr>
<tr>
<td>Total Sulphydryl</td>
<td>1.833 ± 0.010ᵃ</td>
<td>0.76 ± 0.030ᵇ</td>
<td>1.068 ± 0.013ᶜ</td>
<td>1.681 ± 0.018ᵈ</td>
<td>698.637</td>
</tr>
<tr>
<td>XOD</td>
<td>0.861 ± 0.016ᵃ</td>
<td>1.183 ± 0.015ᵇ</td>
<td>1.071 ± 0.016ᶜ</td>
<td>0.836 ± 0.021ᵃ</td>
<td>97.036</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.52 ± 0.020ᵃ</td>
<td>1.321 ± 0.019ᵇ</td>
<td>1.394 ± 0.014ᶜ</td>
<td>1.453 ± 0.011ᵈ</td>
<td>27.269</td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>57.845 ± 0.221ᵃ</td>
<td>43.4 ± 0.235ᵇ</td>
<td>45.06 ± 0.346ᶜ</td>
<td>55.668 ± 0.370ᵈ</td>
<td>593.983</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>2.588 ± 0.016ᵃ</td>
<td>17.19 ± 0.017ᵇ</td>
<td>10.643 ± 0.023ᶜ</td>
<td>5.181 ± 0.016ᵈ</td>
<td>125740.8</td>
</tr>
<tr>
<td>Total Sulphydryl</td>
<td>1.91 ± 0.013ᵃ</td>
<td>0.693 ± 0.019ᵇ</td>
<td>1.39 ± 0.019ᶜ</td>
<td>1.85 ± 0.013ᵈ</td>
<td>1179.18</td>
</tr>
<tr>
<td>XOD</td>
<td>0.951 ± 0.012ᵃ</td>
<td>1.49 ± 0.025ᵇ</td>
<td>1.221 ± 0.016ᶜ</td>
<td>0.938 ± 0.015ᵃ</td>
<td>217.662</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.565 ± 0.024ᵃ</td>
<td>1.2 ± 0.019ᵇ</td>
<td>1.345 ± 0.019ᶜ</td>
<td>1.505 ± 0.023ᵃ</td>
<td>59.23</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan’s Multiple Range Test (DMRT).

Units: Total protein—mg/dl; Protein carbonyls—n moles/mg protein; Total sulphydryl—μg/mg protein; Xanthine oxidase (XOD)—μg of urate formed/h/mg protein; Glucose-6-phosphate dehydrogenase (G6PDH)—units/min/mg protein.
Table 3.11: Effect of ethanol extract of *A. radiata* on protein carbonyl, total sulfhydryl, XOD and G6PDH in brain tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCL₄ treated)</th>
<th>Group III (CCL₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCL₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acute</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>64.423 ± 0.381ᵃ</td>
<td>46.448 ± 0.403ᵇ</td>
<td>54.35 ± 0.414ᶜ</td>
<td>59.497 ± 0.353ᵈ</td>
<td>391.311</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>1.751 ± 0.125ᵃ</td>
<td>5.645 ± 0.228ᵇ</td>
<td>4.648 ± 0.036ᶜ</td>
<td>2.25 ± 0.039ᵈ</td>
<td>200.01</td>
</tr>
<tr>
<td>Total Sulfhydryl</td>
<td>1.343 ± 0.040ᵃ</td>
<td>1.076 ± 0.015ᵇ</td>
<td>1.25 ± 0.040ᵃ</td>
<td>1.345 ± 0.036ᵃ</td>
<td>13.537</td>
</tr>
<tr>
<td>XOD</td>
<td>0.958 ± 0.031ᵃ</td>
<td>1.356 ± 0.036ᵇ</td>
<td>1.143 ± 0.038ᶜ</td>
<td>0.921 ± 0.074ᵃ</td>
<td>17.384</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.645 ± 0.040ᵃ</td>
<td>1.251 ± 0.031ᵇ</td>
<td>1.34 ± 0.035ᵇ</td>
<td>1.445 ± 0.036ᵇ</td>
<td>22.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chronic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>52.917 ± 0.318ᵃ</td>
<td>30.4 ± 0.628ᵇ</td>
<td>41.9 ± 0.458ᶜ</td>
<td>47.462 ± 0.401ᵈ</td>
<td>407.245</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>1.99 ± 0.035ᵃ</td>
<td>9.366 ± 0.208ᵇ</td>
<td>5.796 ± 0.109ᶜ</td>
<td>3.255 ± 0.030ᵈ</td>
<td>740.112</td>
</tr>
<tr>
<td>Total Sulfhydryl</td>
<td>1.643 ± 0.046ᵃ</td>
<td>0.833 ± 0.054ᵇ</td>
<td>1.376 ± 0.080ᶜ</td>
<td>1.561 ± 0.032ᵃ</td>
<td>42.514</td>
</tr>
<tr>
<td>XOD</td>
<td>1.143 ± 0.042ᵃ</td>
<td>1.715 ± 0.053ᵇ</td>
<td>1.423 ± 0.033ᶜ</td>
<td>1.143 ± 0.036ᵃ</td>
<td>42.409</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.638 ± 0.054ᵃ</td>
<td>0.935 ± 0.038ᵇ</td>
<td>1.335 ± 0.033ᶜ</td>
<td>1.496 ± 0.027ᵈ</td>
<td>60.66</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Total protein—mg/dl; Protein carbonyl—nmol/mg protein; Total sulfhydryl—μg/mg protein; Xanthine oxidase (XOD)—μg of urate formed/h/mg protein; Glucose-6-phosphate dehydrogenase (G6PDH)—units/min/mg protein.
Figure 3.16: Effect of ethanol extract of *A. radiata* on the level of XOD in liver, kidney, brain and heart of control and experimental rats against CCl₄ acute toxicity.

Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

Figure 3.17: Effect of ethanol extract of *A. radiata* on the level of XOD in liver, kidney, brain and heart of control and experimental rats against CCl₄ chronic toxicity.
Figure 3.18: Effect of ethanol extract of A. radiata on the level of G-6-PDH in liver, kidney, brain and heart of control and experimental rats against CCl₄ acute toxicity.

Figure 3.19: Effect of ethanol extract of A. radiata on the level of G-6-PDH in liver, kidney, brain and heart of control and experimental rats against CCl₄ chronic toxicity.
Table 3.12: Effect of ethanol extract of *A. radiata* on protein carbonyl, total sulphydryl, XOD and G6PDH in heart tissue of control and experimental rats against CCl₄ induced acute and chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>53.563 ± 0.387ₐ</td>
<td>24.658 ± 0.360ᵇ</td>
<td>41.397 ± 0.332ᶜ</td>
<td>48.305 ± 0.341ᵈ</td>
<td>1251.073</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>2.551 ± 0.280ₐ</td>
<td>8.333 ± 0.352ᵇ</td>
<td>6.586 ± 0.338ᶜ</td>
<td>3.811 ± 0.162ᵈ</td>
<td>80.198</td>
</tr>
<tr>
<td>Total Sulphydryl</td>
<td>1.251 ± 0.036ᵃ</td>
<td>0.935 ± 0.038ᵇ</td>
<td>1.05 ± 0.034ᶜ</td>
<td>1.158 ± 0.040ᶜ</td>
<td>13.729</td>
</tr>
<tr>
<td>XOD</td>
<td>0.748 ± 0.039ᵃ</td>
<td>1.024 ± 0.004ᵇ</td>
<td>0.95 ± 0.040ᶜ</td>
<td>0.838 ± 0.039ᵃ</td>
<td>12.775</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.058 ± 0.037ᵃ</td>
<td>0.75 ± 0.043ᵇ</td>
<td>0.848 ± 0.042ᵃ</td>
<td>0.938 ± 0.041ᵃ</td>
<td>10.256</td>
</tr>
<tr>
<td><strong>Chronic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein</td>
<td>49.435 ± 0.387ᵃ</td>
<td>34.503 ± 0.366ᵇ</td>
<td>43.412 ± 0.368ᶜ</td>
<td>46.452 ± 0.312ᵈ</td>
<td>322.235</td>
</tr>
<tr>
<td>Protein carbonyl</td>
<td>2.610 ± 0.231ᵃ</td>
<td>14.547 ± 0.350ᵇ</td>
<td>8.332 ± 0.343ᶜ</td>
<td>5.427 ± 0.378ᵈ</td>
<td>239.68</td>
</tr>
<tr>
<td>Total Sulphydryl</td>
<td>1.243 ± 0.038ᵃ</td>
<td>0.808 ± 0.049ᵇ</td>
<td>1.052 ± 0.039ᵃ</td>
<td>1.153 ± 0.036ᵃ</td>
<td>20.981</td>
</tr>
<tr>
<td>XOD</td>
<td>0.842 ± 0.039ᵃ</td>
<td>1.162 ± 0.040ᵇ</td>
<td>1.047 ± 0.043ᶜ</td>
<td>0.938 ± 0.037ᵃ</td>
<td>11.995</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.155 ± 0.033ᵃ</td>
<td>0.838 ± 0.034ᵇ</td>
<td>0.947 ± 0.038ᵇ</td>
<td>1.065 ± 0.044ᵇ</td>
<td>13.713</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

Units: Total protein—mg/dl; Protein carbonyls—n moles/mg protein; Total sulphydryl—µg/mg protein; Xanthine oxidase (XOD)—µg of urate formed/h/mg protein; Glucose-6-phosphate dehydrogenase (G6PDH)—units/min/mg protein.
receiving CCl₄ and two doses of *A. radiata* ethanol extract (Group III and Group IV) for a period of 10 days and 8 weeks when compared to Group II rats. This condition may be due to the protective effect of EEAR by scavenging the free radicals and minimizing the oxidative stress condition. Various reports suggested a decreased activity of G-6-PDH at the time of oxidative stress and observed the reversal of this condition to normal with treatment of natural antioxidant supplements (Ip et al., 1995; Ko et al., 1995 and Singab et al., 2005).

