4. DISCUSSION

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4. DISCUSSION

4.1 PRELUDE

Environmental factors have been implicated as causative agents for about 20% of total global cancer incidence and other pathological states (Doll and Peto, 1981; Weinstein, 1981; Stalder, 1986). Biological oxidation when tilted toward peroxidant states by reactive oxygen species (ROS) generators induces epigenetic and genetic changes leading to structural and functional losses (Pryor, 1986; Marnett, 1987; Cerutti, 1985). The peroxidized lipids are deleterious to health as they induce crosslinking of proteins (Gardner, 1979), DNA damage (Vaca and Metsharmana, 1989) and activation of eicosanoid pathways generating inflammatory mediators (Marshall et al., 1987). The oxidized low density lipoproteins are efficiently phagocytosed by macrophages through scavenger receptors. This transforms macrophages to foam cells and initiates the formation of atherosclerotic plaques (Steinberg et al.; Ross, 1986). Thus, the ROS-induced lesions are increasingly being recognized as being solely responsible for the diseased states (Dryor, 1986; Cerutti, 1985). The peroxidation in vivo is influenced by the activity of enzymes like superoxide dismutase, catalase and glutathione peroxidase. Antioxidants like vitamin E, selenium, β-carotene, uric acid and bilirubin can also prevent peroxide formation. Hence, it is of paramount importance to search for exogenous preventive agents in the form of antioxidants to help, sustain and spare endogenous antioxidants such as vitamin E, β-carotene, SOD and catalase (Halliwell and Gutteridge, 1990; Ames, 1983).
Spices are widely used in Indian cooking; they add flavour and enhance the palatability of the food. Certain constituents in Asian diet for example, spice principles like curcumin, capsaicin and extracts of onion and garlic have been shown to have the potential to act as antioxidants (Chawla et al., 1987). Capsaicin and turmeric prevent oxidation of oils and fats (Salimath et al., 1986). Eugenol inhibits Cu²⁺/H₂O₂ and benzoylperoxide/Cu²⁺-induced lipid peroxidation in human erythrocyte membrane (Nagashima, 1989).

Turmeric is a spice that is extensively used both for its colour and flavour. Curcumin, the lipophilic compound, which is the active principle in turmeric has been observed to have strong antioxidant properties (Sharma, 1976). Further, curcumin has been reported to prevent tumour development in animals (Huang et al., 1988; Kuttan et al., 1985) and to have an antimutagenic effect on bacteria in vitro (Nagabhushan et al., 1989). All studies have been carried out either with turmeric or with alcoholic extract of turmeric. Aqueous extracts of turmeric are shown to be potent inhibitors of lipid peroxidation and afford protection to DNA against free radical attack (Shalini and Srinivas, 1987). These studies indicate that turmeric possesses several antioxidants. However, the potential to inhibit lipid peroxidation in vivo has not been evaluated with aqueous extract of turmeric. The present investigation was undertaken to study whether the aqueous extracted principle can protect the peroxidation reactions in in vivo system (a) induced by administration of a hepatotoxin, carbon tetrachloride, which is known to induce liver injury by lipid peroxidation reaction (Recknagel and Glende, 1978) and (b) a nephrotoxin, ethylene glycol, which is known to induce calcium oxalate
deposition, but its induction of lipid peroxidation is not known. However, our earlier studies have shown elevated lipid peroxidation reactions in renal cells of stone forming rats (Selvam and Bijikurien, 1991; Selvam and Ravichandran, 1991).

4.2 **IN VITRO STUDIES WITH AQUEOUS EXTRACTED TURMERIC PROTEIN (AET)**

One of the promising approaches to elucidate and understand the complicated mechanisms of the inhibition of free radical reactions in biological systems is to carry out with the simplified model reactions in vitro. Preliminary studies were carried out in vitro systems containing (a) rat brain homogenate/liver homogenate, ascorbic acid, tBH and CCl₄ (b) RBC and H₂O₂, for investigating the antioxidant properties of the aqueous extract of the turmeric. The aqueous extract of turmeric has been purified and the protein (AET) is found to be homogenous.

4.2.1 a) **Effect of AET on lipid peroxidation - induced Ca²⁺-ATPase inactivation**

When the brain homogenate is used as a substrate, the formation of lipid peroxides is effectively inhibited by AET, even in the presence of promoters of lipid peroxidation. A 50% inhibitory effect on lipid peroxidation is noticed at 50µg protein concentration. Ca²⁺-ATPase activity is inhibited as a function of lipid peroxidation due to loss of thiol groups. Parola et al (1990) have shown that the high affinity Ca²⁺-ATPase is affected in vitro by
4-hydroxynonenal, a major end product of lipid peroxidation, by interacting with the thiol groups. Enhanced NADPH-dependent inhibition of Ca\textsuperscript{2+} uptake due to a decrease in Ca\textsuperscript{2+}-ATPase has been reported (Srivatsava et al., 1990) when microsomes were incubated with CCl\textsubscript{4}. Inactivation of Ca\textsuperscript{2+}-ATPase activity of sarcoplasmic reticulum (Kukreja et al., 1988) and depression of membrane bound Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in kidney (Kako et al., 1988) by oxygen free radicals and brain Na\textsuperscript{+}, K\textsuperscript{+}-ATPase by lipid peroxidation (Sun, 1972) have been reported.

The processes of peroxidation of lipids and inhibition of Ca\textsuperscript{2+}-ATPase activity due to the oxidation of sulphhydryl groups is evidenced by the ability of reducing agents to prevent inhibition. Scherer and Deamer (1986) have reported a similar observation that oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulphhydryl groups in Ca\textsuperscript{2+}-ATPase and the activity is found to be partially restored in the presence of thiol reagents. Moreover, Rafatullah et al., (1990) have also reported the antiulcer activity of turmeric extract by restoring the non-protein sulphhydryl content of the glandular stomachs of rats.

