ANTIOXIDANT PROPERTIES AND CHEMOPREVENTIVE POTENTIAL OF THE BIOACTIVE CONSTITUENTS OF THE ROOTS OF *DECALEPIS HAMILTONII*

Thesis

submitted to the
UNIVERSITY OF MYSORE

For the award of degree of
DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

by
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February 2006

To

My Parents

DECLARATION

I hereby declare that the thesis entitled "**ANTIOXIDANT PROPERTIES AND CHEMOPREVENTIVE POTENTIAL OF THE BIOACTIVE CONSTITUENTS OF THE ROOTS OF *DECALEPIS HAMILTONII***" submitted to the University of Mysore, for the award of the degree of Doctor of philosophy in Biotechnology, is the result of the research work carried out by me in the Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore, under the guidance of Dr. T. Shivanandappa, during the period July, 2001 to February, 2006.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

Anup Srivastava
Senior Research Fellow

Date:
Place: Mysore

CERTIFICATE

I hereby certify that this thesis entitled "**ANTIOXIDANT PROPERTIES AND CHEMOPREVENTIVE POTENTIAL OF THE BIOACTIVE CONSTITUENTS OF THE ROOTS OF *DECALEPIS HAMILTONII***" submitted by Mr. Anup Srivastava for the award of the degree of Doctor of philosophy in Biotechnology, University of Mysore, is the result of the research work carried out by him in the Department of Food Protectants and Infestation Control, Central Food Technological Research Institute, Mysore, under my guidance and supervision during the period July, 2001 to February, 2006.

Dr. T. Shivanandappa
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Place: Mysore

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Anup Srivastava

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SYNOPSIS OF THE THESIS

ANTIOXIDANT PROPERTIES AND CHEMOPREVENTIVE POTENTIAL OF THE BIOACTIVE CONSTITUENTS OF THE ROOTS OF *DECALEPIS HAMILTONII*

Oxidative damage to cells and macromolecules is considered to be the cause of several diseases such as coronary heart disease, arthritis, cataractogenesis, various neurodegenerative diseases including Alzheimer's disease, cancer and aging. Oxidative injury involves free radical-induced damage from both endogenous and exogenous sources. Several studies have shown that dietary antioxidants play an important role in preventing degenerative diseases associated with ageing such as cancer, cardio-vascular diseases, cataract, neurodegenerative diseases and immunological decline. There is a great deal of interest in the natural antioxidants often referred to as "nutraceuticals" in view of their positive health effects. Several studies have shown that the action of the natural antioxidants at the cellular and molecular level involves scavenging of free radicals and modulating apoptosis. In view of this, nutraceuticals are becoming widely accepted as an adjunct to conventional therapies for enhancing the well being. Bioprospecting or the search for newer bioactive compounds from the nations biodiversity for better health is a new thrust area in biotechnology.

Decalepis hamiltonii (Wight and Arn.) known as swallow root (family: Asclepiadaceae) is a monogeneric climbing shrub and a native of the forests of Deccan peninsula and Western Ghats of India. Its tubers are consumed as pickles and the juice for its alleged health promoting properties. The roots of *D. hamiltonii* are used as a flavoring principle, appetizer, blood purifier and as a preservative. Similarly, the roots of this taxon

are considered as “Sariva Bheda” in Ayurveda where these find use as an alternative to the roots of *Hemidesmus indicus* in the preparation of several herbal drugs like *Amrutamalaka taila* (hair tonic), *Drakshadi churna* (general vitalizer), *Shatavari rasayana* (adapatogenic) and *Yeshtimadhu taila* (mild analgesic, rheumatism). The roots contain 92% fleshy matter and 8% woody core. Of late, the highly aromatic roots have been subjected to over exploitation by destructive harvesting that has endangered the survival of this plant. A method for rooting of *D. hamiltonii* for field transfer is reported. In earlier reports it was observed that the aromatic roots of *D. hamiltonii* possess bioinsecticide property on storage pests at lethal and sub-lethal levels. The extracts of these roots have also been shown to be potent antimicrobial agents as well.

Earlier work has shown that the *D. hamiltonii* roots contain aldelydes, inositols, saponins, amyrins, lupeols and volatile flavour compounds such as 2-hydroxy-4methoxybenzaldehyde, vanillin, 2-phenyl ethyl alcohol, benzaldehyde, and others. Essential oil costitiuents of *D. hamiltonii* roots contain: 2-hydroxy-4-methoxy bezaldehyde, β-pinene, benzyl alcohol, γ-hexalactone, 2-hydroxy benzaldehyde, 4-o-methylresorcylaldehyde, α-atlantone, γ-terpinene, 2-phenylethanol, 4-methoxy benzaldehyde, geraniol etc..

Previous studies in our laboratory have shown that the roots of *D. hamiltonii* possess strong antioxidant activity. Recently 2-hydroxy, 4-methoxy benzaldehyde, *p*-anisaldehyde, vanillin, borneol, salicylaldehyde, *bis*-2,3,4,6-galloyl α/βD-glucopyranoside (decalepin) are the antioxidant compounds isolated from the methanolic extract of *D. hamiltonii*. The root extract as well as root powder were not toxic at acute

(upto 1g/ kg b.w.) and subchronic (upto 2.5% in basal diet for 90 days) doses to rats as assessed by the mammalian toxicity experiments.

Since the Ayurvedic practice started in India, plant extracts are being used in the cure of diseases. The exact mechanism of these plant-derived preparations is not well understood which requires to be scientifically investigated. Moreover, the active principles responsible for the alleged health promoting activity need to be isolated to elucidate their exact mode of action. Although the roots of *D. hamiltonii* have been used for their alleged health benefits, there is no scientific investigation in this regard. To fill these scientific lacunae, the present work was undertaken for one such plant, *Decalepis hamiltonii.*

Objectives of the study

a. Isolation and characterization of antioxidant constituents from the aqueous extract of the roots of *D. hamiltonii*.
b. Evaluation of their antioxidative properties, *in vitro.*
c. Chemoprotective effect of the antioxidant compounds on xenobiotic induced cytotoxicity.
d. Assessment of the hepatoprotective & neuroprotective potential of the aqueous extract of *D. hamiltonii* roots *in vivo*.

Chapter I comprising literature survey gives an overview free radicals, oxidative stress, antioxidants and antioxidant protection in the body. Literature on Indian plants showing antioxidant activity and work done on *D. hamiltonii* has been surveyed.