**Antioxidant enzymes and non-enzymic antioxidants:**

Reactive oxygen species, including superoxide, hydroxyl radicals and hydrogen peroxide, are generated and react with biological molecules, eventually damaging membranes and other tissues (Vuillaume, 1987). Antioxidant enzymes - Superoxide dismutase, catalase, Glutathione peroxidase, Glutathione reductase and Glutathione-s-transferase (Halliwell and Gutteridge, 1990) and non-enzymic antioxidants - reduced glutathione, vitamin C and E represent one protection against oxidative tissue damage.

**Superoxide dismutase (SOD):**

Superoxide radical anion (O₂⁻) is an oxygen centered radical with selective reactivity. This species is produced by a number of enzyme systems, by auto-oxidation reactions and by non-enzymatic electron transfer that univalently reduce molecular oxygen (Fridovich, 1989). Excessive production of O₂⁻ in the body can cause oxidative damage to the surrounding tissues. SOD catalyzes the removal of superoxide radical O₂⁻ to H₂O₂. Deficiency of this enzyme causes the accumulation of these ions. A reduction in the activity of this enzyme in CCl₄ toxicity has been well-documented (Recknagel, 1983). Significant (p<0.01 and p<0.001) reduction in the level of SOD was observed in present study in liver, kidney, brain and heart tissues of rats receiving CCl₄ (Group II) for a period of 10 days and 8 weeks. Similar significant reduction of SOD activities in liver of CCl₄ intoxicated rats were reported by Pandit et al., (2004). This may be due to the inactivation of the enzyme by free radicals which are generated by CCl₄. Administration of EEAR during acute and chronic treatment in Group III (exposed to CCl₄ and 250 mg/kg b. wt. of EEAR) and Group IV (exposed to CCl₄ and 500 mg/kg b. wt. of EEAR)
rats significantly (p<0.01 and p<0.001; p<0.001 and p<0.01) increased the levels of this enzyme in dose and time dependent manner when compared to Group II (CCL4 intoxicated) rats and very similar to the values noted in control (Group I) rats. The activity of SOD in liver, kidney, brain and heart tissues of control and experimental rats during acute and chronic treatments are represented in Tables 3.13-3.20. This suggests a protective effect by the A. radiata ethanol extract, which is a very encouraging finding and may be due to preventing the formation of superoxide radical anion. The results in the present findings are in accord with the reports of Semiz and Sen (2007).

Catalase:
In addition to SOD, catalase also plays an important role in protecting the tissues against oxidants. CAT is a hemeprotein, localized in the peroxisomes or the microperoxisomes. This enzyme catalyses the decomposition of H2O2 to water and oxygen and thus protecting the cell from oxidative damage by H2O2 and OH' (Young and Woodside, 2001). Reduction in the activity of this enzyme is associated with the accumulation of hydrogen peroxide, which exerts its deleterious effects on the biological system. Administration of CCL4 leads to generation of peroxo radical, O2O , which is associated with inactivation of CAT in various organs studied. In the present study, a significant (p<0.01) decrease in the activity of CAT was found in liver, kidney, brain and heart tissues of rats challenged with CCL4 (Group II) for a period of 10 days and 8 weeks. The reduced activity of CAT (relative to control) observed in the present investigation in rats administered with CCL4 is consistent with the results of Sundaram and Mitra (2007). In rats receiving CCL4 and two doses of A. radiata ethanol extract (Group III and Group IV) for a period of 10 days and 8 weeks, the activity of CAT was significantly (p<0.001 and p<0.01) higher than in Group II rats, and very similar to the values noted in control (Group I) rats. Tables 3.13-3.20 represent the effect of EEAR during acute and chronic treatments on the activity of CAT in liver, kidney, brain and heart tissues of control and experimental rats. This suggests that ethanol extract of A. radiata confers its protective effect by scavenging H2O2. Gupta et al., (2004) reported that the administration of Ervatamia coronaria extract increased the activity of CAT in CCL4 induced rats. So also in the present investigation, administration of A. radiata ethanol extract appears to have
brought about a remarkable improvement in the activity of this antioxidant enzyme in liver, kidney, brain and heart tissues of CCl₄ intoxicated rats.

**Glutathione peroxidase:**

GPx is an equally important antioxidant enzyme, which reacts with hydrogen peroxide thus preventing intracellular damage caused by the free radicals. GPx also catalyzes the reduction of organic hydroperoxides or lipid peroxides to their respective alcohols in the presence of GSH. GPx coupled with glutathione reductase, catalyses the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) and simultaneously NADPH is oxidized to NADP⁺. Singh *et al.*, (1999) reported that GPx activity was significantly reduced after CCl₄ treatment in rats, which indicates the damage to the living cells. The deficiency of this enzyme leads to accumulation of oxidised glutathione which affects the glutathione redox status. In the present study, GPx activity was significantly (p<0.001 and p<0.01) reduced in CCl₄-treated rats (Group II) for 10 days and 8 weeks in liver, kidney, brain and heart tissues. Treatment with EEAR in two different doses, 250 and 500 mg/kg b.wt (Group III and IV) resulted in significant (p<0.001 and p<0.01; p<0.001 and p<0.01) increase in the activity of GPx when compared to Group II (CCl₄ challenged) rats. The reversal of the GPx activity to normal after treatment with plant extract is due to antioxidant activity by scavenging/ detoxifying the endogenous metabolic peroxides generated during CCl₄ injury in the internal organs (Tables 3.13-3.20). The results are in correlation with the reports of Shui-Xiang He (2006), who reported the ameliorative effect of *Ginkgo biloba* on carbon tetrachloride-induced liver injury in rats by increasing the activity of GPx. Chao-Yun Wang *et al.*, (2007); Gao and Zhou, (2005) also reported similar pattern of results.

**Glutathione reductase:**

Glutathione reductase is one of the GSH-related enzymes which play detoxifying and antioxidant roles in metabolizing xenobiotics through the conjugation with glutathione or reduction of free radicals. GR is responsible for the re-generation of GSH, and GPx worked together with GSH in disintegrating hydrogen peroxide or other organic hydroperoxides (Hatono *et al.*, 1996). GR is concerned with the maintenance of cellular level of GSH (especially in the reduced state) by effecting fast reduction of oxidised glutathione to reduced form. Deficiency of this enzyme will affect the redox status of
GSH in biological system and could not protect tissues from oxidative damage. Lee et al., (2007) reported decreased GR activity during CCl₄-induced oxidative damage. In a similar manner significant (p<0.001 and p<0.01) decrease in GR activity was observed in present investigation in Group II (CCl₄ challenged) rats during both acute and chronic treatment. Treatment with EEAR in two different doses, 250 and 500 mg/kg b.wt (Group III and IV) resulted in significant (p<0.001 and p<0.01; p<0.001 and p<0.01) increase in the activity of GR when compared to Group II (CCl₄ challenged) rats and the values reached to near to that of controls (Tables 3.13-3.20). This suggests that EEAR reduces the oxidative damage generated due to CCl₄ metabolites by restoring the activity of GR in turn maintaining the GSH levels. The results of present study are in accord with the findings of Lee et al., (2007) and Venu Kumar and Latha (2002), who reported that supplementation of Camellia oleifera Abel. and Curculigo orchioides reduced oxidative damage caused by CCl₄ intoxication by replenishing the activity of glutathione reductase.

Glutathione-s-transferase
Glutathione-s-transferases (GST) are a multigene superfamily of dimeric, multifunctional, and soluble enzymes that play an essential role in protecting organisms from oxidative damage to DNA and lipids. For instance, GSH S-transferases conjugate glutathione (GSH) with electrophilic centers in a variety of compounds (e.g., epoxides) in the first step of the mercapturic acid pathway. Under oxidative stress, the peroxidase activity of GPx reduces lipid hydroperoxides by nucleophilic attack of GSH on the electrophilic oxygen. The oxidized GSH (GSSG) is subsequently recycled via a coupled reaction involving GR and G-6-PDH (Huang et al., 2007). Chemicals like chloroform and CCl₄ alter the hepatic GST activity (Aniya and Anders, 1985). In the present study, GST level was significantly (p<0.001 and p<0.01) reduced in CCl₄-treated rats (Group II) for 10 days and 8 weeks in liver, kidney, brain and heart tissues. Similar decrease in GST levels was reported by Aysel Guven and Necati Kaya, (2005) in Goose liver cells with damage induced by carbon tetrachloride and ethanol. An upward reversal was observed (Tables 3.13-3.20) after treatment with the A. radiata extract of two different doses, 250 and 500 mg/kg b.wt (Group III and IV). This may be attributed to a direct action of plant extract on the GST activation, the mechanism of which is not known. The increase in GST activity by the plant extract may be due to the increase in GSH levels so that GST activity is increased to detoxify the toxic metabolites. Aneja et al., (2005); Hwang et al.,
(2007) and Guven et al., 2003 reported similar increase in GST levels in tissues of rats and mice during CCl₄ induced oxidative damage when treated with natural antioxidants like Phytoestrogens, Puerarin and Kefir respectively which coincide with our findings.