The protection of Ca\textsuperscript{2+}-ATPase from inactivation in the presence of promoters of lipid peroxidation by AET shows that the antioxidant activity may be mediated through the protection of the thiol groups of the enzymes. GSH has also been shown to protect the CCl\textsubscript{4} mediated lipid peroxidation (Srivatsava et al., 1990).
4.2.2 b) **Effect of AET on hydrogen peroxide - induced hemolysis and lipid peroxidation of human erythrocytes**

Normal canine erythrocytes are oxidized by hydrogen peroxide, and hemolysis and lipid peroxidation of erythrocyte membranes have been observed (Otomo and Fujisawa, 1970).

The effect of AET on lipid peroxidation and hemolysis has been compared with α-tocopherol and curcumin. Lipid peroxidation and hemolysis are inhibited by all the three compounds with varying degree of inhibition. However, AET is found to have a better inhibitory ratio than the other two. The low inhibitory ratio seen with the α-tocopherol on lipid peroxidation and hemolysis may be due to partial association with the membranes during the experimental conditions.

Inhibition of lipid peroxidation and hemolysis of human erythrocytes by curcuminoids (from turmeric) have been reported by Toda et al., (1988). Park and Boo (1991) have observed inhibition of the light induced hemolysis of the rabbit erythrocytes at 50 μM concentration of curcumin. The inhibitory effect has been suggested to be due to the presence of the methoxy groups (Toda et al., 1988) and curcuminoids structure (Tappel, 1965). The inhibition of AET may be due to its association with the membranes and thereby protecting the enzyme activity as well as hemolysis.
Based on the *in vitro* effects of AET, further studies have been extended to *in vivo* systems, using two experimental conditions, in order to assess its efficacy as an antioxidant.

4.3 **EFFECT OF AET ON CCl₄ - INDUCED HEPATOTOXICITY AND ETHYLENE GLYCOL - INDUCED HYPEROXALURIA**

4.3.1 Body weight changes

AET pretreatment prevents CCl₄-induced body weight loss. The observed slight decrease in body weight in the ethylene glycol fed animals is also normalized on AET treatment. Similar observations have been reported by Krishnan and Stenger (1966) and Diaz Gomez *et al.*, (1975). Roberts *et al.*, (1991) have reported that CCl₄ treatment itself depresses food consumption leading to a starvation-like increase in responsiveness to CCl₄.

4.3.2 Histopathology studies

Histopathology studies of the tissues of the CCl₄ fed animals have revealed centrilocular necrosis and the ethylene glycol fed animals, focal necrosis. AET supplemented animals show no drastic pathological damage though complete protection has not been effected. Similar pathological changes like focal necrosis with calcium oxalate crystal deposition and its prevention by feeding vitamin E or methionine have been observed in glycolic acid fed rats (Selvam and Bijikurien, 1991; Selvam and Ravichandran, 1991).
4.3.3 Effect of AET on CCl₄ - induced lipid peroxidation

An increased formation of TBARS has been observed in the liver and kidney of the CCl₄ and ethylene glycol fed rats, with and without promoters such as ascorbate, ferrous ion and tBH. However, AET pretreatment is found to inhibit the CCl₄-induced TBARS formation significantly when compared to that of simultaneous treatment of AET along with CCl₄. Similar observations have also been noticed in the ethylene glycol fed rats.

Stimulation of lipid peroxidation by CCl₄ has been demonstrated in liver homogenates (Comporti et al., 1965; Poli et al., 1983, 1985) in liver microsomal suspensions (Slater and Sawyer, 1971) in isolated hepatocytes (Poli et al., 1979) and in vivo (Recknagel and Ghoshal, 1966; Jose and Slater, 1972). Yuasa et al., (1986) have observed a five fold increase of in vivo lipid peroxidation in the liver of the CCl₄ administered rats and a correlation between membrane peroxidation and membrane breakdown.

In the liver, CCl₄ is metabolized by the cytochrome-P-450 system to reactive intermediates and radicals which can attack polyunsaturated fatty acids ultimately resulting in lipid peroxidation of hepatocyte membranes (Cunningham et al., 1985; Brault et al., 1985; Berger et al., 1986; Andrews and Snyder, 1986; Dogterom et al., 1988). However, peroxidation of lipids is not demonstrable with most other toxicants (Clawson, 1989). A relationship between lipid peroxidation and hepatotoxicity has been suggested (Pessaye et al., 1982; Shertzer and Sainsbury, 1988; DeGroot et al., 1988; Danni et al., 1988).
Several substances like tritoqualine, vitamin E, (Yuasa et al., 1986), sulphhydryl compounds like N-acetyl cysteine and dithiothreitol (Gonskii et al., 1991) and adenosine and inosine (Singh et al., 1992) have been shown to suppress the CCl₄-induced hepatotoxicity. These substances show beneficial effect in restoring the biochemical as well as pathological alterations. Cystamine, cysteine and 2-diethyl amino ethyl 2,2 diphenyl valerate (SKF-525 A) protect against CCl₄ necrosis (Ferreira et al., 1977). Pantothine, pantothenic acid and cystamine have been shown to confer significant protection against the hepatotoxic and peroxidative actions of CCl₄ in rats (Nagiel - Ostaszewski and Lau-Cam, 1990). Dietary administration of sesamin to rats has been shown to prevent the ethanol-induced changes in mouse blood parameters such as SGOT, SGPT, total cholesterol, triglyceride and total bilirubin and liver function (Akimoto et al., 1993). Similarly, sesamin feeding also prevents liver lipid accumulation caused by CCl₄ administration to mice (Akimoto et al., 1993). Likewise, curcumin and its derivatives have been shown to be effective in preventing CCl₄-induced cytotoxicity in primary cultured rat hepatocytes (Kiso et al., 1983). Soundamini et al., (1992) have reported an inhibition in the formation of peroxides by the administration of curcumin in CCl₄ toxicity.