Chapter II describes the isolation and characterization of antioxidant constituents from the aqueous extract of *D. hamiltonii* roots and evaluation of their antioxidant properties. Sequential extraction of the root powder was done with different solvents with increasing polarity i.e. hexane, chloroform, ethyl acetate, acetone, methanol and water. Among the extracts, maximum antioxidant activity was shown by the aqueous extracts and therefore, was chosen for further study. Six compounds showing antioxidant activity were isolated using silica gel, LH-20 and thin layer chromatography. The purified compounds were characterized by spectroscopic analysis (UV, IR, NMR, LC-MS). The compounds were designated as DHA I-VI. (DHA I: 4-hydroxy isophthalic acid; DHA II: ellagic acid; DHA III: 14- aminotetradecanoic acid; DHA IV: 4-(1-hydroxy-1-methylethyl)-1-methyl-1, 2-cyclohexane diol; DHA V: 2-hydroxymethyl-3-methoxybenzaldehyde; DHA VI: 2,4,8 trihydroxybicyclo [3.2.1]octan-3-one). The compounds exhibited high antioxidant activity as measured by free radical scavenging assays, metal chelating potential, reducing power and inhibition of human LDL oxidation. All the compounds isolated from the aqueous extract of the roots of *D. hamiltonii* are novel antioxidants reported for the first time.

In **Chapter III**, we have examined the ameliorative potential of the antioxidant compounds isolated from the aqueous extract of the roots of *D. hamiltonii* against xenobiotic-induced oxidative injury in (a) EAT cells and (b) rat primary hepatocytes, and the possible mechanism(s) underlying their protective effects. Cell culture systems are suitable for the study of cytotoxicity mediated by free radicals and the cytoprotective effect of antioxidants. Since cytotoxic injury is believed to be integral to toxicological manifestation, agents that ameliorate cytotoxic injury are considered to possess health

promoting potential. Antioxidants prevent the damage to macromolecules and cells by interfering with the free radicals. Ehrlich Ascites tumor (EAT) cells derived from the mice mammary glands, can be conveniently cultured in the mice peritoneum cavity. EAT cells offer a good model to study LPO and ROS induction by xenobiotics. Isolated hepatocytes are also a useful *in vitro* model for pharmacological and toxicological studies of xenobiotics. Hexachlorocyclohexane (HCH), carbon tetrachloride (CCl_4), ethanol and cumene hydroperoxide (CHP) are well known inducers of oxidative stress in cell systems. We have employed these xenobiotics to induce oxidative stress in EAT cells and primary hepatocytes from rat and observed amelioration by the phytochemical antioxidants.

The antioxidant compounds isolated from *D. hamiltonii* ameliorate xenobiotic-induced cell injury/death. Relative cytoprotective action of the antioxidant compounds in EAT cells was in the following order: (a) HCH-induced cytotoxicity - DHA-II > DHA-III > DHA-V > DHA-I > DHA-VI > DHA-IV; (b) CCl_4-induced cytotoxicity - DHA-I > DHA-III > DHA-II > DHA-V > DHA-VI > DHA-IV; (c) CHP-induced cytotoxicity - DHA-VI > DHA-III > DHA-II > DHA-I > DHA-V > DHA-IV. Amelioration of cytotoxicity in hepatocytes by the isolated compounds was in the following order: DHA-II > DHA-I > DHA-V > DHA-III > DHA-VI > DHA-IV for both ethanol and CCl_4 induced cytotoxicity. All the antioxidant compounds from *D. hamiltonii* were not toxic to the cells at the highest concentration used

The mechanism of cytoprotective action appears to involve maintaining the intracellular GSH, suppression of ROS induction and inhibition of LPO. These novel

antioxidant compounds could be potential candidates as therapeutic agents for preventing or delaying degenerative diseases.

In **Chapter IV**, we have investigated (a) the hepatoprotective potential of *D. hamiltonii* aqueous extract (DHA) pretreatment (single and multiple doses) against CCl_4 and ethanol induced hepatotoxicity in rats by histopathological observations and biochemical changes.

Chronic liver damage is a widespread pathology characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. Various xenobiotics are known to cause hepatotoxicity, carbon tetrachloride (CCl_4) and ethanol are among them. The hepatotoxic effect of CCl_4 and ethanol is attributed to oxidative stress. Oxidative damage to biological molecules is an important event in the development of a variety of degenerative diseases that result from overwhelming the biological defense system against oxidative stress, drugs and carcinogens. Intake in the human diet of antioxidant compounds that ameliorate or enhance the biological antioxidant mechanisms can prevent and in some cases help in the treatment of some oxidative-related disorders and carcinogenic events. Natural products have been used for this purpose since ancient times and a tendency is emerging today for their increased use. As the oxidative stress plays a central role in liver pathologies and progression, the use of antioxidants have been proposed as therapeutic agents, as well as drug coadjuvants, to counteract liver damage. A number of investigators have previously demonstrated that antioxidants prevent CCl_4 and ethanol hepatotoxicity by inhibiting lipid peroxidation, suppressing alanine aminotransaminase (ALT) and aspartate aminotransaminase activities and increasing antioxidant enzyme activity.

This study demonstrates for the first time, that DHA effectively prevented CCl_4 and ethanol induced hepatotoxicity. The hepatoprotective activity of DHA could, atleast in part, be attributed to the free radical scavenging or inhibition of inflammatory mediators in CCl_4 and ethanol mediated lipid peroxidation. The bioactive antioxidant principles present in the aqueous extract could be responsible for the observed hepatoprotective effect *in vivo*. Further, DHA exhibits antioxidant activity *in vivo* by inhibition of lipid peroxidation and enhancement of the antioxidant status of cells by induction of the antioxidant enzymes and GSH. These results provide a scientific basis for the hepatoprotective effect and perhaps may underlie many other health promoting attributes of *D. hamiltonii*.

In **Chapter V**, we examined the neuroprotective potential of the aqueous extract of the roots of *D. hamiltonii* (DHA) in rats against (a) ethanol-induced oxidative alterations in the brain, (b) HCH-induced oxidative stress in the brain regions, and (c) DDVP-induced AChE inhibition in the different regions of brain.

Brain is considered highly sensitive to oxidative damage since it is rich in easily peroxidizable fatty acids, consumes an inordinate fraction (20%) of the total oxygen for its relatively small weight (2%) and is relatively deficient in its antioxidant defenses. There is a substantial evidence that oxidative stress is a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis as well as in cases of stroke, trauma and seizures. Decreased level of antioxidant activity and increased lipid peroxidation and oxidative modifications of DNA and proteins especially in substantia nigra of the brain have been reported in patients with Parkinson's disease. A number of *in*

vitro studies have shown that antioxidants- both endogenous and dietary- can protect nervous tissue from damage by oxidative stress.

This study shows that DHA positively affects the antioxidant status of the rat brain and exhibits neuroprotective potential, *in vivo*. Since excessive or chronic oxidative stress is considered an important factor in the etiology of many diseases, effects of DHA on the enhancement of the antioxidant status could provide an explanation for the health promoting properties attributed to it.