**Reduced glutathione (GSH):**
Reduced glutathione (GSH) a non-enzymic antioxidant constitutes the first line of defense against free radicals. GSH is widely distributed in cells. GSH is an intracellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. Glutathione peroxidase catalyses the reduction of peroxides and converts GSH to its oxidized, disulphide form (GSSG), which is then reduced back to GSH by glutathione reductase. Indeed, GSH depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects, for example, a decrease in the rate of gluconeogenesis or an increase in glycogenolysis. The concept of a glutathione- SH threshold for drug detoxification was discussed by Jollow (1980). GSH is a naturally occurring substance that is abundant in many living creatures. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury. For example, liver injury included by consuming alcohol or by taking drugs like acetaminophen, lung injury by smoking and muscle injury by intense physical activity (Leeuwenburgh and Ji, 1995), all are known to be correlated with low tissue levels of GSH. From this point of view, exogenous EEAR supplementation might provide a mean of recover reduced GSH levels and to prevent tissue disorders and injuries. In the present study, we have demonstrated the effectiveness of EEAR by using CCl₄ induced rats, which are known models for both tissue GSH depletion and injury. In view of its importance, in the present study, the effect of EEAR during acute and chronic treatments on the levels of GSH in liver, kidney, brain and heart tissues of control and experimental rats was estimated (Tables 3.13-3.20). Acute and chronic CCl₄ injected rats (Group II) showed a significant (p<0.01 and p<0.001) reduction in the levels of GSH when compared with control animals (Group I). These changes were reversed to near normal values in EEAR treated animals (group III and group IV). There was a remarkable (p<0.001; p<0.01; p<0.001 and p<0.01) increase in the levels of GSH in group III and group IV rats (CCl₄ and two doses of EEAR treated) when compared with the CCl₄ induced rats (Group II). Reduction in GSH levels of
various organs (liver, kidney, brain and heart) in acute and chronic CCl₄-treated rats as observed in this study indicates the damage to the organs. Administration of A. radiata ethanol extract promoted the conversion of GSSG (oxidized glutathione) into GSH by the reactivation of glutathione reductase enzyme in the organs of acute and chronic CCl₄-treated rats (Fig. 3.20 and 3.21). Various reports suggested a decreased level of GSH at the time of oxidative stress and observed the reversal of this condition to normal with treatment of natural antioxidant supplements (Malaya Gupta et al., 2004; Dahiru et al., 2005; Bhandarkar and Khan 2004). The results of present study are in correlation with these reports.

Vitamin C and E:
Antioxidants such as vitamins C and E are non-enzymic scavengers of free radicals. Vitamin C is a powerful water-soluble, chain-breaking antioxidant that has several antioxidant effects including the ability to donate electrons to most water-soluble radicals and oxidants (Niki, 1987; Jialal et al., 1990). Vitamin E reacts with lipid peroxy radicals, acting as a chain terminator of lipid peroxidation, and protects the cellular structures from attack by free radicals (Arivazhagan et al., 2000). Vitamin C facilitates the maintenance of vitamin E levels at optimum concentrations. A decrease in ascorbic acid level in brain, heart and kidney of rats during oxidative stress has been reported by Kazumi et al., (2003). Decrease in vitamin E level in the liver of acute CCl₄ treated rats has also been reported by Kamalakkannan et al., (2005). In the present study, significantly (p<0.01 and p<0.001) lower (relative to control rats) levels of vitamins C and E were recorded (Tables 3.13-3.20) in both chronic and acute CCl₄ (Group II) treated rats; however, in rats receiving CCl₄ and two doses of A. radiata ethanol extract (Group III and Group IV) for a period of 10 days and 8 weeks, the levels of these vitamins were significantly (p<0.001; and p<0.01; p<0.01 and p<0.001) reverted to the levels in control rats (Group I). A similar increase in vitamins C and E levels in liver of CCl₄ intoxicated rats with the administration of Nigella sativa L and Urtica dioica, N-Acetyl Cysteine was reported by Kanter et al., (2005) and Kamalakkannan et al., (2005).
Table 3.13: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in liver of control and experimental rats against CCL₄ induced acute toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCL₄ treated)</th>
<th>Group III (CCL₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCL₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.481 ± 0.014ᵃ</td>
<td>0.213 ± 0.001ᵇ</td>
<td>0.341 ± 0.01⁰ᶜ</td>
<td>0.415 ± 0.001ᵈ</td>
<td>180.327</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>68.23 ± 0.547ᵃ</td>
<td>49.586 ± 0.468ᵇ</td>
<td>52.348 ± 0.300ᶜ</td>
<td>64.533 ± 0.690ᵈ</td>
<td>305.379</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>55.398 ± 0.623ᵃ</td>
<td>33.385 ± 0.595ᵇ</td>
<td>40.881 ± 0.891ᶜ</td>
<td>45.675 ± 0.289ᵈ</td>
<td>209.932</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>11.336 ± 0.128ᵃ</td>
<td>8.875 ± 0.068ᵇ</td>
<td>9.65 ± 0.012ᶜ</td>
<td>10.2 ± 0.024ᵈ</td>
<td>197.144</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>1.351 ± 0.017ᵃ</td>
<td>0.718 ± 0.007ᵇ</td>
<td>0.87 ± 0.009ᶜ</td>
<td>1.178 ± 0.014ᵈ</td>
<td>538.12</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>7.03 ± 0.014ᵃ</td>
<td>2.568 ± 0.120ᵇ</td>
<td>4.316 ± 0.032ᶜ</td>
<td>6.555 ± 0.074ᵈ</td>
<td>814.713</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>2.621 ± 0.206ᵃ</td>
<td>1.463 ± 0.014ᵇ</td>
<td>1.725 ± 0.014ᵇ</td>
<td>2.64 ± 0.014ᵃ</td>
<td>34.279</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.773 ± 0.012ᵃ</td>
<td>0.645 ± 0.017ᵇ</td>
<td>1.148 ± 0.016ᶜ</td>
<td>1.618 ± 0.023ᵈ</td>
<td>848.406</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan’s Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR- μmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Table 3.14: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in liver of control and experimental rats against CCl₄ induced chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.501 ± 0.002ᵃ</td>
<td>0.205 ± 0.003ᵇ</td>
<td>0.278 ± 0.008ᶜ</td>
<td>0.478 ± 0.002ᵈ</td>
<td>1122.781</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>74.59 ± 0.571ᵃ</td>
<td>34.441 ± 0.296ᵇ</td>
<td>54.151 ± 0.273ᶜ</td>
<td>65.873 ± 0.772ᵈ</td>
<td>1112.42</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>64.761 ± 0.459ᵃ</td>
<td>30.113 ± 0.422ᵇ</td>
<td>45.97 ± 0.321ᶜ</td>
<td>58.721 ± 0.208ᵈ</td>
<td>1757.368</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>11.005 ± 0.036ᵃ</td>
<td>6.718 ± 0.017ᵇ</td>
<td>8.026 ± 0.019ᶜ</td>
<td>9.811 ± 0.018ᵈ</td>
<td>6320.395</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>1.38 ± 0.015ᵃ</td>
<td>0.523 ± 0.020ᵇ</td>
<td>0.773 ± 0.012ᶜ</td>
<td>1.203 ± 0.017ᵈ</td>
<td>575.917</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>5.273 ± 0.015ᵃ</td>
<td>1.27 ± 0.015ᵇ</td>
<td>3.093 ± 0.052ᶜ</td>
<td>4.488 ± 0.102ᵈ</td>
<td>906.662</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>3.255 ± 0.068ᵃ</td>
<td>2.131 ± 0.012ᵇ</td>
<td>2.698 ± 0.020ᶜ</td>
<td>3.080 ± 0.019ᵈ</td>
<td>180.313</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.778 ± 0.133ᵃ</td>
<td>0.708 ± 0.024ᵇ</td>
<td>1.273 ± 0.017ᶜ</td>
<td>1.69 ± 0.021ᵃ</td>
<td>50.347</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan’s Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR- μmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Table 3.15: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in kidney tissue of control and experimental rats against CCl₄ induced acute toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.543 ± 0.002a</td>
<td>0.281 ± 0.005b</td>
<td>0.382 ± 0.002c</td>
<td>0.468 ± 0.012d</td>
<td>315.526</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>75.266 ± 0.596a</td>
<td>55.921 ± 0.302b</td>
<td>62.805 ± 0.370c</td>
<td>69.333 ± 0.499d</td>
<td>334.545</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>34.545 ± 0.315a</td>
<td>22.433 ± 0.254b</td>
<td>29.55 ± 0.337c</td>
<td>32.643 ± 0.448d</td>
<td>237.194</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>14.33 ± 0.094a</td>
<td>9.698 ± 0.021b</td>
<td>10.895 ± 0.027c</td>
<td>12.823 ± 0.019d</td>
<td>1620.923</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>0.22 ± 0.015a</td>
<td>0.067 ± 0.013b</td>
<td>0.133 ± 0.015c</td>
<td>0.175 ± 0.018a</td>
<td>17.809</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>10.568 ± 0.013a</td>
<td>3.32 ± 0.021b</td>
<td>6.903 ± 0.017c</td>
<td>9.126 ± 0.017d</td>
<td>33070.49</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.445 ± 0.026a</td>
<td>0.795 ± 0.021b</td>
<td>1.173 ± 0.020c</td>
<td>1.39 ± 0.020a</td>
<td>181.677</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.308 ± 0.021a</td>
<td>0.713 ± 0.020b</td>
<td>1.063 ± 0.015c</td>
<td>1.171 ± 0.025d</td>
<td>158.47</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR - μmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Table 3.16: Effect of ethanol extract of *A. radix* on enzymic and non-enzymic antioxidants in kidney tissue of control and experimental rats against CCl₄ induced chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.590 ± 0.003</td>
<td>0.108 ± 0.002</td>
<td>0.292 ± 0.002</td>
<td>0.440 ± 0.004</td>
<td>4708.507</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>76.185 ± 0.337</td>
<td>41.513 ± 0.387</td>
<td>53.34 ± 0.267</td>
<td>67.361 ± 0.250</td>
<td>2355.348</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>37.465 ± 0.344</td>
<td>19.256 ± 0.406</td>
<td>28.238 ± 0.327</td>
<td>33.046 ± 0.169</td>
<td>581.187</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>15.438 ± 0.321</td>
<td>7.58 ± 0.299</td>
<td>10.406 ± 0.324</td>
<td>13.416 ± 0.321</td>
<td>118.348</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>0.225 ± 0.017</td>
<td>0.042 ± 0.002</td>
<td>0.107 ± 0.021</td>
<td>0.207 ± 0.019</td>
<td>27.508</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>11.445 ± 0.282</td>
<td>1.496 ± 0.021</td>
<td>7.643 ± 0.315</td>
<td>10.285 ± 0.358</td>
<td>257.133</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.678 ± 0.016</td>
<td>0.921 ± 0.019</td>
<td>1.263 ± 0.013</td>
<td>1.5 ± 0.015</td>
<td>441.165</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.428 ± 0.020</td>
<td>0.746 ± 0.019</td>
<td>1.128 ± 0.023</td>
<td>1.366 ± 0.020</td>
<td>226.204</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan’s Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR - μmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Table 3.17: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in brain tissue of control and experimental rats against CCl₄ induced acute toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.524 ± 0.003ᵃ</td>
<td>0.365 ± 0.004ᵇ</td>
<td>0.414 ± 0.004ᶜ</td>
<td>0.494 ± 0.003ᵈ</td>
<td>383.298</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>30.181 ± 0.376ᵃ</td>
<td>18.555 ± 0.372ᵇ</td>
<td>20.671 ± 0.394ᶜ</td>
<td>26.355 ± 0.322ᵈ</td>
<td>208.22</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>34.336 ± 0.308ᵃ</td>
<td>26.098 ± 0.251ᵇ</td>
<td>29.305 ± 0.324ᶜ</td>
<td>32.448 ± 0.411ᵈ</td>
<td>121.484</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>2.353 ± 0.039ᵃ</td>
<td>1.445 ± 0.037ᵇ</td>
<td>1.838 ± 0.040ᶜ</td>
<td>2.146 ± 0.036ᵈ</td>
<td>106.845</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>0.238 ± 0.039ᵃ</td>
<td>0.073 ± 0.021ᵇ</td>
<td>0.155 ± 0.030ᵃ</td>
<td>0.226 ± 0.044ᵃ</td>
<td>5.436</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>11.305 ± 0.341ᵃ</td>
<td>6.445 ± 0.305ᵇ</td>
<td>9.348 ± 0.305ᶜ</td>
<td>10.403 ± 0.265ᵈ</td>
<td>47.937</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.54 ± 0.035ᵃ</td>
<td>0.851 ± 0.036ᵇ</td>
<td>1.206 ± 0.060ᶜ</td>
<td>1.446 ± 0.037ᵃ</td>
<td>50.425</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.178 ± 0.031ᵃ</td>
<td>0.931 ± 0.038ᵇ</td>
<td>1.025 ± 0.026ᵇ</td>
<td>1.138 ± 0.031ᵃ</td>
<td>12.273</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan’s Multiple Range Test (DMRT).