A significant increase in malondialdehyde release has been observed in the liver and kidney of the calcium oxalate-induced and vitamin B₆ deficient urolithic rats. The peroxide formation has been found to be enhanced in the presence of promoters, NADPH, ascorbate, tBH (Bijlkuiren and Selvam, 1989; Selvam and Ravichandran, 1991). Methionine feeding has been found to
prevent kidney stone deposition by restoration of free radical mediated changes in experimental rat urolithiasis (Selvam and Bijikurien, 1991). Supplementation of methionine or vitamin E along with B₆ deficient diet has also been found to restore the levels of antioxidants in blood to near normal and also protect against oxidative stress (Selvam and Ravichandran, 1991).

The inhibitory action of the AET protein on lipid peroxidation may be by scavenging the diol epoxides as has been suggested by Polasa et al., (1991). Many antioxidants are known to act in this way (Ames et al., 1981). Further, Polasa et al., (1991) have suggested that the mechanism of action of curcumin in inhibiting lipid peroxidation is due to its prevention of initiation reactions by interacting with the toxic metabolite binding to macromolecules, similar to the action of plant phenols (Sayer et al., 1982; Wood et al., 1982). Because of this antioxidant potency of AET to inhibit peroxidation in vivo, one can advocate it for therapeutic use in hepatotoxicity as well as kidney stone disease similar to that of vitamin E or methionine.

4.3.4 Antioxidants

As part of their normal diet or living environment, all organisms are constantly exposed to harmful chemicals or xenobiotics, which are of no functional value. Consequently, the development of protection mechanisms against the stress imposed by exposure to xenobiotics has been an essential part of the evolutionary process (Hayes and Wolf, 1990). The antioxidants that have received the most attention in biological systems include α-tocopherols, ascorbic acid and thiol containing compounds.
Therefore, the concentration of reduced glutathione, ascorbic acid, total protein and non-protein thiols have been determined in the toxicity induced conditions.

4.3.4.1 Reduced glutathione (GSH)

Reduced glutathione concentration is found to be significantly decreased in the liver and kidney of the CCl₄ treated and ethylene glycol fed rats. AET pretreatment is able to restore the GSH to near normal levels in both experimental conditions.

GSH has been attributed with a number of cellular functions and roles, including a central pivotal role in cellular reductive processes and protection/detoxification from harmful oxidative species and xenobiotics. There is now unequivocal evidence that cellular GSH levels are a determinant in sensitivity to cytotoxic drugs as well as oxidative and radical induced damage. As a direct consequence of the role of glutathione in cytoprotection it is expected that the enzymes which interact with this peptide (involved in its homeostasis) will play a role in drug resistance (Black and Wolf, 1991).

The liver is the major source of extracellular GSH (Lauterburg et al., 1984). GSH may arise from two sources, one which is exogenous and derived from intake in the diet, the other involving endogenous synthesis in the liver (Leah et al., 1986). The maintenance of GSH level depends on the GSH generating enzyme, glutathione reductase and GSH utilizing enzymes, such as glutathione peroxidase and glutathione-S-transferase. The NADPH required
for GSH generation (via glutathione reductase) is supplied by glucose-6-phosphate dehydrogenase (G6PD).

The present study shows a moderate increase in G6PD activity in both liver and kidney of the CCl₄ and ethylene glycol fed animals. This may be to furnish the reducing equivalents of the cell which is under great oxidative stress.

Roberts et al., (1991) have observed an impaired detoxification through a depletion of hepatic glutathione content in CCl₄ intoxicated rats. α-adrenoreceptors including epinephrine are capable of producing a significant depression of hepatocellular glutathione levels (James et al., 1998). It has been observed that glutathione depression by diethylmaleate or phorone increased the toxicity of CCl₄ (Siegers et al., 1977; Siegers et al., 1985). Irreversible binding of acetaminophen and bromobenzene derived active intermediates to tissue macromolecules has also been found to increase with concomitant decrease in tissue levels of GSH (Mehendale, 1981).

GSH is known to undergo conjugation reactions with electrophilic reactants. Increased toxicities associated with depleted GSH levels have been explained on the basis of diversion of the biotransformation pathway via, the increased accumulation of active intermediates which interacted with tissue macromolecules to cause toxicity. Interestingly, the presence of a [GSH⁺-CCl₃] radical adduct has been identified by spin trapping and the detection of this new radical establishes the reaction of GSH with a CCl₄-derived free radical as a significant event in the metabolism of CCl₄ (Connor et al., 1990).
Decreased levels of GSH has also been reported in the urolithic rat liver and kidney by feeding either vitamin B₆ deficient diet or sodium glycolate supplemented diet (Selvam and Ravichandran, 1991; Kurien and Selvam, 1989). However, the depleted glutathione concentrations in the experimental urolithic animals has been found to be recovered on methionine treatment (Selvam and Kurien, 1991, 1992; Selvam and Ravichandran, 1991).

Polasa et al., (1991) have observed decreased mutagenicity with a concomitant increase in the cellular glutathione levels on feeding vitamin A and turmeric. Azuine and Bhide (1992) have observed significant inhibition of BP-induced forestomach tumours and DMBA-induced skin tumours in mice with an increase in GSH and GST activity by feeding 2-5% turmeric diet.

4.3.4.2 Ascorbic acid

The antioxidant property of ascorbate is often associated with the ability to regenerate vitamin E or vitamin E radical (Niki et al., 1984). Nishikimi (1975) has shown that ascorbate reacts with $O_2^-$ to give $H_2O_2$ and dehydroascorbate. Within the body, ascorbic acid, can in theory be maintained in the reduced form by the shuttle of dehydroascorbate across the erythrocyte membranes for conversion to ascorbate (Orringer and Roer, 1979).