CONCLUSIONS

- Natural antioxidants are thought to prevent or slow down free radical induced oxidative stress and therefore possess health promoting potential.
- Six antioxidant compounds were isolated from the edible roots of *D. hamiltonii* and five of them are novel antioxidant molecules, reported for the first time. All the compounds showed free radical scavenging activity, reducing power, metal chelation and inhibited protein carbonylation and human LDL oxidation.
- The antioxidant compounds isolated from *D. hamiltonii* ameliorated xenobiotic-induced cytotoxicity in EAT cells and rat primary hepatocytes. The mechanism of cytoprotective action appears to involve inhibition of lipid peroxidation, suppression of ROS and maintaining GSH level.
- The aqueous extract of the roots of *D. hamiltonii* showed hepatoprotective potential in rats. The root extract protected against hepatotoxicity induced by CCl_4 and ethanol in rats. Further, the extract boosted the antioxidant status of the liver.

- The root extract also shows neuroprotective potential; it protected the rat brain against xenobiotic (ethanol, HCH, DDVP)-induced neurotoxicity and enhanced the antioxidant status of the brain.
- The bioactive principles of the aqueous extract of *D. hamiltonii* could be responsible for the protective effect by enhancing the antioxidant status in vivo.
- The edible roots of *D. hamiltonii* are a source of novel nutraceuticals.

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Chapter I

INTRODUCTION

Free radicals in Health and Disease

A free radical is defined as any molecular species that contains an unpaired electron in the atomic orbital (Halliwell and Gutteridge, 1999). Radicals are highly reactive that either donate an electron to or extract an electron from other molecules, and therefore, behave as oxidants or reductants. As a result of their high reactivity, most radicals have a very short half life (10^{-6} seconds or less) in biological systems (Halliwell and Gutteridge, 1999). The most important free radicals produced in the body are oxygen derivatives, particularly superoxide and the hydroxyl radical. Examples of free radicals and reactive oxygen species include: superoxide anion radical, hydroxyl radical, nitric oxide, thiyl radical, trichloromethyl radical, hypochlorite radical, hypochlorous acid, and also some potentially dangerous non-radicals such as hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone. Radical production in the body occurs by both endogenous and environmental factors (Fig 1.1.).

Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids. Lipoprotein particles or membranes characteristically undergo the process of lipid peroxidation, giving rise to a variety of products including short chain aldehydes such as malondialdehyde or 4-hydroxynonenal, alkanes and alkenes, conjugated dienes and a variety of hydroxides and hydroperoxides (Esterbauer, 1996). Oxidative damage to proteins and nucleic acids similarly gives rise to a variety of specific damage products as a result of modifications of amino acids or nucleotides (Griffiths et al., 2002). Such oxidative damage might also lead

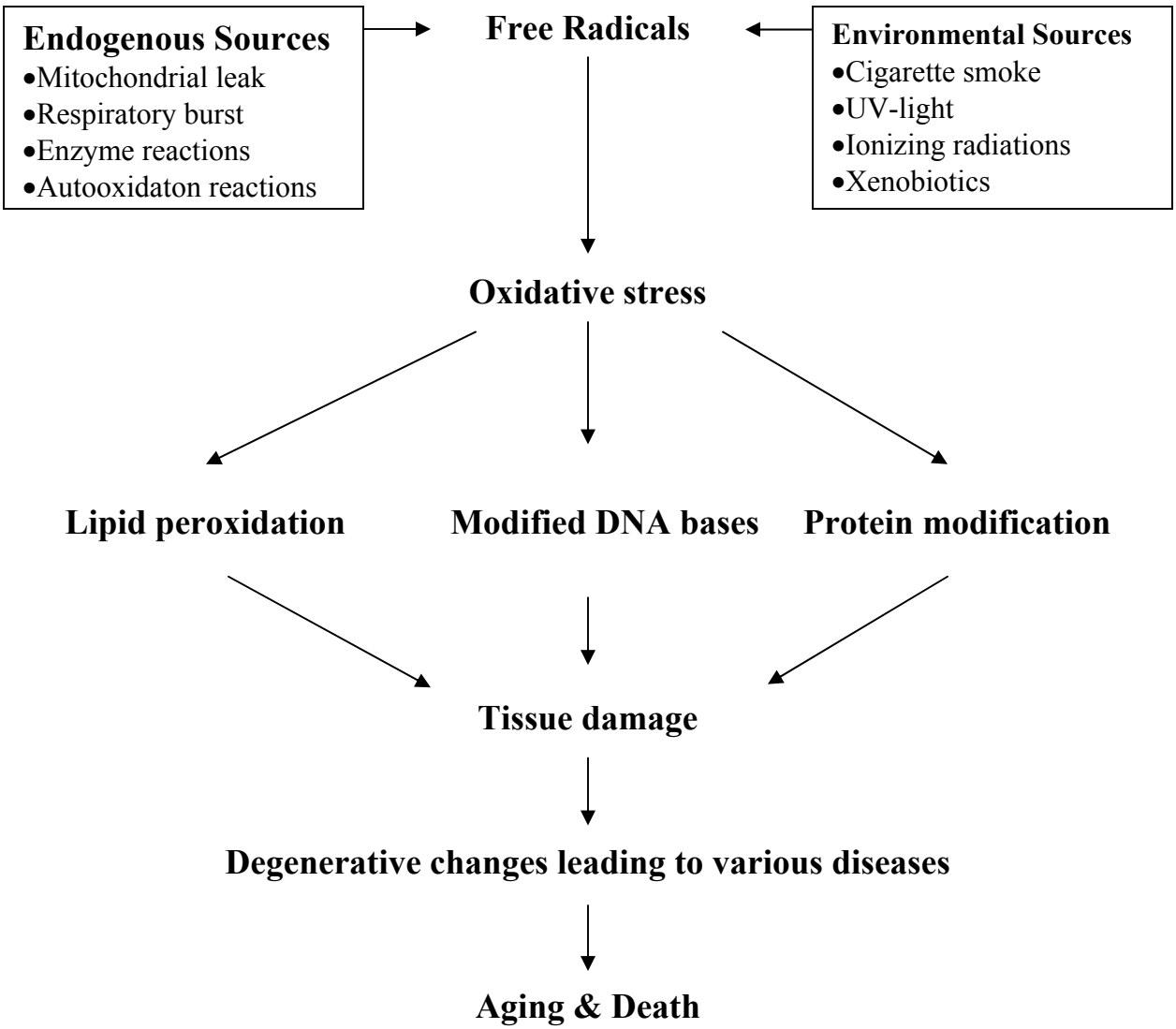


Figure 1.1.: Major sources of free radicals in the body and the consequences of free radical damage.

to cellular dysfunction and contribute to the pathophysiology of a wide variety of diseases.