CAT - µmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - µmoles of GSH oxidized/min/mg protein; GR - µmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - µg of reduced glutathione/mg protein; Vitamin C - µg/mg protein and Vitamin E - µg/mg protein.
Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

Figure 3.20: Effect of ethanol extract of A. radiata on the level of GSH in liver, kidney, brain and heart of control and experimental rats against CCl₄ acute toxicity.

Group-I: Control; Group-II: CCl₄; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

Figure 3.21: Effect of ethanol extract of A. radiata on the level of GSH in liver, kidney, brain and heart of control and experimental rats against CCl₄ chronic toxicity.
Table 3.18: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in brain tissue of control and experimental rats against CCl₄ induced chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.548 ± 0.005a</td>
<td>0.224 ± 0.010b</td>
<td>0.392 ± 0.011c</td>
<td>0.513 ± 0.007d</td>
<td>289.895</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>33.221 ± 0.313a</td>
<td>15.286 ± 0.188b</td>
<td>22.486 ± 0.350d</td>
<td>28.375 ± 0.363d</td>
<td>616.422</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>37.638 ± 0.388a</td>
<td>18.473 ± 0.212b</td>
<td>28.495 ± 0.401c</td>
<td>34.411 ± 0.350d</td>
<td>593.058</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>3.020 ± 0.086a</td>
<td>0.946 ± 0.056b</td>
<td>1.645 ± 0.040c</td>
<td>2.466 ± 0.045d</td>
<td>235.102</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>0.365 ± 0.042a</td>
<td>0.068 ± 0.006b</td>
<td>0.166 ± 0.019c</td>
<td>0.248 ± 0.029d</td>
<td>21.255</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>12.741 ± 0.164a</td>
<td>5.131 ± 0.098b</td>
<td>8.836 ± 0.026c</td>
<td>11.613 ± 0.277d</td>
<td>404.227</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>2.188 ± 0.120a</td>
<td>1.676 ± 0.038b</td>
<td>1.883 ± 0.018c</td>
<td>1.928 ± 0.045c</td>
<td>9.755</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.316 ± 0.059a</td>
<td>1.133 ± 0.085b</td>
<td>1.175 ± 0.017a</td>
<td>1.255 ± 0.031a</td>
<td>2.257</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR - μmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Table 3.19: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in heart tissue of control and experimental rats against CCl₄ induced acute toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.245 ± 0.034ᵃ</td>
<td>0.143 ± 0.018ᵇ</td>
<td>0.164 ± 0.004ᵇ</td>
<td>0.245 ± 0.025ᵃ</td>
<td>5.398</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>41.353 ± 0.437ᵃ</td>
<td>30.591 ± 0.281ᵇ</td>
<td>33.66 ± 0.413ᶜ</td>
<td>40.395 ± 0.374ᵃ</td>
<td>187.939</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>46.345 ± 0.294ᵃ</td>
<td>25.67 ± 0.367ᵇ</td>
<td>33.511 ± 0.359ᶜ</td>
<td>44.616 ± 0.380ᵈ</td>
<td>768.145</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>4.308 ± 0.261ᵃ</td>
<td>2.44 ± 0.202ᵇ</td>
<td>3.536 ± 0.200ᵃ</td>
<td>4.078 ± 0.104ᵃ</td>
<td>17.382</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>0.163 ± 0.036ᵃ</td>
<td>0.075 ± 0.004ᵇ</td>
<td>0.084 ± 0.004ᶜ</td>
<td>0.145 ± 0.033ᶜ</td>
<td>3.154</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>9.221 ± 0.33ᵃ</td>
<td>3.471 ± 0.35ᵇ</td>
<td>7.453 ± 0.32ᵃ</td>
<td>8.43 ± 0.36ᵃ</td>
<td>53.865</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.548 ± 0.037ᵃ</td>
<td>0.848 ± 0.040ᵇ</td>
<td>1.281 ± 0.028ᶜ</td>
<td>1.34 ± 0.034ᶜ</td>
<td>70.742</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.166 ± 0.036ᵃ</td>
<td>0.948 ± 0.036ᵇ</td>
<td>1.07 ± 0.044ᵃ</td>
<td>1.076 ± 0.059ᵃ</td>
<td>4.022</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR- μmoles of GSH utilized/min/mg protein; GST – units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Table 3.20: Effect of ethanol extract of *A. radiata* on enzymic and non-enzymic antioxidants in heart tissue of control and experimental rats against CCl₄ induced chronic toxicity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I (control)</th>
<th>Group II (CCl₄ treated)</th>
<th>Group III (CCl₄ + 250 mg/kg b. wt. of EEAR)</th>
<th>Group IV (CCl₄ + 500 mg/kg b. wt. of EEAR)</th>
<th>ANOVA F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>0.346 ± 0.003ᵃ</td>
<td>0.110 ± 0.002ᵇ</td>
<td>0.253 ± 0.004ᶜ</td>
<td>0.315 ± 0.004ᵈ</td>
<td>1012.34</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>44.368 ± 0.348ᵃ</td>
<td>25.223 ± 0.403ᵇ</td>
<td>33.445 ± 0.400ᶜ</td>
<td>41.598 ± 0.374ᵈ</td>
<td>511.825</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx)</td>
<td>51.525 ± 0.351ᵃ</td>
<td>20.437 ± 0.321ᵇ</td>
<td>37.485 ± 0.409ᶜ</td>
<td>46.428 ± 0.347ᵈ</td>
<td>1452.01</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td>4.548 ± 0.039ᵃ</td>
<td>1.732 ± 0.034ᵇ</td>
<td>2.950 ± 0.042ᶜ</td>
<td>4.053 ± 0.046ᵈ</td>
<td>955.105</td>
</tr>
<tr>
<td>Glutathione-s-transferase (GST)</td>
<td>0.185 ± 0.004ᵃ</td>
<td>0.048 ± 0.010ᵇ</td>
<td>0.075 ± 0.004ᶜ</td>
<td>0.145 ± 0.004ᵈ</td>
<td>105.654</td>
</tr>
<tr>
<td>Reduced Glutathione (GSH)</td>
<td>11.448 ± 0.439ᵃ</td>
<td>2.492 ± 0.290ᵇ</td>
<td>8.363 ± 0.386ᶜ</td>
<td>10.560 ± 0.318ᵃ</td>
<td>123.29</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.363 ± 0.038ᵃ</td>
<td>0.750 ± 0.041ᵇ</td>
<td>1.005 ± 0.023ᶜ</td>
<td>1.250 ± 0.034ᵈ</td>
<td>61.43</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.247 ± 0.038ᵃ</td>
<td>0.835 ± 0.040ᵇ</td>
<td>1.065 ± 0.033ᶜ</td>
<td>1.152 ± 0.031ᵃ</td>
<td>24.371</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of six rats in each group. Means having same superscript in each row do not differ significantly at 0.01 level by Duncan's Multiple Range Test (DMRT).

CAT - μmoles of H₂O₂ utilized/min/mg protein; SOD - units/min/mg protein; GPx - μmoles of GSH oxidized/min/mg protein; GR - μmoles of GSH utilized/min/mg protein; GST - units/min/mg protein; GSH - μg of reduced glutathione/mg protein; Vitamin C - μg/mg protein and Vitamin E - μg/mg protein.
Although toxic effects of CCl₄ were shown in the brain, heart and kidney tissues, the major injury after CCl₄ intoxication was investigated in the liver tissue. In the present study, CCl₄ intoxication in both acute and chronic treatment was observed in tissues in a decreasing order - liver > kidney > brain > heart. The alterations in the biochemical parameters and depletion in the levels of enzymic and non-enzymic antioxidants was more pronounced in liver and kidney tissues followed by brain and heart tissues during CCl₄ toxicity in both acute and chronic studies and these alterations was dose dependently protected by EEAR.