The ascorbate levels are found to be decreased in the liver and kidney of the CCl₄ and ethylene glycol treated rats. Supplementation of AET partially restores the ascorbate levels in the ethylene glycol fed rats. However, complete recovery is seen in the kidney of the CCl₄ fed rats.
Ascorbate has been shown to provide important antioxidant protection to plasma and lipid membranes (Frei et al., 1988, 1989) of ocular tissues (Varma, 1987) and degenerative diseases including cancer (Block and Menkes, 1989), heart disease (Ginter, 1982) as well as a variety of other diseased conditions (Clemetson, 1989).

Decreased levels of ascorbic acid has also been reported in experimental kidney stone forming conditions and methionine supplementation to these urolithic rats is found to restore the levels to near normal (Selvam and Kurien, 1991, 1992; Selvam and Ravichandran, 1991).

4.3.4.3 Total, protein and non-protein thiols

The total protein and non-protein sulphydryl content is significantly decreased in both CCl₄ as well as ethylene glycol treated conditions. AET pretreatment restores the thiol content to near normal in CCl₄ treated rats but only partially in the ethylene glycol fed group.

Cellular GSH has been suggested to act as a buffer against the oxidation of protein sulphydryls (PSH) which are critical in maintaining normal cellular function (DiMonte et al., 1984). Depletion of cellular GSH below a certain level may result in the oxidation of critical PSH and result in cellular toxicity. Enzymes which are highly sensitive to oxidative stress i.e. the accumulation of oxygen radicals and alteration of cellular redox state, include the Ca²⁺-ATPases which function to maintain cytosolic Ca²⁺ within narrow limits (Bellomo et al., 1983). Numerous important cellular functions are regulated by
cytosolic Ca\textsuperscript{2+} (Duncan 1976) and perturbation of cytosolic Ca\textsuperscript{2+} may result in cell death (Di Monte et al., 1984a).

Formaldehyde and acrolein have been found to decrease both cytosolic and mitochondrial GSH and PSH significantly prior to causing cell death, resulting in an increase of cytosolic Ca\textsuperscript{2+}, by a PSH-independent and PSH-dependent mechanism respectively. Formaldehyde toxicity has been, however, correlated with depletion of PSH (Ku and Billings, 1986). Horman et al., (1989) have observed an initial depletion of GSH, a subsequent increase in MDA and decrease in PSH and an eventual loss in membrane integrity in allyl alcohol-induced cytotoxicity.

Thus, the decreased PSH concentration observed in CCl\textsubscript{4} and ethylene glycol treated animals may be due to the depletion of GSH. Restoration of PSH is associated with the normalization of GSH in AET treatment.

Sulphydryl compounds like N-acetyl cysteine and dithiothreitol are shown to protect against CCl\textsubscript{4} toxicity by delaying the depletion of GSH and thereby preventing loss of PSH and protecting the cells against viability loss. Szabo et al., (1981) have found that ethanol exposure lowers the concentration of non-protein sulphhydrlys in gastric mucosa, and that thiols induce gastric cytoprotection by increasing non-protein sulphhydrly levels.

Decreased total thiol content is also observed in the experimental calcium oxalate induced and vitamin B\textsubscript{6} deficient stone forming rats (Selvam and Ravichandran 1991; Kurien and Selvam, 1989). Feeding methionine to the
stone forming rats is found to prevent the thiol loss and restore it to near normal (Selvam and Kurien, 1991; 1992).

Rafatullah et al., (1990) have observed an increase in the gastric wall mucus and restoration of non-protein sulphydryl (NPSH) content in the glandular stomachs of the rats on feeding turmeric extract.

4.3.5 Antioxidant enzymes

4.3.5.1 Catalase (CAT), Glutathione peroxidase (GPx) and superoxide dismutase (SOD)

A significant decrease in the activities of CAT, GPx and SOD are observed in the CCl₄ and ethylene glycol fed rats. When the CCl₄ and ethylene glycol intoxicated animals are treated with AET, the antioxidant enzyme levels (CAT, GPx, SOD) are brought back to near normal in the CCl₄ treated animals, while in the ethylene glycol treated animals, it prevents the loss of enzyme activities only partially.

Catalase has been shown to be responsible for the detoxification of significant amounts of hydrogen peroxide (Brenner and Alison, 1953; Nicholls, 1965). Catalase normally exists in the form of inactive complex II (Leibowitz and Cohen, 1968). The conversion of complex II to active catalase is dependent on the availability of NADPH concentration (Eaton et al., 1972). Under conditions of increased oxidative stress, the NADP/NADPH ratio will switch in favour of NADP. The paucity of NADPH will in turn reduce catalase activity.
So the observed decreased catalase activity in the CCl₄ and EG fed animals may be due to the impaired redox system. Another possibility is that reactive oxygen radicals can themselves inhibit the activity of CAT and GPx (Hodgson and Fridovich, 1975; Searle and Wilson, 1980).

GPx uses glutathione to reduce various hydroperoxides and is involved in the protection of lipid membranes and the detoxification of their break down products. Peroxidase reactions are catalysed predominantly by the selenium dependent peroxidase and by the alpha class glutathione-S-transferases. (Landenstein, 1984). The ability of GPx to detoxify hydroperoxides makes it a potentially important enzyme in drug resistance. The activity of selenium dependent enzyme is completely dependent on the presence of selenocysteine at its active site which interestingly is encoded by a UGA stop codon (Reddy et al., 1988).