Oxidative stress has been implicated in the etiology of a host of degenerative diseases including cardiovascular disease, diabetes, cancer, alzheimer's disease, neurodegenerative disorders and in aging (Scalbert et al., 2005b). In addition, they also play a role not only in acute conditions such as trauma, stroke and infection but also in physical exercise and stress (Sahnoun et al., 1998).

Since free radicals are causally involved in the disease state, it is believed that antioxidants should be effective in preventing or delaying their occurrence. Indeed, investigations at the cellular, tissue and whole animal level as well as epidemiological studies, strongly support the concept that nutritional antioxidant status is inversely related to the occurrence of free radical–mediated diseases (Glantzounis et al., 2005; Poeggeler, 2005).

Antioxidants

An antioxidant is defined as: "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell, 1995). The physiological role of antioxidants is to prevent damage to cellular constituents arising as a consequence of chemical reactions involving free radicals.

An ideal antioxidant: An ideal antioxidant should have the following attributes -

- No harmful physiological effects.
- Effective in low concentration. Fat-soluble.
- Carry-through effect.

- Not contribute an objectionable flavor, odor or color to the food.
- No destruction during processing.
- Readily-available.
- Economical

Antioxidant Synergy: Combination of antioxidants is more effective than the sum of the individual effects (Liu, 2004). Combined interaction improves effectiveness in several ways, for example: Vitamin E and C – Ascorbate can reduce Vit E, so in a lipid oxidation system Vit E and C together will be more effective than adding the effects of each alone. In biological samples synergy is also referred to as co -antioxidants. Antioxidant synergy is the key to the overall antioxidant defense system of living systems (Closa and Folch-Puy, 2004).

The antioxidant defense system

Since radicals have the capacity to react in an indiscriminate manner leading to damage to almost any cellular component, an extensive range of antioxidant defenses, have evolved to protect the cell from the free radical induced damage (Nordberg and Arner, 2001). The cellular antioxidants could be divided into three main groups: antioxidant enzymes, non-enzymatic antioxidants, and transition metal binding proteins (Halliwell and Gutteridge, 1999) (Fig 1.2.).

The antioxidant enzymes

Catalase: Catalase was the first antioxidant enzyme to be characterized; it catalyses the two stage conversion of hydrogen peroxide to water and oxygen:

$$\text{Catalase-Fe (III)} + H_2O_2 \longrightarrow A$$

$$A + H_2O_2 \longrightarrow \text{Catalase-Fe (III)} + 2H_2O + O_2$$

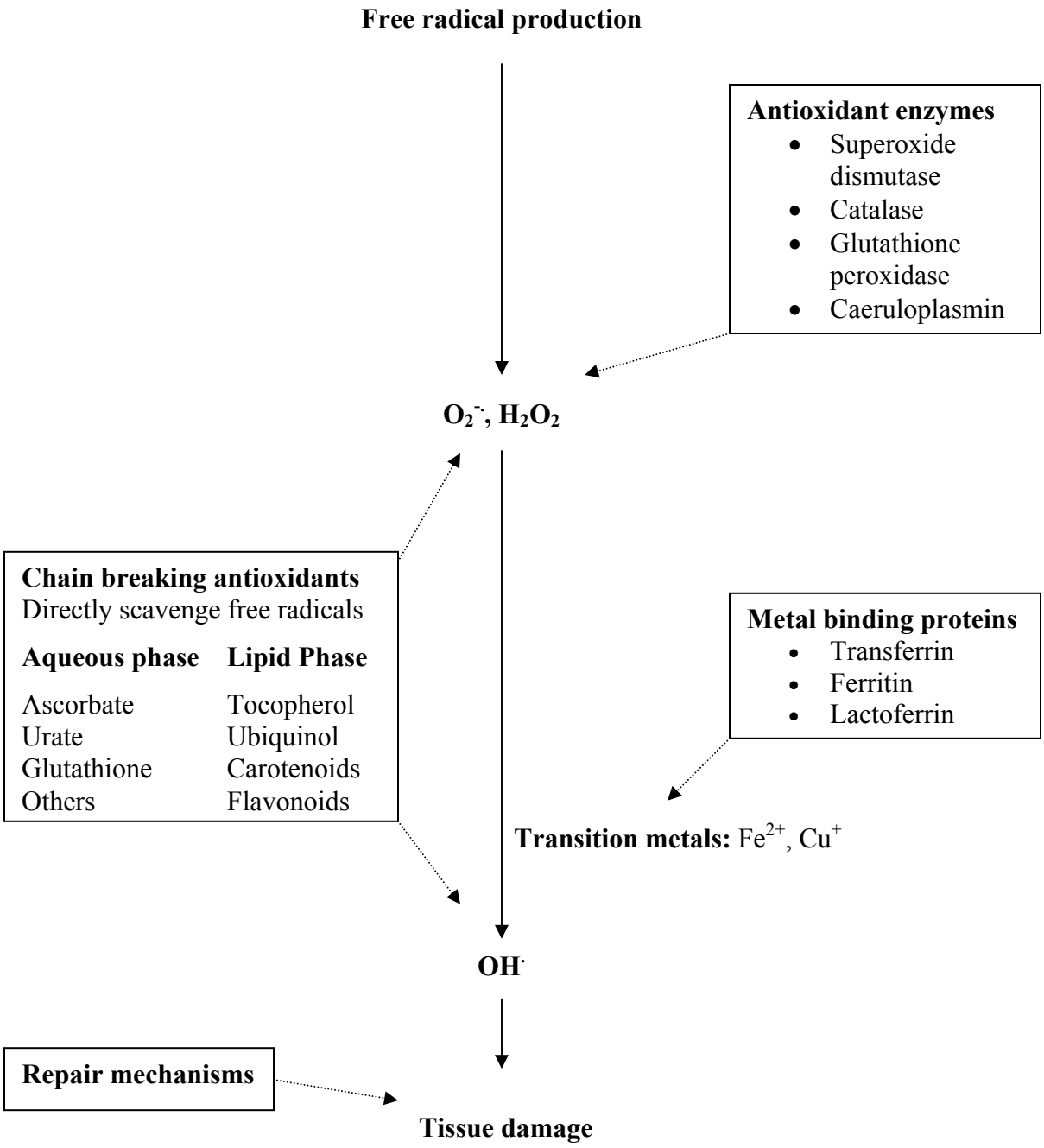


Figure 1.2.: Antioxidant defenses against free radical attack

Catalase consists of four protein subunits, each containing a haem group and a molecule of NADPH (Kirkman et al., 1999). The rate constant for the reactions described above is extremely high (~10^7 M/sec), implying that it is virtually impossible to saturate the enzyme *in vivo*. Catalase is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide.