Isozyme pattern of antioxidant enzymes:
The 1964 Committee recommended (IUPC-IUB, 1977) that "multiple enzyme forms" in a single species should be known as isoenzymes (or isozymes). It is known that enzymes catalyzing essentially the same reaction may differ in various ways. Isozymes were first described by Hunter and Markert (1957) who defined them as "different variants of the same enzyme having identical functions and present in the same individual". This definition encompasses: 1) enzyme variants that are the product from different genes and thus represent different loci (described as isozymes) and 2) enzymes that are the product of different alleles of the same gene (described as allozymes). Isozymes share a common catalytic activity. Each isozyme has a specific role in the metabolic pathway and functions in harmony with other enzymes within the organizational framework of cells. Isozymes often exhibit tissue or cell specificity (Miroslav Zeidler, 2000; Yli-Mattila et al., 1997; Zeidler, 1999).

Isozymes are usually the result of gene duplication, but can also arise from polyplopidisation or hybridization. Over evolutionary time, if the function of the new variant remains identical to the original, then it is likely that one or the other will be lost as mutations accumulate, resulting in a pseudo gene. However, if the mutations do not immediately prevent the enzyme from functioning, but instead modify either its function, or its pattern of gene expression, then the two variants may both be favored by natural selection and become specialised to different functions (Weeden and Wendel, 1990).

Isozymes (and allozymes) are variants of the same enzyme. Unless they are identical in terms of their biochemical properties, for example their substrates and enzyme kinetics, they may be distinguished by a biochemical assay. However, such
differences are usually subtle (particularly between allozymes which are often neutral variants). This subtlety is to be expected, because two enzymes that different significantly in their function are unlikely to have been identified as isozymes (Wendel and Weeden, 1990). Whilst isozymes may be almost identical in function, they may differ in other ways. In particular, amino acid substitutions that change the electric charge of the enzyme (such as replacing aspartic acid with glutamic acid) are simple to identify by gel electrophoresis, and this forms the basis for the use of isozymes as Molecular markers. To identify isozymes, a crude protein extract is made by grinding animal or plant tissue with an extraction buffer, and the components of extract are separated according to their charge by gel electrophoresis. Electrophoresis is a versatile biochemical technique to detect genetic variation (Crawford, 1989).

Protein molecules migrate in an electric field because they are charged (Hamrick and Godt, 1990). When an electrical gradient is applied, the molecules migrate toward the electrode with the charge opposite to their own, with the result that the initial single boundary formed by the mixture of molecules is broken into several boundaries according to the relative mobilities of the mixture. This technique is useful for separating and analyzing complex proteins mixtures (May, 1994).

Isoenzyme patterns and activities have recently drawn a considerable attention in oxidative stress correlated studies of carcinogenesis, aging and smoking (Cook and McNamara., 1980; El-Zayat and Amer, 2002; Jolitha et al., 2006; Pompili et al., 2003; Sapone et al., 2003; Yao et al., 2004). Isozymic profiles of several important enzymes can be used as useful tools to detect variable responses to stressing factor (Wang et al., 2004). In view of this, in the present study isozyme pattern of antioxidant enzymes (SOD, CAT and GPx) were analyzed in the liver of rats as molecular markers to evaluate the protective effect of EEAR against oxidative stress.

In the present investigation an analysis of the electrophoretic pattern of SOD isozymes revealed two isozymes in the liver tissue (Figs. 3.22a-3.22b) of all four groups of rats during acute treatment. Isoform SOD1 appeared as a band of essentially similar intensity in the liver of all four groups (band area 142, 139.8, 140.2 and 141.3 in Groups I, II, III and IV respectively), whereas in the case of SOD2, the staining intensity was decreased in Group II (band area 115.7) when compared to Group I (band area 124.2) rats. A dose-dependent increase in the staining intensity of SOD2
was observed in Group III (band area 122.99) and Group IV (band area 123.4) when compared with Group II (CCL₄ treated) rats. In a similar way two isoforms of SOD were detected in the liver of all four groups of rats during chronic treatment. Isozyme SOD₁ appeared as a band of essentially similar intensity in the liver (band area 143.7, 140.6, 141.2 and 142.4) of rats of Groups I, II, III and IV, respectively, whereas in the case of SOD₂, the staining intensity was decreased in Group II band area 109.6 when compared to Group I (band area 127.4) rats. A dose-dependent increase in the staining intensity of SOD₂ was observed in Group III (band area 119.8) and Group IV (band area 124.3) when compared with Group II (CCL₄ treated) rats (Figs. 3.23a-3.23b).

An analysis of the electrophoretic pattern of catalase isozymes in the liver tissue during acute treatment (Fig. 3.15 and 3.15a) revealed single band with slight variations in the staining intensity of the band among the four groups of rats; the liver of control (Group I) rats exhibited high intensity (band area 240.12), whereas Group II (CCL₄ treated) rats revealed a single band of notably decreased intensity (band area 221.14). Group III (CCL₄ + 250mg/kg b.wt of EEAR extract) and Group IV (CCL₄ + 500mg/kg b.wt of EEAR extract) rats exhibited increased intensity (band area 230.66 and 237.42 respectively) in a dose dependent manner when compared with Group II rats. In a similar fashion the electrophoretic pattern of catalase isozymes in the liver tissue during chronic treatment (Fig. 3.16 and 3.16a) revealed single band with slight variations in the staining intensity of the band among the four groups of rats; the liver of control (Group I) rats exhibited high intensity (band area 247.32), whereas Group II (CCL₄ treated) rats revealed a single band of notably decreased intensity (band area 216.14). Group III (CCL₄ + 250mg/kg b.wt of EEAR extract) and Group IV (CCL₄ + 500mg/kg b.wt of EEAR extract) rats exhibited increased intensity (band area 233.52 and 241.61 respectively) in a dose dependent manner when compared with Group II rats.

Five isozymes of GPx enzyme were noted in the liver tissue of all four groups of rats during acute treatment. The staining intensity of the GPx₁, GPx₄ and GPx₅ isozymes was essentially similar in all four groups of rats (Figs. 3.26a and 3.26b). However, the staining intensity of the GPx₂ isozyme was less in Group II rats (band area 203.4) compared to that in Group I (control) rats (band area 225.4). Group III (treated with 250 mg/kg b. wt of EEAR) rats (band area 223.8) and Group IV (treated
Figure 3.22a: Electrophoretic pattern of SOD isoenzymes in the liver tissue of control and experimental rats during acute treatment.

Figure 3.22b: Densit

L1—Control; L2—CCL₄ alone;
L3—CCL₄ + 250 mg/kg b. wt of EEAR; L4—CCL₄ + 500 mg/kg b. wt of EEAR.
Figure 3.23a: Electrophoretic pattern of SOD isoenzymes in the liver tissue of control and experimental rats during chronic treatment.

Figure 3.23b:

L1—Control;  
L2—CCL₄ alone;  
L3—CCL₄ + 250 mg/kg b. wt of EEAR;  
L4—CCL₄ + 500 mg/kg b. wt of EEAR.
Figure 3.24a: Electrophoretic pattern of catalase isoenzymes in the liver tissue of control and experimental rats during acute treatment.

Figure 3.24b: Ds

L1- Control; 
L3- CCl₄ + 250 mg/kg b. wt of EEAR; 

L2- CCl₄ alone; 
L4 - CCl₄ + 500mg/kg b. wt of EEAR.
Figure 3.25a: Electrophoretic pattern of catalase isoenzymes in the liver tissue of control and experimental rats during chronic treatment.

Figure 3.25b: Density of catalase isoenzymes in liver tissue in

L1—Control;  
L2—CCl₄ alone;  
L3—CCl₄ + 250 mg/kg b. wt of EEAR;  
L4—CCl₄ + 500mg/kg b. wt of EEAR.
Figure 3.26a: Electrophoretic pattern of GPx isoenzymes in the liver tissue of control and experimental rats during acute treatment.

Figure 3.26b: Densitometry of GPx isoenzymes in liver tissue in control and treated rats.

L1–Control;  \( \text{L2} \)– \( \text{CCl}_4 \) alone;  \( \text{L3} \)– \( \text{CCl}_4 + 250 \text{ mg/kg b. wt of EEAR} \);  \( \text{L4} \)– \( \text{CCl}_4 + 500 \text{mg/kg b. wt of EEAR} \).
Figure 3.27a: Electrophoretic pattern of GPx isoenzymes in the liver tissue of control and experimental rats during chronic treatment.

L1—Control; L2—CCL₄ alone; L3—CCL₄ + 250 mg/kg b. wt of EEAR; L4—CCL₄ + 500mg/kg b. wt of EEAR
with 500 mg/kg b. wt of EEAR) rats (band area 224.5) showed increased staining intensity of GPX2, dose-dependently when compared to Group II (CCL4 treated) rats. The GPX3 isozyme exhibited a similar phenomenon (reduced staining intensity (band area 98.02) in Group II rat livers compared to Group I (band area 122.6), Group III (band area 109.36) and Group IV (band area 116.4) rats). In a similar pattern, during chronic treatment five isozymes of GPx enzyme were noted in the liver tissue of all four groups of rats. The staining intensity of the GPX1, GPX4 and GPX5 isozymes was essentially similar in all four groups of rats (Figs. 3.26a and 3.26b). However, the staining intensity of the GPX2 isozyme was less in Group II rats (band area 218.8) compared to that in Group I (control) rats (band area 230.4). Group III (treated with 250 mg/kg b. wt of EEAR) rats (band area 222.7) and Group IV (treated with 500 mg/kg b. wt of EEAR) rats (band area 226.9) showed increased staining intensity of GPX2, dose-dependently when compared to Group II (CCL4 treated) rats. The GPX3 isozyme exhibited a similar phenomenon (reduced staining intensity (band area 106.3) in Group II rat livers compared to Group I (band area 127.4), Group III (band area 118.6) and Group IV (band area 123.3) rats).