The impairment of tissue GPx after in vivo treatment with oxidizing agents has been proposed as a specific and sensitive index of tissue damage (Tappel, 1980). The decreased GPx activity observed in the present study is likely to be the result of CCl₄- and ethylene glycol dependent cell injury. Manno et al. (1985) and Slater (1982), have observed that GPx alone has little protection against CCl₄-dependent lipid peroxidation, since this enzyme is present in cytosol and mitochondria but not in microsomal membrane, the site of CCl₄ activation.

SOD on the other hand, is an effective defense of the hepatocyte and most other cells against both endogenous and toxicologically induced
generation of \( \text{O}_2^- \) (Brawn and Fridovich, 1980). A decrease in SOD activity is observed in the CCl\(_4\) fed animals and also in the ethylene glycol treated ones. This may in fact be the result of the attack of CCl\(_4\) or EG on the cytosolic enzyme. The SOD activity determined, however, accounts only for the cytosolic SOD. Similar reports on the CCl\(_4\)-dependent inhibition of SOD and GPx activities have been observed in the liver extracts of CCl\(_4\)-treated animals (Manno et al., 1985).

Impaired activity of SOD, CAT and GPx with increased free radical lipid peroxidation and a marked decrease in the thiol concentrations have been observed in the CCl\(_4\) intoxicated rats (Gonskii et al., 1991). Sharma et al. (1991) also have reported a pronounced elevation in lipid peroxidation, associated with a significantly greater inhibition of the antioxidant enzymes GPx, GR and SOD with a concomitant decrease in the GSH and total thiols in the cadmium and ethanol exposed rats. GPx, SOD and CAT inactivation by peroxides and oxygen derived free radicals have been discussed by Pigeot et al., (1990), wherein, they have suggested the possibility of the inhibition of the antioxidant enzymes under higher oxidative stress leading to an irreversible autocatalytic process in which the production rate of the oxidants will continuously increase, leading to cell death.

Selvam and Kurien (1987) and Kurien and Selvam (1989) have observed decreased activity of catalase and GPx in experimental urolithiasis and the decreased activity of catalase has been attributed due to the inhibition of oxalate. Similar observations have also been made by Selvam and Ravichandran (1993) in vitamin B\(_6\) deficient rats. Decreased activity of SOD
has been observed in the B₈ deficient urolithic rats. Methionine and vitamin-E supplementation to the experimental urolithic rats is found to prevent the inhibition of the SOD, CAT and GPx activities, by combating the free radical mediated changes (Selvam and Kurien 1991; 1992; Ravichandran and Selvam, 1993). Apparently, the protective action of the antioxidant defense system requires a complex use of water and lipid soluble antioxidants. It has been found that tocopherol and/or dimethyl sulfoxide could efficiently repair the impaired antioxidant enzymic activities (SOD, CAT, GPx and GR) partially or completely (Gonskii et al., 1991).

The restoration of the antioxidant enzymes by AET pretreatment may be due to the improvement in the redox status as seen by the increment in the levels of the endogenous thiol compounds under this condition.

4.3.5.2 Glutathione-S-transferase (GST)

The glutathione-S-transferases are a group of multifunctional proteins involved in the transformation of xenobiotics (Jakoby, 1978; Jakoby and Habig 1980). These enzymes catalyze the conjugation of electrophilic compounds with glutathione and also play a role in the binding and storage of toxic compounds such as bilirubin and azo-dye carcinogens (Ketley et al., 1975; Ketterer et al., 1987). In rat liver cytosol, several GST's have been identified and designated as AA, A, B, C and D + E (Habig et al., 1974).

A marked decrease in the GST activity of the liver and kidney is observed both in the CCl₄ and ethylene glycol treated animals. It is found that
AET pretreatment partially or completely has restored the impaired enzyme status in both the CCl₄ and ethylene glycol fed animals.

It has been reported that hepatic glutathione-S-transferases are altered by hepatotoxic chemicals (chloroform, carbon tetrachloride etc.) and are released into the serum (Aniya and Anders, 1985a,b; Fukai et al., 1989). CCl₄ intoxication leads to a marked increase in the plasma GST activity and a decrease in the hepatic GST activity. Analysis of hepatic GSTs immuno histochemically has revealed the leakage of GSTs from the centrilobular zone where AA and A GSTs are found to be localized dominantly in the normal rat liver. Massive cell necrosis is also seen at the same regions. Yeung (1991) has observed decreases in hepatic and renal GST activities in the glycerol-induced acute renal failure rats. Acute renal failure causes an increase in the GSSG/GSH ratio - indicative of acute oxidant stress with a decrease in the GSSG reductase and GPx activity which are essential for the protection against lipid peroxidation. Hepatic GST is significantly decreased in the short term diabetic rats and this decrease is additive when chloroform is administered (Aniya et al., 1989). Hepatic cytosolic GST activity decreased on chloroform treatment and only GST D + E has been detected. These alternations of GST on chloroform treatment have been prevented by treatment with cysteine or SKF-525A (Aniya and Anders, 1985); these agents also inhibit the covalent binding of chloroform metabolites to macromolecules (Pohl et al., 1980; Stevens and Anders, 1981).

The observed decrease in GST activity may be due to the increase in the GSSG/GSH ratio, as GSH depletion is observed in the toxin treated animals.
4.3.6 Glucose-6-phosphate dehydrogenase (G6PD)

Increased activity of G6PD has been observed in the CCl₄ administered rats. The liver of the ethylene glycol fed animal has not shown any marked alterations while the kidney alone registered a moderate increase in G6PD activity. The increased activity is found to be restored to near normal in the AET treated animals of the CCl₄ and ethylene glycol treated rats.

Glucose-6-phosphate dehydrogenase is the first and also the rate limiting enzyme of the hexose monophosphate shunt, furnishing the reducing equivalents NADPH, for the cell. NADPH is utilized by glutathione reductase for the production of GSH from GSSG. G6PD is activated by an increased NADP/NADPH ratio and by GSSG, by mixed disulphide formation (Brigelius, 1985).