Glutathione peroxidases and glutathione reductase: Glutathione peroxidases catalyze the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxide (Takahashi et al., 1987):

$$ROOH + 2GSH \longrightarrow GSSG + 2H_2O + ROH$$

Other peroxides, including lipid hydroperoxides, also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. Glutathione peroxidases require selenium at the active site (Nakane et al., 1998). The predominant subcellular distribution is in the cytosol and mitochondria suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in subcellular compartments. The activity of the enzyme is dependent on the constant availability of reduced glutathione (Masella et al., 2005). The ratio of reduced to oxidised glutathione is usually kept very high as a result of the activity of the enzyme glutathione reductase:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

The NADPH required by this enzyme to replenish the supply of reduced glutathione is provided by the pentose phosphate pathway. Glutathione reductase is a flavine nucleotide dependent enzyme and has a similar tissue distribution to glutathione peroxidase (Rana et al., 2002).

Superoxide dismutase: The enzyme superoxide dismutases catalyse the dismutation of superoxide to hydrogen peroxide:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O2$$

The hydrogen peroxide must then be removed by catalase or glutathione peroxidase, as described above. There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution.

1. Copper zinc superoxide dismutase (CuZnSOD): CuZnSOD is found in the cytoplasm and organelles of virtually all mammalian cells (Liou et al., 1993). It has a molecular mass of approximately 32 000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom.
2. Manganese superoxide dismutase (MnSOD): MnSOD is found in the mitochondria of almost all cells and has a molecular mass of 40 000 kDa (Abe and Okazaki, 1987). It consists of four protein subunits, each probably containing a single manganese atom. The amino acid sequence of MnSOD is entirely dissimilar to that of CuZnSOD and it is not inhibited by cyanide, allowing MnSOD activity to be distinguished from that of CuZnSOD in mixtures of the two enzymes.

3. Extracellular superoxide dismutase (EC-SOD): EC-SOD was described by Marklund (1982) and is a secretory copper and zinc containing SOD distinct from the CuZnSOD described above. EC-SOD is synthesised by only a few cell types, including fibroblasts and endothelial cells.

Non-enzymatic antioxidants

Whenever a free radical interacts with another molecule, secondary radicals may be generated that will further react with the available targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralised by an antioxidant (de Zwart et al., 1999). Antioxidants are molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts (Halliwell et al., 1995). Such antioxidants can be conveniently divided into aqueous phase and lipid phase antioxidants.

Lipid phase antioxidants: These antioxidants scavenge radicals in membranes and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E (Singh et al., 2005). It quickly reacts with a peroxyl radical to form a relatively stable tocopheroxyl radical, with the excess charge associated with the extra electron being dispersed across the chromanol ring. The carotenoids are a group of lipid soluble antioxidants based around an isoprenoid carbon skeleton (Dembitsky, 2005). The most important of these is ß-carotene, although at least 20 others may be present in membranes and lipoproteins. They are particularly efficient scavengers of singlet oxygen, but also trap peroxyl radicals at low oxygen pressure with an efficiency at least as great as that of α-tocopherol (Moong-Ngarm et al., 2004). As peroxidising conditions prevail in biological tissues, the carotenoids might play a role in preventing *in vivo* lipid peroxidation (Tapiero et al., 2004). The other important role of certain carotenoids is as precursors of an antioxidant, vitamin A (retinol). Flavonoids are a large group of polyphenolic antioxidants found in many fruits, vegetables and beverages

such as tea and wine (Scalbert et al., 2005a; Carratu and Sanzini, 2005; Dore, 2005). Over 4000 flavonoids have been identified and they are divided into several groups according to their chemical structure, including flavonols (quercetin and kaempherol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein). There is evidence that augmenting the intake of flavonoids might improve biochemical indices of oxidative damage (Havsteen, 2002; Scalbert et al., 2005b). Epidemiological studies suggest an inverse relation between flavonoid intake and incidence of chronic diseases such as coronary heart disease (CHD) (Yao et al., 2004; Woodman and Chan, 2004). Ubiquinol-10, the reduced form of coenzyme Q10, is an effective lipid soluble chain breaking antioxidant (Thomas et al., 1997; Niki, 1997). Coenzyme Q can scavenge lipid peroxyl radicals with higher efficiency than either α-tocopherol or the carotenoids, and can also regenerate membrane bound α-tocopherol from the tocopheryl radical (Thomas et al., 1999). Indeed, whenever plasma or isolated low density lipoprotein (LDL) cholesterol is exposed to radicals generated in the lipid phase, ubiquinol-10 is the first antioxidant to be consumed, suggesting that it might be of particular importance in preventing the propagation of lipid peroxidation (Gordon, 1996).

Aqueous phase antioxidants: These antioxidants scavenge radicals present in the aqueous compartment. Qualitatively the most important antioxidant of this type is vitamin C (ascorbate) (Goldenberg, 2003). Ascorbate has been shown to scavenge superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals and singlet oxygen. Ascorbate undergoes a two electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate during its antioxidant action (Birlouez-Aragon and Tessier, 2003). Uric acid efficiently scavenges radicals,

being converted in the process to allantoin. Urate provides protection against certain oxidizing agents, such as ozone (Becker, 1993). Indeed, it has been suggested that the increase in life span that has occurred during human evolution might be partly explained by the protective action provided by uric acid in human plasma (Benzie, 2000; Glantzounis et al., 2005). Albumin bound bilirubin is also an efficient radical scavenger, and it has been suggested that it might play a particularly crucial role in protecting the neonate from oxidative damage (Quinlan et al., 2005; Oliveira, 2005). The other major chain breaking antioxidants in plasma are the protein bound thiol groups. The sulfhydryl groups present in plasma proteins function as chain breaking antioxidants by donating an electron to neutralise a free radical, with the resultant formation of a protein thiyl radical. Albumin is the predominant plasma protein and a major contributor to plasma sulfhydryl groups, although it also has several other properties. In addition, albumin has the capacity to bind copper ions and therefore inhibits copper dependent lipid peroxidation and hydroxyl radical formation. It is also a powerful scavenger of the phagocytic product hypochlorous acid, and provides the main defense in the plasma against this oxidant (Quinlan et al., 2005).

Reduced glutathione (GSH) is a major source of thiol groups in the intracellular compartment (Wu et al., 2004; Nialsson and Norgren, 2005). GSH could function directly as an antioxidant, scavenging a variety of radical species, as well as participating in the reactions of glutathione peroxidase. Thioredoxin also functions as a key intracellular antioxidant, particularly in redox-induced activation of transcription factors (Haddad and Harb, 2005).