The induction of stress-related isoenzymes is probably related to the level of ROS, which cause oxidative damage of various cellular components, such as proteins, membrane lipids and nucleic acids (Halliwell and Gutteridge, 1989). The activation of the existing isoenzymes suggests that the same isoenzymes detoxifying ROS produced under normal conditions function also under stress when an overproduction of ROS occurs. In agreement with many authors (Scandalios, 1994; Tsang et al., 1991; Wang et al., 2004) our results indicate that different isoforms of the antioxidant enzymes respond to different stress factors and only those isoforms which are needed to protect a particular cell compartment are expressed. It is well known that antioxidant isoenzymes are differentially compartmentalized depending on the tissue, likely to respond differently to biotic and abiotic stresses. In the present investigation isozyme pattern of antioxidant enzymes in liver was done, as it is the organ of paramount importance which plays an essential role in the metabolism and disposition of chemicals (xenobiotics) and their metabolites from the body. The dose dependent increase in the band area and staining intensity of these isoenzymes in EEAR treated (Group III and Group IV) rats when
compared to CCl₄ intoxicated rats may be due to the potent antioxidant efficacy of EEAR by scavenging the free radicals of CCl₄ through enhancement of the synthesis of these isoenzymes. Similar pattern of differential expression of antioxidant enzyme isozymes has been noted in barley shoot and root exposed to saline stress (Sang et al., 2005). Similar pattern of differential expression of antioxidant enzyme isozymes has also been noted in rats with aging stress (Jayakumar et al., 2007). Jayakumar et al., (2006) has also reported the similar pattern of differential expression of antioxidant enzyme isozymes by supplementation of the oyster mushroom, Pleurotus ostreatus, during CCl₄-induced liver injury in rats.

**Effect of EEAR on gene expression of SOD, CAT and GPx**

When oxidative stress occurs, cells function to counteract the resulting oxidant effects and to restore the redox balance. All organisms have adaptive responses to oxidative stress, with antioxidant defense enzymes being induced by changes in the levels of H₂O₂ or O₂⁻, leading to the activation or silencing of genes encoding defensive enzymes, transcription factors and structural proteins (Dalton et al., 1999). ROS have also been proposed to function as second messengers independent of oxidative stress and to signal such cellular fates as cell proliferation, necrosis, and apoptosis. Although various observations have led to the suggestion that cells have the means to sense ROS and to induce specific responses (Scandalios, 1997), the underlying mechanisms are still not fully understood.

Cells exposed to a non-lethal increase in free radical activity, induced by either environmental insult or a specific intracellular generation mechanism (e.g. tumor necrosis factor a-induced intracellular reactive oxygen species) have been shown to respond to these increases by the induction of a number of genes. These responses can be placed into two overlapping groups, the cellular adaptation to free radical insult, and the use of free radicals as intracellular messengers. The mechanism of induction of these genes and the absolute number of genes involved remains to be clarified. However, various studies have demonstrated some specific signalling pathways used and the genes which are activated. The genes induced by increased free radical activity are activated by similar pathways, but the exact pathway differs between genes. Generally, an increase in free radical
activity triggers a signal transduction pathway which terminates in the production of active transcription factors. These factors are then able to bind to the promoter region of their target genes and activate transcription. It would also appear that the final event of transcription factor binding to DNA can be modulated by the redox status of the cell.

Carbon tetrachloride is a well-investigated hepatotoxin, and several micro array studies have been published describing gene expression changes caused by acute CCl₄ toxicity (Bulera et al., 2001; Harries et al., 2001). These gene expression profiles have catalogued the molecular responses to acute CCl₄ toxicity and revealed the genetic basis of hepatic toxicity. However, these acute studies have provided information regarding only acute phase responses and instant adaptation of the liver to toxic insults. Changes in gene expression profiles in response to a long-term exposure to toxicants have a fundamental impact on disease development (Jiang et al., 2004). It is important to investigate whether the changes in gene expression would be reversible, if so to what extent, upon removal of the toxic insult by the administration of supplements. To address these questions, we investigated changes in hepatic gene expression of antioxidant enzymes in control and experimental animals.

The gene expression of the antioxidant enzymes SOD, Catalase and GPx in the liver tissue of control and experimental rats during chronic treatment was measured by RT-PCR using gene specific primers (Table-5). The yields of SOD, CAT, GPx, and β-actin PCR products were determined by quantifying the integrated optical density of the gel. The integrated optical densities of SOD, catalase, GPx, and β-actin PCR products were used to quantify the contents of SOD, catalase, and GPx mRNAs. To determine whether gene transcription of SOD, catalase and GPx was induced or suppressed by EEAR treatment, the ratio of SOD or catalase or GPx mRNA to β-actin mRNA was quantified in control and experimental rats.

The effect of EEAR on the level of gene expression of SOD in the liver is presented in Fig. 3.28a-c. The representative gel in Fig. 3.28a illustrates the partial results of RT-PCR analyses of SOD and β-actin gene expression, and Figs. 3.28b-c presents the overall results. Chronic treatment of CCl₄ induced oxidative stress in Group II rats resulted in a reduced mRNA level of SOD by 50.30 % when compared to control (Group I) rats (98.48 %). On treatment with EEAR, a significant increase in SOD mRNA
transcript level was observed in Group III (treated with 250 mg/kg b. wt. of EEAR) and Group IV (treated with 500 mg/kg b. wt. of EEAR) rats by 65.24 % and 90.13 % respectively when compared to Group II (received CCl₄ alone) rats.

The effect of EEAR on the gene-expression level of liver catalase is presented in Fig. 3.29a-c. A representative gel (Fig. 3.29a) illustrates the partial results of RT-PCR analyses of catalase and β-actin gene expression, and Figs. 3.29b-c presents the overall results. The mRNA transcript level of catalase in Group II (CCl₄ induced) rats were found to be decreased by 44.8 % when compared to control (Group I) rats (90.4 %). On treatment with EEAR, a significant increase in catalase mRNA transcript level was observed in Group III (treated with 250 mg/kg b. wt. of EEAR) and Group IV (treated with 500 mg/kg b. wt. of EEAR) rats by 63.5 % and 82.4 % respectively when compared to Group II (received CCl₄ alone) rats.

The effect of EEAR on the level of gene expression of GPx in the liver is presented in Fig. 3.30a-c. The representative gel in Fig. 3.30a illustrates the partial results of RT-PCR analyses of GPx and GAPDH gene expression, and Figs. 3.30b-c presents the overall results. Chronic treatment of CCl₄ induced oxidative stress in Group II rats resulted in a reduced mRNA level of GPx by 54.87 % when compared to control (Group I) rats (95.3 %). On treatment with EEAR, a significant increase in GPx mRNA transcript level was observed in Group III (treated with 250 mg/kg b. wt. of EEAR) and Group IV (treated with 500 mg/kg b. wt. of EEAR) rats by 66.8 % and 80.4 % respectively when compared to Group II (received CCl₄ alone) rats.

Oxidative stress specifically down-regulates the expression of various genes. The low activity of antioxidant enzymes in liver tissue during CCl₄ toxicity seems to be a result not only of tissue structure oxidative damage but also damage of enzymatic structure, function, and enzymatic gene expression (Szymonik-Lesiuk et al., 2003). Oxidative stress in liver tissue caused by CCl₄ intoxication would lead to damage of antioxidant enzymes such as SOD, CAT and GPx, or reactive intermediates formed in the course of bioactivation of CCl₄ may bind to those enzymes that are responsible for their inactivation. It was found that the mechanism responsible for the regulation of their gene expression was depressed after CCl₄ intoxication (Sanzgir et al., 1997).
Figure 3.28: Effect of EEAR on mRNA expression of SOD in liver tissue control and experimental rats during chronic CCl₄ toxicity.

(a) Agarose gel representing the RT-PCR analysis of SOD and β-actin mRNA expression in control and experimental rats. 
L₁ - Control; L₂ - CCl₄ alone; L₃ - CCl₄ + 250 mg/kg b.wt of EEAR; L₄ - CCl₄ + 500 mg/kg b. wt of EEAR.

(b) The ratio of SOD mRNA to β-actin mRNA expression in control and experimental rats. 
Group-I: Control; Group-II: CCl₄ alone; Group-III: CCl₄ + 250 mg/kg b. wt of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt of EEAR.

(c) Histogram of relative transcript level of SOD in control and experimental rats.
Figure 3.29: Effect of EEAR on mRNA expression of CAT in liver tissue of control and experimental rats during chronic CCl₄ toxicity.

(a) Agarose gel representing the RT-PCR analysis of CAT and β-actin mRNA expression in control and experimental rats. L1 – Control; L2 – CCl₄ alone; L3 – CCl₄ + 250 mg/kg b.wt of EEAR; L4 – CCl₄ + 500 mg/kg b. wt of EEAR.

(b) The ratio of CAT mRNA to β-actin mRNA expression in control and experimental rats. Group-I: Control; Group-II: CCl₄ alone; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

(c) Histogram of relative transcript level of CAT in control and experimental rats.
(a) Agarose gel representing the RT-PCR analysis of GPx and β-actin mRNA expression in control and experimental rats. L1 - Control; L2 - CCl4 alone; L3 - CCl4 + 250 mg/kg b.wt of EEAR; L4 - CCl4 + 500 mg/kg b. wt of EEAR.

(b) The ratio of GPx mRNA to β-actin mRNA expression in control and experimental rats. Group-I: Control; Group-II: CCl4 alone; Group-III: CCl4 + 250 mg/kg b. wt of EEAR; Group-IV: CCl4 + 500 mg/kg b. wt. of EEAR.