It has been reported by several others that the first metabolic reaction that takes place in a hepatocyte or any cell, subsequent to administration of xenobiotics or chemicals, is the depletion of the cellular glutathione levels, which may in turn lead to an increase in the NADP/NADPH ratio due to the increased utilization of GSH for the toxic metabolite covalent binding to elicit its effects. This may be one of the reasons for an increase in activity seen in the present study, under the toxin treated conditions. The partial or complete restoration seen when fed with AET may be by its efficiency to inhibit peroxidation and improve the cellular thiols thereby elevating the redox status of the cell.
Renal G6PD has been found to increase in renal cortex of rats with alloxan-induced diabetes (Anderson and Stowring, 1973). Similarly, the activity is also found to increase in the jejunal mucosa of rats with streptozotocin-induced diabetes (Murphy and Anderson, 1974).

4.3.7 Glucose-6-phosphatase (G6Pase)

The activity of the microsomal G6Pase is found to be decreased in the liver and kidney of both the CCl₄ and ethylene glycol fed animals. The activity is found to be partially or completely normalized by AET pretreatment.

Early centrilobular suppression of G6Pase and transitory influx of calcium into midzonal liver parenchymal cells have been observed in rats following CCl₄ intoxication (Reynolds, 1963; Reynolds and Yee, 1968).

Lipid peroxidation either associated with or resulting from free radical cleavage of CCl₄ has been postulated to be a vector in the suppression of G6Pase activity after CCl₄ poisoning. (Recknagel and Ghoshal, 1965). CCl₄ and lipid peroxidation have been demonstrated to have synergistic effects on the suppression of G6Pase in vitro (Recknagel and Ghoshal, 1965) 4-hydroxy nonenal a degradative product of lipid peroxidation has been shown to inhibit G6Pase activity (Benedetti et al., 1980). de Groot et al., (1966) have observed a decline in the G6Pase activity and attributed the alterations to be due to an irreversible damage of the phosphorylase active site of the G6Pase system and further suggested the crucial role of low pO₂ in hepatotoxicity of haloalkanes.
The observed decreased activity of G6Pase may be due to inactivation of the enzyme by lipid peroxidation and hypoxia. AET treatment has restored the activity to normal by counter acting the oxidative insult.

Achudume (1991) has reported an increase in the serum ALT and AST activities with a decrease in G6Pase in the CCl₄ administered rats. Dimethyl sulfoxide pretreatment has protected the animals from CCl₄ toxicity. The decreased activity of G6Pase is also found to be normalized by methionine supplementation to the stone forming rats (Biji Kurien, 1989).

4.3.8 Aspartate and alanine amino tranferases (AST and ALT)

Liver AST and ALT are significantly elevated in the CCl₄-induced animals, but remained unaltered in the ethylene glycol treated rats. On treatment with AET, the altered activities are brought back to normalcy.

AST and ALT are both present in high concentrations in the liver cell. AST is located both in the mitochondria and cytosol, while ALT is located solely in the cytosol. (Clermont and Chalmers, 1976). An increase in mitochondrial AST has been shown to occur in alcoholic liver disease. (Nishimura et al., 1986; Golberg et al., 1986). The decrease in liver oxygen supply seen in alcoholic liver disease initially results in leakage of cytosolic ALT. Further, continued liver damage with break down of the tight barrier of the mitochondrial membrane results in AST leakage (Sekiya et al., 1975). Elevation in the liver and serum AST and ALT activities have been reported after CCl₄ administration (Pappas - 1986). CCl₄ is capable of causing severe
hepatocellular damage with 9 and 17 fold increase in serum AST and ALT respectively.

4.3.9 Lactate dehydrogenase (LDH)

LDH of the liver and kidney is significantly enhanced in CCl₄ treated rats while in ethylene glycol treated rats only liver LDH activity is increased. AET pretreatment is found to restore the activities to near normal in the CCl₄ rats, but partially in ethylene glycol fed rats.

LDH plays a primary role in the synthesis of oxalate. Increased LDH activity in the experimental stone forming rats has been observed by Selvam and Kurien (1992) and the activity is not restored by citrate or methionine feeding. Elevated activity of RBC-LDH in calcium oxalate stone formers has been reported by Selvam and Rengaraju (1986). LDH of the rat liver is found to be elevated by male sex hormone and testosterone and decreased by the female sex hormone, estradiol (Murthy et al., 1982).

The major role of LDH in the liver is the oxidation of lactate to pyruvate for utilization in gluconeogenesis. The oxidation of glyoxylate to oxalate by LDH utilizes NAD as the coenzyme. The increased LDH activity observed in the liver of the ethylene glycol fed hyperoxaluric rats suggests an increase in the NAD/NADH ratio. This indicates the role of LDH in oxalate synthesis (Richardson and Farinelli, 1981).

Release of LDH from the cells has been notably increased by CCl₄ addition (Yuasa et al., 1986). Masuda and Nakamura (1990) have reported
increased TBARS with leakage of K⁺ and LDH in the isolated perfused livers of the CCl₄-induced hepatotoxicity due to plasma membrane permeability changes.

Tritoqualine and vitamin-E are found to reduce the increase in LDH activation.

4.3.10 Membrane bound ATPases

ATPases are lipid dependent membrane bound enzymes (Fourcans and Jain, 1974) and any alterations in membrane lipid lead to changes in membrane fluidity which in turn alters the ATPase activities and cellular functions (Tanaka and Strictland, 1965).

The total, magnesium, calcium and sodium - potassium dependent ATPases have registered a marked loss in activity in CCl₄ treated group while kidney Ca²⁺-ATPase registered an increase in the ethylene glycol treated group. AET pretreatment of the animals has shown partial or complete restoration of the activity.