The transition metal binding proteins

Transition metal binding proteins (ferritin, transferrin, lactoferrin, and caeruloplasmin) act as a crucial component of the antioxidant defense system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical (Wilkinson et al., 2003; Burkitt, 2001). The copper binding protein, caeruloplasmin, could also function as an antioxidant enzyme that can catalyse the oxidation of divalent iron (Vassiliev et al., 2005).

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

Fe^{2+} is the form of iron that drives the Fenton reaction and the rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant effect.

Table1.1.: Cellular defense against oxidative damage

1. **Preventive antioxidants**: suppress the formation of free radicals

(a) Quenching of active oxygen species:

Superoxide dismutase	Disproportionation of superoxide $2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$
Vitamin E, vitamin C, urate, etc.	Quenching of free radicals

(b) Non-radical decomposition of hydroperoxides and hydrogen peroxide:

Catalase	Decomposition of hydrogen peroxide: $2H_2O_2 \longrightarrow 2H_2O + O_2$
Glutathione peroxidase (cellular)	Decomposition of hydrogen peroxide and free fatty acid hydroperoxides: $H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$ $LOOH + 2GSH \longrightarrow LOH + H_2O + GSSG$
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phosholipid hydroperoxides: PLOOH + 2GSH → PLOH + H2O + GSSG
Phospholipid hydroperoxide glutathione peroxidase	Decomposition of lipid hydroperoxide.
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxides: $LOOH + AH_2 \longrightarrow LOH + H_2O + A$ $H_2O_2 + AH_2 \longrightarrow 2H_2O + A$
Glutathione-S-transferase	Decomposition of lipid hydroperoxides.

(c) Sequestration of metals by chelation

Transferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of haemoglobin
Haemoplexin	Stabilisation of haem
Caeruloplasmin, albumin	Sequestration of copper

2. **Radical scavenging antioxidants**: scavenge radicals to inhibit chain initiation and break chain propagation.
(a) Hydrophilic: Vitamin C, Uric acid, bilirubin, albumin
(b) Lipophilic: Vitamin E, ubiquinol, carotenoids, flavonoids

3. **Repair and *de novo* enzymes**: repair the damage and reconstitute membrane lipase, protease, DNA repair enzymes, transferase, etc.

4. **Adaptation**: generate appropriate antioxidant enzymes.

Antioxidant Phytochemicals

Phytochemicals are non-nutritive plant-derived chemicals with biological effects. There are over a thousand phytochemicals found in foods and one serving of a fruit or vegetable may have as many as 100 different phytochemicals (Carratu and Sanzini, 2005). Fruits and vegetables that are bright in color--yellow, red, green, blue, and purple--usually have the most phytochemicals (carotenoids) and nutrients. It is well known that plants produce these chemicals to protect itself against oxidative insult but recent research has demonstrated that many phytochemicals could protect humans against diseases (Collins, 2005; Nishino et al., 2005). Phytochemicals exhibit diversified physiological and pharmacological effects, viz., inactivate cancer-causing substances, stimulate the immune system, protect the heart from free radical attack, and help prevent cataracts in the eye lens (Liu, 2004; Campbell et al., 2004). Phytochemicals promote human health by strengthening the immune system and blood vessels, by fighting tumors, and through other effects (Borek, 2004; Kundu and Surh, 2004). Many of the non-nutritive antioxidant substances show potent biological activity that may work towards lowering risk for chronic diseases (Awika and Rooney, 2004; Hannum, 2004). Some of the more common types of phytochemicals include carotenoids, flavonoids, indoles, isoflavones, capsacin and protease inhibitors (Graf et al., 2005).

Living organisms are equipped with defense systems to deal with the ‘harmful’ action of oxygen. Plants are especially susceptible to damage by the ‘active’ oxygen (exposed to radiation, UV light), which is why they have developed numerous antioxidant defense systems in the form of antioxidants (Shetty and Wahlquist, 2004). Besides plants, many of microbial and animal products as well as fermented products, seaweeds, protein

hydrolysates contain powerful antioxidants (Strobel et al., 2004). Foods such as vegetables, fruit, tea and wine contain a wide variety of free radical scavenging molecules, thus are products rich in natural antioxidant compounds (Urquiaga and Leighton, 2000). Among numerous antioxidants, the following plant secondary products are of particular interest (Osawa, 1999): Plant phenolics (phenylproponaoids, coumarines, flavonoids), polyphenolics (tannins, proanthocyanidins), nitrogen containing compounds, alkaloids, nonprotein aminoacids, isothiocyanate, indoles, phytosterols, carotenoids, chlorophyll derivatives, etc. Much of the interest in the bioactivity of plant phenolics has been spurred by the dietary anomaly referred to as the "*French paradox*" the apparent compatibility of a high fat diet with a low incidence of coronary atherosclerosis, which is attributed to the red wine which is a part of their diet. Red wine is rich in antioxidants (Sun et al., 2002). Currently, there is a great deal of interest in the study of natural compounds with free radical scavenging capacity and their role in human health.

SOME WELL-KNOWN ANTIOXIDANT HERBALS

- Alchemilla
- Artichoke
- Bilberry fruit
- Cocoa
- Garlic
- Green tea
- Jojoba
- Lemongrass
- Olive leaf
- Propolis
- Rosemary
- Sour cherry
- Willard water
- All spices
- Astragalus
- Chaparral
- Cumin
- Ginger
- Jiaofulan
- Lemon
- Milk thistle
- Onion
- Red bush tea
- Sage
- Tumeric
- Wine (red grapes)

ACTIVE PRINCIPLES

- Alpha-lipoic acid
- Tocopherols and tocotrienols
- Ascorbic acid
- Glutathione
- Flavonoids
- Carotenoids
- Allylic sulfides
- Guggulsterones
- Selegiline
- N-acetylcysteine

Table 1.2.: An overview of some classes of phytochemicals and the food sources associated with them.