(c) Histogram of relative transcript level of GPx in control and experimental rats.

Figure 3.30: Effect of EEAR on mRNA expression of GPx in liver of control and experimental rats during chronic CCl4 toxicity.
The variation in the antioxidant enzyme isoform profile observed in the present investigation during CCl₄ induced oxidative damage may possibly be due to an alteration in gene expression. The modification in the pattern of enzyme isoforms during stress has been attributed to some shift in gene expression (El-baky et al., 2003). Since direct genomic action of exogenous antioxidants against oxidative stress has been well-documented (El-Gendy et al., 1994; Young et al., 2007; Steinhiller et al., 1995).

Supplementation with exogenous antioxidants during oxidative damage has been proposed as a means of increasing the activity of antioxidant defense system gene with a view to promoting the synthesis of antioxidant enzymes (Ng et al., 2005). In the present study, a similar phenomenon possibly occurred, resulting in the increased levels of antioxidant enzyme isoforms and thereby increasing the mRNA expression levels of these enzymes following supplementation with EEAR. The results of present study are in accord with the results reported by Ng et al., (2005).

Protective effect of EEAR against DNA damage:
Maintenance of DNA integrity is of paramount importance for all living organisms, and this is reflected in the very efficient and intricate mechanisms that exist for the protection of the genetic material and the consequent low mutational rate (Shugart, 1999; Steinert, 1999). Molecular changes in DNA and/or damage to its integrity may potentiate a cascade of detrimental effects from the cellular to the organism level and ultimately to the population level (Forbes, 1999; Shugart, 1999; Moustacchi, 2000). Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radicals, are generated both from endogenous sources and from the reactions of chemicals. The oxidation of DNA bases is produced ROS (Ames, 1989; Frenkel 1992). Physical agents, e.g. solar radiation, X-rays, and a variety of chemical compounds can damage the DNA of living cells. Examples of DNA lesions produced by chemical and physical agents include strand breaks, modified bases, DNA–DNA cross links and DNA–protein cross links. Strand breaks may be introduced directly by genotoxic compounds, through the induction of apoptosis or necrosis, secondarily through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of excision repair enzymes (Eastman and Barry, 1992; Park et al., 1991; Speit and Hartmann, 1995). Chemical compounds can cause base alterations in the DNA sequence. Many electrophilic
chemicals can bind covalently to DNA, forming bulky adducts (Lutz, 1979). Monofunctional alkylating agents can add alkyl groups to DNA. Alkylated bases can also lead to secondary alterations in DNA, e.g. an alkyl adduct labilize the bond that connects the base to deoxyribose, thereby stimulating base loss. Base loss from DNA leaves an apurinic or apyrimidinic site, commonly called an AP site. A subsequent insertion of an incorrect base into the AP site can cause a mutation (Laval et al., 1990). Polyfunctional alkylating agents, can also alkylate DNA and induce various types of damage, including DNA/DNA cross links (Erickson et al., 1980; Crook et al., 1986; Little and Mirkes, 1987; Anderson et al., 1994).

The comet assay, which is also called the single-cell gel electrophoresis technique, is a simple, sensitive, and rapid method that can be used to estimate DNA damage at the individual cell level through strand breaks, open repair sites, cross-links, and labile sites (Singh et al., 1988; Tice et al., 2000; Hartmann et al., 2003). The assay works on the principle that free radicals such as reactive oxygen species (ROS) cause breaks in the DNA and/or base oxidation. It is well known that CCl₄ may exert its toxicity by generating reactive oxygen species (ROS); resulting in various types of oxidative damage, including DNA strand breakage and lipid peroxidation (DiRenzo et al., 1982). To date, the comet assay has been used for a variety of applications including studies on toxicology (Cotelle and Ferard, 1999), pollution (Calderon-Garciduenas et al., 1996; 1997), aging (Singh et al., 1990; 1991), exercise (Hartmann et al., 1994; Selman et al., 2002), training (Niess et al., 1996), and measurement of cell-growth and DNA-repair mechanisms (Duthie and Collins, 1997; Collins and Horvathova, 2001). The comet assay has also been used to study the effects of diet and antioxidant supplementation on oxidative DNA damage (Moller and Loft, 2002).

In the present investigation comet assay was carried out to determine the protective role of the EEAR against CCl₄ induced DNA damage in whole blood of animals from each experimental group during acute and chronic treatments. The percentage of damaged cells and the average tail lengths of comets in each experimental group during acute and chronic treatments were measured.
Percentage of damaged cells in each Group:

Percent of damaged cells in each experimental group during acute treatment was represented in Table 3.21. Blood drawn from the animals of group I (control group) during acute treatment showed very few (3.79 %) damaged cells at the time of experiment, whereas in group II (CCl₄ induced) 49.92 % cells showed a distinct comet tail which came down significantly to 32.74 % in group III (CCl₄ and 250 mg/kg b. wt. of EEAR treated) and 16.54 % in group IV (CCl₄ and 500 mg/kg b.wt. of EEAR treated) animals.

Table 3.22 depicts the per cent of damaged cells in each experimental group during chronic treatment. At the time of experiment, the blood cells collected from group I rats showed very few (4.12 %) damaged cells, whereas in group II (CCl₄ induced) 61.08 % of the blood cells showed comet tail formation, which was reduced by the treatment with 250 mg/kg b. wt. of EEAR to 37.34 % (group III) and 500 mg/kg b. wt. of EEAR to 21.44 % (group IV) respectively.

Average tail length due to DNA migration in each Group:
In the control group (Group I) the magnitude of the comet tail was very low and increased after CCl₄ injection during both acute and chronic treatments. It might cause DNA damage of the cells that involved in greater elongation and diffused comet tail formation, resulting in an increase of tail momentum in single-cell-gel electrophoresis.

The microscopical image resulting from CCl₄ induced damaged blood cells (groups II) are comets with small or non-existent head and large, diffused tails during both acute and chronic treatments. The average tail length was increased about 90.61 % and 91.31% in group II (CCl₄ injected) rats during acute and chronic treatments respectively in comparison with group I, whereas the tail lengths decreased by 38.78 % in group III (250 mg/kg b.wt. of EEAR treated) and 65.46 % in group IV (500 mg/kg b.wt. of EEAR treated) respectively during acute treatment in comparison to the CCl₄ control (group II) (Table 3.21; Figs 3.31A-D). The average tail length decreased by 40.87 % in group III (250 mg/kg b.wt. of EEAR treated) and 61.64 % in group IV (500 mg/kg b.wt. of EEAR treated) respectively during chronic treatment in comparison to the respective CCl₄ control (group II) (Table 3.22; Figs. 3.32A-D).
Table 3.21: Assessment of the protective activity of EEAR against CCl₄ induced DNA damage in blood lymphocytes of rats during acute treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Damaged cells showing comet (%)</th>
<th>Average tail length (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3.79 ± 0.62⁹</td>
<td>5.923 ± 0.81⁹</td>
</tr>
<tr>
<td>II</td>
<td>49.92 ± 2.73⁸</td>
<td>56.99 ± 2.18⁸</td>
</tr>
<tr>
<td>III</td>
<td>32.74 ± 4.12⁷</td>
<td>34.89 ± 2.04⁷</td>
</tr>
<tr>
<td>IV</td>
<td>16.54 ± 1.44⁸</td>
<td>19.69 ± 3.08⁸</td>
</tr>
</tbody>
</table>

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 (DMRT).

Group-I: Control; Group-II: CCl₄ alone; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.

Table 3.22: Assessment of the protective activity of EEAR against CCl₄ induced DNA damage in blood lymphocytes of rats during chronic treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Damaged cells showing comet (%)</th>
<th>Average tail length (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.12 ± 0.16⁸</td>
<td>6.23 ± 0.03⁸</td>
</tr>
<tr>
<td>II</td>
<td>61.08 ± 4.61⁸</td>
<td>71.64 ± 3.92⁸</td>
</tr>
<tr>
<td>III</td>
<td>37.34 ± 3.03⁷</td>
<td>42.36 ± 3.64⁷</td>
</tr>
<tr>
<td>IV</td>
<td>21.44 ± 2.11⁷</td>
<td>27.48 ± 3.33⁷</td>
</tr>
</tbody>
</table>

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 (DMRT).

Group-I: Control; Group-II: CCl₄ alone; Group-III: CCl₄ + 250 mg/kg b. wt. of EEAR; Group-IV: CCl₄ + 500 mg/kg b. wt. of EEAR.
Figure 3.1: Effect of ESR on DNA damage in blood lymphocytes during acute CCL toxicity by cancer cause.

(d) Photomicrographs showing DNA damage after 24 h in lymphocytes from blood of control group (100 X).
(e) Photomicrographs showing DNA damage after 24 h in lymphocytes from blood of control group (100 X).
(f) Photomicrographs showing DNA damage after 24 h in lymphocytes from blood of control group (100 X).
(g) Photomicrographs showing DNA damage after 24 h in lymphocytes from blood of control group (100 X).
(h) Photomicrographs showing DNA damage after 24 h in lymphocytes from blood of control group (100 X).

Diabetic control cell formation 100 X. (c) Photomicrographs showing DNA damage after 24 h in lymphocytes from blood of control group (100 X).

10 days showed no significant DNA migration (damage) (Group I). 100 X.
Figure 3.32: Effect of EEAR on DNA damage in blood lymphocytes during chronic CCl₄ toxicity by comet assay.

(A) Microphotograph of lymphocytes from blood of control treated rats with vehicle for 8 weeks showed no significant DNA migration (damage) (Group I), 100 X.

(B) Microphotograph showing DNA damage after 24 h in lymphocytes from blood of rats treated with CCl₄ alone for 8 weeks (Group II). Arrowhead showing highly migrated DNA and a distinct scattered comet tail and small diffused heads, 100 X.

(C) Microphotograph showing DNA damage after 24 h in lymphocytes from blood of rats treated with CCl₄ and 250mg/kg b.wt of EEAR extract for 8 weeks (Group III). Arrowheads showing less migration of DNA with short less diffused comet tail, 100 X.