Muriel and Mourelle (1990) have observed that significant decreases in sodium-potassium and calcium activated ATPase activities in CCl₄ treated animals have been restored by silymarin treatment by inhibiting lipid peroxidation. The radical induced inactivation of kidney sodium, potassium-ATPase and its sensitivity to membrane lipid peroxidation are found to be protected by vitamin-E (Thomas and Reed, 1990).
Magnesium-ATPase is found to be inhibited by tetrachloroethylene potentiation of carbon tetrachloride toxicity in isolated rat hepatocytes (Kefalas and Stacey, 1991; Rufeger and Frimmer, 1978). Sodium, potassium-ATPase and magnesium-ATPase activities in hepatocyte plasma membrane preparations are found to be inhibited by solvent exposure (Kukongviriyapan et al., 1990). Erythrocyte calcium-ATPase is inhibited by activated oxygen through thiol- and lipid dependent mechanisms (Hebbel et al., 1986).

The observed increase in calcium-ATPase activity in the kidney of the ethylene glycol fed hyperoxaluric rats may be due to the peroxide induced damage. Oxidized fatty acids are shown to facilitate the movement of calcium across liposomal membranes. Calcium-ATPase is responsible for the transport of calcium across the cell membrane and calcium accumulation is observed with increased enzyme activity (Sarkadi, 1980).

Normalization of lipid peroxidation process as well as calcium-ATPase in the methionine supplemented calculi producing diet fed rat liver and kidney have been shown (Biji Kurien, 1989).

4.3.11 Glycolic acid oxidase (GAO)

The biosynthesis of oxalate has been shown to be a contributing factor in primary hyperoxaluria (Williams and Smith, 1968a and 1968b; Williams and Smith, 1972), kidney stone formation (Nordin and Hodgkinson, 1967) and possibly ethylene glycol toxicity (Chou and Richardson, 1978).
Glycolic acid oxidase of the liver of the ethylene glycol intoxicated animals is significantly increased. The enhanced activity is not normalized even after treatment with AET. Only partial but a significant recovery compared to ethylene glycol fed rats is achieved.

Murthy et al., (1982, 1983) have reported a similar observation of increased GAO in rats fed with pyridoxine deficient diet or glycolate. GAO is elevated by testosterone and decreased by estradiol. The increased liver GAO levels in vitamin B₆ deficiency is attributed to hyperoxaluria (Sharma et al., 1990; Varalakshmi and Richardson, 1983). Increased GAO activities were also reported by Selvam and Ravichandran (1991) and Selvam and Kurien (1992) in the experimental stone forming rats. Citrate feeding (Selvam and Kurien, 1992) is found to partially restore the enhanced enzyme activities.

Glyoxylic acid has been identified as the major immediate precursor of oxalate (Williams and Smith, 1972). One of the pathways for the formation of glyoxylate is the reaction catalysed by glycolic acid oxidase, which further catalyses the oxidation of glyoxylate to oxalate (Richardson and Tolbert, 1981). The major endogenous source of glycolic acid is glycoaldehyde which is derived from ethanolamine via the glycine-serine pathway which approximately contributes to 33% of the urinary oxalate (Dean et al., 1968). Oxidation of ethylene glycol occurs by way of glycoaldehyde and glycolic acid (Gessner et al., 1961).
4.3.12 Oxalate and lipid peroxidation

The induction of lipid peroxidation is considered to be an important process in the aetiology of many pathogenic diseases. Increased lipid peroxidation in rat liver and kidney has been reported in experimental urolithiasis (Selvam and Bijikurien, 1987; Bijikurien and Selvam, 1989). Further, induction of lipid peroxidation by oxalate as well as calcium oxalate has been reported (Selvam and Biji Kurien, 1987).

Ethylene glycol fed rats show significant accumulation of oxalate in liver and kidney. It is interesting to note that AET treatment prevented oxalate increase considerably. A complete restoration is seen in the liver and only a partial restoration in the kidney. Therefore, the increased lipid peroxidation observed in the ethylene glycol fed rats may be due to the direct influence of oxalate. The fact that calcium oxalate promotes lipid peroxidation is remarkable because it is the main constituent of kidney stones. Since, calcium is ineffective in inducing lipid peroxidation, it is assumed that calcium contributes little or practically nothing towards peroxidative damage to lipids. Accumulation of oxalate may be associated with the increase in the GAO and LDH activities in ethylene glycol treated group.

The reduction in the accumulation of oxalate on AET feeding may be due to the inhibition of lipid peroxidation and oxalate synthesis. Both LPO and accumulation of oxalate are found to correlate positively. Feeding vitamin E or methionine supplemented diet to stone forming rats is shown to prevent the
accumulation of oxalate as well as calcium oxalate deposition (Selvam and Kurien, 1991; Selvam and Ravichandran, 1991).

4.3.13 Tissue calcium and magnesium

Significant increase in calcium content of liver and kidney is seen in the ethylene glycol treated rats and the level is restored partially on treatment with AET. Increased renal calcium content has been observed in the stone forming rats due to ethylene glycol feeding (Lee et al., 1992; Miyazawa et al., 1989).

However, magnesium level remains unaltered in the liver while a slight decrease in the kidney is noted. On treatment with AET, the levels of Ca and Mg are restored to near normal.

4.3.14 Urinary excretion

The present study shows increased concentrations of oxalate (hyperoxaluria), calcium (hypercalciuria), phosphorous (hyperphosphaturia) and decreased magnesium content in urine of the ethylene glycol fed rats for 7 or 15 days. Supplementation of AET to these animals affects a partial normalization of these ions.

Hyperoxaluria is one of the major risk factors for the formation of urinary calcium oxalate stones (Khan et al., 1989). Hyperoxaluria and hypocitraturia with increase in pH of the urine and renal calcium content by 7.9 fold have been reported in ethylene glycol fed rats (Lee et al., 1992). Khan
et al., (1989) have suggested that the renal injury may be induced by lithogenic challenges and the resulting hyperoxaluria.