Phytochemical Class	Food Source
Carotenoids	Yellow/orange vegetables and fruits, dark-green leafy vegetables
Dithiolthiones	Cruciferous vegetables – broccoli, cauliflower, Brussels sprouts, etc.
Glucosinolates/indoles	Cruciferous vegetables – broccoli, cauliflower, Brussels sprouts, etc.
Isothiocyanates/ thiocyanates	Cruciferous vegetables – broccoli, cauliflower, Brussels sprouts, etc.
Coumarins	Vegetables and citrus fruits
Flavonoids	Vegetables and citrus fruits
Phenols	Most fruits and vegetables, green tea, wine
Protease inhibitors	Seeds and legumes, particularly soy
Plant sterols	Vegetables
Isoflavones	Soybeans
Saponins	Plants, particularly soybeans
Inositol hexaphosphate	Plants, particularly soybeans and cereals
Allium compounds	Onions, garlic, leeks, chives
Limonene	Citrus fruits

(from: Hasler, 1996)

Action of antioxidants at the cellular level: Cytoprotective potential

Human cells are continuously subjected to physiological and external influences, which can give rise to cytotoxic, genotoxic and oxidative damage (Sastra et al., 2000; Crapo, 2003). Cells have evolved sophisticated mechanisms for counteracting and minimizing damage (Hayes and McLellan, 1999). In recent years there has been increasing understanding that dietary patterns and constituents can modulate these forms of toxicity in cells (Borek, 2004; Surh et al., 2005). A considerable body of epidemiological evidence indicates that diets high in fruits and vegetables are inversely related to risk of chronic, degenerative diseases such as coronary heart disease and certain cancers (Khachik et al., 2002; Zenebe and Pechnova, 2002; Anderson, 2003). Much research effort has focused on the identification of phytochemicals in fruits and vegetables that exert beneficial effects and the elucidation of the mechanisms by which they inhibit cellular injury and degeneration (Park and Surh, 2004; Hensley et al., 2004). Various experimental model systems (e.g. membrane systems, cell culture, animal models, human clinical trials) are used to study the bioactivity of these plant-derived compounds. There are, of course, advantages and limitations of the *in vitro* systems. Cell-culture models have a number of advantages over other *in vitro* cell free systems, including the ethical issues relating to animals or human studies, ability to cryopreserve cell lines, to conduct mechanistic studies at the molecular level, ease of control of the experimental environment and cost. However, cell-culture systems cannot replicate *in vivo* conditions, systemic functions such as the nervous and endocrine systems are missing. Thus, control of cellular metabolism may be amenable for control *in vitro* and the cultured cells but may not fully be representative of the tissue from which they were derived. Keeping the

limitations of the model in view, cell culture is a valuable experimental tool (Robertson and Orrenius, 2000).

There is an increasing body of evidence that non-nutrient dietary components (such as flavonoids) are significant bioactive compounds and exert significant modulatory effects on cytotoxicity, genotoxicity and oxidative reactions in cellular systems (Gosslau and Chen, 2004). Scientific advances from diverse models, over time, help in further identifying cellular mode of action of beneficial antioxidants and their role in human health.

Chemoprevention by antioxidants

a) *Hepatoprotective activity of antioxidants:*

Many of the herbal preparations used in traditional medicine and the ancient Indian medicine (Ayurveda) have used medicinal plants as rejuvenating agents that play the role of "purifying" the body with hepatoprotective action. Several medicinal plants such as *Silybum marianum*, *Picrrorhiza kurroa*, *Andrographis paniculata*, *Phyllanthus niruri* and *Eclipta alba* have been suggested to show hepatoprotective potential in experimental animals (Levy et al., 2004; Dhiman and Chawla, 2005). These plants are used widely in hepatoprotective preparations and extensive studies have been done on them.

Curcuma longa: Turmeric has been shown to protect the liver in experimental animals from a variety of hepatotoxic substances, including carbon tetrachloride, galactosamine, pentobarbitol, 1-chloro-2,4-dinitrobenzene, 4-hydroxy-nonenal and paracetamol. Diarylhepatonoids including curcumin is the active constituent of the plant (Ammon and Wahl, 1991; Luper, 1999).

Taraxacum officinale: Traditionally *Taraxacum officinale* has been used as a remedy for jaundice and other disorders of the liver and gallbladder, and as a remedy for counteracting water retention. Generally, the roots of the plant have the most activity regarding the liver and gallbladder. Oral administration of extracts from the roots of *Taraxacum officinale* has been shown to act as a cholagogue, increasing the flow of bile. Bitter constituents like taraxecerin and taraxcin are active constituents of the medicinal herb (Weisser, 1955; Onal et al., 2005).

Cichorium intybus: *Cichorium intybus* is a popular Ayurvedic remedy for the treatment of liver diseases. It is commonly known as kasni and is part of polyherbal formulations used in the treatment of liver diseases (Huseini et al., 2005). In mice, liver protection was observed at various doses of *Cichorium intybus* but optimum protection was seen with a dose of 75 mg/kg given 30 minutes after CCl_4 intoxication. In preclinical studies an alcoholic extract of the *Cichorium intybus* was found to be effective against chlorpromazine-induced hepatic damage in adult albino rats. A bitter glucoside, cichorin has been reported to be the active constituent of the herb (Gazzani et al., 2000; Ahmed et al., 2003).

Solanum nigrum: In Ayurveda, the drug is known as “kakamachi”. Aromatic water extracted from the drug is widely used for liver disorders. Although clinical documentation is scarce as far as hepatoprotective activity is concerned, but some traditional practitioners have reported favorable results with powdered extract of the plant (Sultana et al., 1995; Raju et al., 2003).

Glychyrrhiza glabra: *Glychyrrhiza glabra*, commonly known as licorice contains triterpene saponin, known as glycyrrhizin, which has potential hepatotprotective activity

(Shibata, 2000). It belongs to a group of compounds known as sulfated polysaccharides. Several studies carried out by Japanese researchers have shown glycyrrhizin to be anti-viral and it has potential for therapeutic use in liver disease (Sitohy et al., 1991). Experimental hepatitis and cirrhosis studies on rats found that it can promote the regeneration of liver cells and at the same time inhibit fibrosis. Glycyrrhizin can alleviate histological disorder due to inflammation and restore the liver structure and function from the damage due to carbon tetrachloride. The effects including: lowering the SGPT, reducing the degeneration and necrosis, and recovering the glycogen and RNA of liver cells. Effects of glycyrrhizin has been studied on free radical generation and lipid peroxidation in primary cultured rat hepatocytes (Nakamura et al., 1985; van Rossum et al., 1998; Ploeger et al., 2001; Nakagiri et al., 2003).

b) ***Neuroprotective potential of antioxidants***

Most of the literature on health promoting effects of phytochemicals and plant extracts is on their anticancer or hepatoprotective effects. There is relatively little published work on the neuroprotective effects of herbal extracts or natural phytochemicals. Phytochemicals, including flavonoids, other polyphenols and organosulfur compounds, have neuroprotective effects, as shown experimentally in cell and animal studies (Parihar and Hemnani, 2003; Heo and Lee, 2005). Observational studies in humans on the beneficial effects of diets rich in vegetables and fruits cannot be related to any specific phytochemical, however, with the exception of studies on *Ginkgo biloba*, clinical trials testing the effects of phytochemicals on cognition are limited (Bastianetto and Quirion, 2002). Indirectly, though, antioxidants reduce stroke risk by improving circulation and

helping prevent atherosclerosis, both factors prevent neural death and dementia (Ahlemeyer and Krieglstein, 2003; Tai et al., 2005; Cooper et al., 2005).