(D) Microphotograph showing DNA damage after 24 h in lymphocytes from blood of rats treated with CCl₄ and 500mg/kg b.wt of EEAR extract for 8 weeks (Group IV). Arrowhead showing no or less comet tail formation due to less DNA migration, 100 X.
It was observed that DNA damage caused by CCl₄ injection was considerably counteracted by treatment with the EEAR in dose and time dependent manner with the maximum level of protection being noted in those groups that received the treatment with the 500 mg/Kg b. wt. of EEAR (Table 3.32; Fig. 3.32 A-D) as evident from the appearance of a shorter tail length of the cells in the comet assay. These results demonstrate that EEAR provides a beneficial effect against CCl₄ induced oxidative DNA damage, although the mechanism is not entirely clear. The protective effect of EEAR on oxidative DNA damage may be due to the polyphenols present in it which contribute major antioxidant capacity to scavenge free radicals. Wu et al., (2006) reported that Lycium barbarum possesses antioxidative properties and can ameliorate DNA damage in non insulin dependent diabetes mellitus rats. Various authors reported that supplementation with natural antioxidants exerts their protective effects against the DNA damage induced during oxidative stress (Moller and Loft, 2002, DiRenzo et al., 1982), Rajat Kumar Das et al., (2007) reported the protective effect of diphenyl methyl selenocyanate against DNA damage caused by CCl₄. Similar pattern of results are observed in the present study, suggesting and providing the evidence for the protective effect of EEAR against CCl₄ induced DNA damage.

**Histopathological examination**

The purpose of histological staining methods by Hematoxylin and Eosin is to visualize and differentiate between tissue components in normal, pathological and treatment conditions.

**Liver:**

When compared to the histoarchitecture of liver tissue of Group I (control) animals (Fig. 3.33A and 3.34A), liver cells of group II rats (exposed to CCl₄) revealed extensive damage, characterized by the disruption of the lattice nature of the hepatocyte, damaged cell membranes, degenerated nuclei, disintegrated central vein and damaged hepatic sinusoids during both acute and chronic treatment (Figs. 3.33B and 3.34B). In Group III (exposed to CCl₄ and 250 mg/kg b. wt. of EEAR) and Group IV (exposed to CCl₄ and 500 mg/kg b. wt. of EEAR) rats, only minimal disruption of the hepatic cellular structure was observed during both acute and chronic treatments (Fig. 3.33C-D and 3.34 C-D).

**Kidney:**

When compared to the histoarchitecture of kidney tissue of Group I (control) animals (Fig. 3.35A and 3.36A), liver cells of Group II rats (exposed to CCl₄) revealed extensive
damage, characterized by the disruption of the glomerular cells with severe congestion of blood vessels, damaged cell membranes and degenerated tubular epithelial cells during both acute and chronic treatment (Fig. 3.35B and 3.36B). In Group III (exposed to CCl₄ and 250 mg/kg b. wt. of EEAR) and Group IV (exposed to CCl₄ and 500 mg/kg b. wt. of EEAR) rats, only minimal disruption of the kidney cell structure was observed during both acute and chronic treatments (Fig. 3.35C-D and 3.36C-D).

**Brain:**

Histoarchitecture of brain tissue of Group II (exposed to CCl₄) rats (Figs. 3.37B and 3.38B) revealed extensive damage to neuronal cells, characterized by degenerative nuclei and severe hemorrhage and damaged cell membranes of neurons when compared to control group (Group I) of rats (Figs. 3.37A and 3.38A) during both acute and chronic treatment. Minimal disruption of neuronal cells were observed in Group III (exposed to CCl₄ and 250 mg/kg b. wt. of EEAR) and Group IV (exposed to CCl₄ and 500 mg/kg b.wt. of EEAR) rats (Figs. 3.37C-D and 3.38A-D) both acute and chronic treatment.

**Heart:**

Histoarchitecture of heart tissue of Group II (exposed to CCl₄) rats (Fig. 3.39B and 3.40B) revealed extensive damage to cardiac cells, characterized by degenerative nuclei and severe hemorrhagic cardiac cells when compared to control group (Group I) of rats (Fig. 3.39A and 3.40A) during both acute and chronic treatment. Minimal disruption of cardiac cells was observed in Group III (exposed to CCl₄ and 250 mg/kg b. wt. of EEAR) and Group IV (exposed to CCl₄ and 500 mg/kg b. wt. of EEAR) rats (Fig. 3.39C-D and 3.40C-D) both acute and chronic treatment.

Identification and confirmation of histopathological changes in the present study was performed to provide direct evidence of the toxicity of CCl₄, and of the protective effect of EEAR on liver, kidney, brain and heart tissues. These results are correlated with Jayakumar et al., (2007) who reported protective effect of oyster mushroom against CCl₄ toxicity. Marked disruption of the cellular structure of liver, kidney, brain and heart was noted in Group II (challenged with CCl₄) rats during both acute and chronic treatments. Only minimal disruption of the cellular structure of these organs were noted in two doses of EEAR treated group of rats (Group III and Group IV) during both acute and chronic treatments, this minimal disruption of cellular structure of all tissues in the study complemented the results of direct and indirect oxidative stress markers.
(A) Control rat liver section showing normal architecture showing central vein without any changes, normal sinusoids and normal hepatocytes, H & E, 10X.

(B) Liver section of CCl₄ received rat for 10 days showing highly conjugated blood vessels and hepatocytes with numerous vacuoles, H & E, 10X.

(C) Liver section of CCl₄ + 250 mg/kg b. wt. of EEAR treated rat showing regenerative normal hepatocytes, central cirrhosis and regenerative sinusoids, H & E, 10X.

(D) Liver section of CCl₄ + 500 mg/kg b. wt. of EEAR treated rat showing regenerative central vein, sinusoids and hepatocytes, H & E, 10X.
Figure 3.34: Liver section of control and experimental rats during chronic treatment.

(A) Control rat liver section showing normal architecture normal hepatocytes, sinusoids and normal centrilobular space, H & E, 10X.

(B) Liver section of CCl₄ received rat for 8 weeks showing highly conjugated blood vessels and hepatocellular diffused ballooning with numerous vacuoles, H & E, 10X.

(C) Liver section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing mild and focal fatty changes and regenerative normal hepatocytes, H & E, 10X.

(D) Liver section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing regenerative central vein, sinusoids and hepatocytes, H & E, 40X.
Figure 3.35: Kidney section of control and experimental rats during acute treatment.

(A) Control rat kidney section showing normal architecture showing normal glomeruli and tubular epithelial cells, H & E, 40X.

(B) Kidney section of CCl₄ received rat for 10 days showing glomeruli with congestion, blood vessel and RBC damage and damaged renal tubules (without tubular epithelial cells), H & E, 40X.

(C) Kidney section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing regenerative renal tubular epithelial cells, H & E, 40X.

(D) Kidney section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing regenerative glomeruli and renal tubular epithelial cells, H & E, 10X.
Figure 3.36: Kidney section of control and experimental rats during chronic treatment.

(A) Control rat kidney section showing normal architecture showing normal glomeruli and tubular epithelial cells, H & E, 40X.

(B) Kidney section of CCl₄ received rat for 8 weeks showing inter tubular hemorrhage and damaged tubules (without tubular epithelial cells), H & E, 40X.

(C) Kidney section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing regenerative renal tubular epithelial cells, H & E, 10X.

(D) Kidney section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing regenerative glomeruli and renal tubules, H & E, 10X.
Figure 3.37: Brain section of control and experimental rats during acute treatment.

(A) Control rat brain section showing normal architecture showing normal neuronal cells, H & E, 10X.

(B) Brain section of CCl₄ received rat for 10 days showing damaged neuronal cells and disruption of the typical layered appearance of the cerebral cortex, H & E, 10X.

(C) Brain section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing neuronal cells with mild damage, H & E, 10X.

(D) Brain section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing neuronal cells with almost normal architecture, H & E, 10X.
Figure 3.38: Brain section of control and experimental rats during chronic treatment.

(A) Control rat brain section showing normal architecture showing normal neuronal cells, H & E, 10X.

(B) Brain section of CCl₄ received rat for 8 weeks showing showing gross disorganization of the layered appearance of cerebral cortex and damaged neuronal cells, H & E, 10X.

(C) Brain section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing neuronal cells with mild damage, H & E, 10X.

(D) Brain section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing neuronal cells with almost normal architecture, H & E, 10X.
Figure 3.39: Heart section of control and experimental rats during acute treatment.

(A) Control rat heart section showing normal architecture showing normal cardiac cells and myocardial fibers, H & E, 40X.

(B) Heart section of CCl₄ received rat for 10 days showing showing damaged cardiac cells characterized by degenerative nuclei, marked edema and focal destruction of myocardial fibres, H & E, 10X.

(C) Heart section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing minimal disruption of cardiac cells, moderate edema and occasional loss of myocardial fibers, H & E, 40X.

(D) Heart section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing minimal disruption of cardiac cells, mild edema and occasional loss of myocardial fibers, H & E, 40X.
Figure 3.40: Heart section of control and experimental rats during chronic treatment.

(A) Control rat heart section showing normal architecture showing normal cardiac cells and myocardial fibers, H & E, 10X.

(B) Heart section of CCl₄ received rat for 8 weeks showing damaged cardiac cells characterized by degenerative nuclei, marked edema and focal destruction of myocardial fibres, H & E, 10X.

(C) Heart section of CCl₄ + 250 mg/kg b. wt. of EEAR received rat showing minimal disruption of cardiac cells, moderate edema and occasional loss of myocardial fibers, H & E, 40X.

(D) Heart section of CCl₄ + 500 mg/kg b. wt. of EEAR received rat showing minimal disruption of cardiac cells, mild edema and occasional loss of myocardial fibers, H & E, 10X.