Damage to the proximal tubular epithelium is generally associated with shedding of the brush border membrane and damaged epithelium facilitates crystal retention (Gill et al., 1979; Khan et al., 1984). Selvam and Biji Kurien (1991, 1992) have reported increased calcium and oxalate and decreased magnesium excretion in experimental urolithiasis. Further, citrate feeding to stone forming rats is found to restore the levels of calcium phosphorous and magnesium to near normal (Selvam and Biji Kurien, 1992).

Fluoride is shown to inhibit stone formation induced by ethylene glycol in rats (Zhang et al., 1992). Sulphydryl compounds like cysteine are shown to rapidly and markedly decrease the urinary oxalate excretion in the ethylene glycol treated rats (Bais et al., 1991). Feeding 0.5% curcumin (turmeric) supplemented with the lithogenic diet for 10 weeks is found to reduce the incidence of gall stone formation (Hussain and Chandrasekhara, 1992). Leakovian (1983) has reported that di- and polycarboxylic acids and their derivatives are capable of dissolving the urinary calculi. Dyes like turmeric ion-exchange resins are shown to have inhibitory effects on oxalic acid absorption in the intestine.

The observed increased excretion of oxalate in the urine of the experimental groups may be due to the increased activity of the oxalate synthesizing enzymes. Several reports suggest that the primary cause of hyperoxaluria is hyperabsorption of dietary oxalate from the gut (Hodgkinson
and Zarembaki, 1968). The prevention or the inhibitory effect of AET on hyperoxaluria may be by inhibiting the ethylene glycol absorption from the gut and also by inhibiting the oxalate synthesizing enzymes GAO and LDH.

The observed decreased accumulation of oxalate in the renal cells of AET pretreated rats under ethylene glycol feeding, may be due to the inhibitory effect of this protein (AET) on oxalate binding as evidenced by its 80% inhibition of mitochondrial oxalate binding.

Increased phosphate and calcium excretion appears to be critical in determining stone formation in hyperoxaluric subject, although hyperphosphaturia does not appear to be a significant factor in stone formation (Cotet and Vittu, 1955; Hodgkinson and Pyrah, 1958).

Magnesium appears to play a dual role in calculus formation. There is increasing evidence that in sterile acid urine, magnesium increases the solubility of calcium salts (Hammersten, 1929; Vermeulen et al., 1958; Mukai, Howard and Thomas 1968). Magnesium forms a more soluble complex with oxalate than with calcium (Desmars and Tawashi, 1973). Decreased excretion of magnesium which occurs during orthophosphate administration and phytate therapy enhance stone formation (Hennenman et al., 1956).
4.4 PROPOSED REACTION MECHANISM OF AET PROTECTION IN CCl₄ AND ETHYLENE GLYCOL TOXICITY (Figure 4A)

a) In CCl₄ - induced liver injury

CCl₄ administered rats showed increased formation of TBARS in both liver and kidney in presence of promoters of LPO suggesting high susceptibility. Due to the enhanced peroxidation, the levels of the antioxidants namely ascorbate, GSH, total, protein and non-protein thiols were depleted. Further, the reaction was accentuated by the lowered activities of the antioxidant enzymes GPx, CAT, SOD and GST. Due to the increased peroxidative damage, the cellular enzymic activities, G6PD, AST, ALT, G6Pase and LDH and membrane bound enzymes (ATPases) were altered.

On AET supplementation, the elevated lipid peroxides were significantly inhibited and also the depleted antioxidants and antioxidant enzymes were restored to near normal. This led to the normalization of activities of cellular and membrane bound enzyme activities.

Thus, the mechanism of prevention of cell necrosis due to CCl₄ intoxication by AET may be possibly due to its restoration of the antioxidants and antioxidant enzymes which prevent membrane peroxidation and thereby normalizing the Ca²⁺ influx into organelles.

Therefore, AET treatment can be advocated to prevent/protect the hepatotoxic conditions.
1. Metabolic activation of CCl$_4$ and ethylene glycol

SOD

2. $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

3. $H_2O_2 + Fe^{2+} \rightarrow OH^0 + OH^- + Fe^{3+}$ (Fenton reaction)
   $O_2^- + H_2O_2 \rightarrow OH^0 + OH^- + O_2$ (Haber - Weiss reaction)

GAO

4. Glyoxylic acid + $O_2 \rightarrow$ oxalic acid + $H_2O_2$

CAT

6. $2H_2O_2 \rightarrow 2H_2O + O_2$

GST

7. GSH + (X$^0$) \rightarrow mercapturic acid

GPx

8. $H_2O_2 + 2GSH \rightarrow 2H_2O + 2GSSG$

Protection by AET

(a) Antioxidant reactions - (5)

(b) Antioxidant enzymes - (6), (7) and (8)

(c) Inhibition of cellular damage like lipid peroxidation, membrane fluidity
   $Ca^{2+}$ accumulation and calcium oxalate retention.
b) In ethylene glycol-induced hyperoxaluria

Administration of ethylene glycol to rats exhibited increased lipid peroxide formation and decreased levels of antioxidants and antioxidant enzymes. The increased peroxidative reactions induced the membrane and cell damage causing marked alterations in the cellular and membrane bound enzymes studied. Further, retention of oxalate due to increased oxalate binding protein activity was observed along with increased excretion of oxalate, calcium and phosphorous in urine, causing hyperoxaluria.

AET administration significantly inhibited the free radical induced damages and restored the antioxidants and antioxidant enzymes, thereby preventing cellular damage and oxalate and calcium accumulation which results in reduced calcium oxalate retention.

These studies suggest that AET can be recommended for therapy of stone disease. However, further studies are needed to assess its antioxidant activity in human stone formers.