Ginkgo biloba: Ginkgo (*Ginkgo biloba*) has been shown to improve cognition in people with certain forms of dementia (Bidzan et al., 2005). In a double-blind, randomized, placebo-controlled multicenter study, 202 patients with severe dementia, some with alzheimer's disease, received 120 mg of standardized ginkgo extract daily for 52 weeks. Patients taking ginkgo showed improved performance and social functioning compared with those on placebo. The authors suggest the effects were attributable to ginkgo's antioxidant effects (Le Bars et al., 1997; Oken et al., 1998; Scdneider et al., 2005).

Camellia sinensis: Green and black tea (*Camellia sinensis*) contains catechins, which have active oxygen-scavenging effects (Zhu et al., 2004). Results of studies in cell cultures show tea catechins protect brain cells of newborn mice from death by oxygen radicals; furthermore, injecting a particular catechin into mice improved memory impaired by a previous injection of a free radical-producing compound, suggesting tea may be useful for protecting humans from senile disorders such as dementia (Okello et al., 2004). In a small study involving 19 people, investigators found drinking 400 ml of black tea or coffee three times daily increased alertness and information processing within 10 minutes after consumption, compared with those who took a caffeine-free placebo. The effects of tea and coffee were similar in all measures and not due entirely to caffeine (Hindmarch et al., 2000).

Allium sativus: Aged garlic extract (kyolic), an odorless form of organic garlic, is rich in water-soluble organosulfur compounds with antioxidant activity, such as S-allyl cysteine, that are low or absent in fresh garlic. Aged garlic extract is antiatherosclerotic and as such

can potentially help against stroke, dementia and alzheimer's disease (Youdim and Joseph, 2001). Aged garlic extract protects blood vessels by inhibiting the stickiness and aggregation of blood platelets that lead to clots (Chang et al., 2005). Results of a recent clinical study showed aged garlic extract prevent plaque build-up in arteries, an important factor in preventing heart disease and stroke (Beretz and Cazenave, 1991). Studies on humans subjects show it reduces cholesterol, decreases blood pressure and increases blood circulation, factors that may help prevent stroke and the dementia that can follow (Turner et al., 2004). Results of experimental studies show aged garlic extract is also neuroprotective—it reduces homocysteine, prevents beta-amyloid neurotoxicity, protects neurons from apoptosis and improves learning and memory in senility-prone mice (Edwards et al., 2005; Tattelma, 2005). Curcumin, a component of the spice turmeric, is a potent polyphenolic antioxidant with anti-inflammatory effects (Sharma et al., 2005). Studies on an alzheimer's disease mouse model show dietary curcumin reduced oxidative damage and decreased beta-amyloid peptides in the brain by 43 percent to 50 percent. In studies on a neuron cell model, curcumin was more effective than alpha-tocopherol in preventing beta-amyloid toxicity (Park and Kim, 2002).

Vitis vinifera: Resveratrol, a natural polyphenol found in grapes (mostly in the skin) is cardioprotective and may play a role in lowering alzheimer's disease risk (Delmas et al., 2005; Dore, 2005). Moderate intake of resveratrol-rich red wine is a good way to get this antioxidant, as well as a wide range of flavonoids. Studies in cell cultures show resveratrol's neuroprotective effects on cells may be attributable to its antioxidant effect and its ability to prevent neuronal death (Bhat et al., 2001; Sun et al., 2002; Pervaiz, 2003).

Table 1.3.: Common traditional plants, their uses and constituent phytochemicals.

Plant (Family)	Phytochemicals present	Traditional uses
Canariumpaniculatum (Burseraceae)	Phenols, anthocyanins, tannins, terpenes, coumarins	Rheumatism, skin ulcerations
Senecio ambavilla (Asteraceae)	Phenols, flavonoids, tannins	Rheumatism, gout, urinary infections, renal functions
Grangeria borbonica (Chrysobalanaceae)	Phenols, flavonoids, tannins	Stomach pains, asthama
Cnestis glabra (Connaraceae)	Flavonoids, tannins, proanthocyanidins, saponins	Fever
Erythroxylum sideroxyloides (Erythroxyloides)	Phenols, flavonoids, saponins, tannins, triterpenes, alkaloides	Fever, renal stones, throat infections
Phyllanthus casticum (Euphorbiaceae)	Phenol, flavonoids, proanthocyanidins	Fever, diarrhoea, dysentery, jaundice
Aphloia theiformis (Flacourtiaceae)	Phenols, flavonoids, proanthocyanidins, tannins	Dysentery, fever, rheumatism, intestinal infections, jaundice
Foetidia mauritiana (Lecythidaceae)	Alkaloids, proanthocyanidins, saponins, tannins	Laxative, emmenagogue, purgative, diuretic
Leea guinensis (Leeaceae)	Phenols, flavonoids, tannins	Oedemas, antiseptic, colds
Embelia angustifolia (Myrsinaceae)	Alkaloids, phenols, flavonoids, saponins	Liver complaints, dysentery, urinary tract infections
Turraea casimiriana (Meliaceae)	Phenols, flavonoids, proanthocyanidins, tannins	Boils, hypotensive, emmenagogue
Chassalia coriaceae (Rubiaceae)	Anthocyanin, saponins, triterpenes, iridoids, flavonoids	Astringent
Molinae alternifolia (Sapindaceae)	Phenols, flavonoids, flavones, saponins, tannins	Dysentery, throat infections
Mimusops maxima (Sapotaceae)	Phenols, flavonoids, flavones, proanthocyanidins, tannins	Diarrhoea, dysentery, astringent
Premna corymbosa (Verbenaceae)	Alkaloids, saponins, flavonoids, phenols	Cough, influenza

(from: Heinrich, 2000; Govindarajan, 2005)

Table 1.4.: Mechanism of actions of phenolic compounds in various pathophysiological conditions.

Phenolic compounds	Pathology	Mechanism of actions	References
Quercetin, Kaempferol, genistein, resveratrol	Colon cancer	Suppresses COX-2 expression by inhibiting tyrosine kinases important for induction of COX-2 gene expression	Lee et al., 1998
Catechins	Neurodegenerative diseases	Enhance activity of SOD and catalase	Levites et al., 2001
(+)-EGCG	Neurodegenerative conditions	Decreases the expression of proapototic genes (bax, bad, caspase-1 and -6, cyclin dependent kinase inhibitor) thus maintaining the integrity of the mitochondrial membrane	Levites et al., 2003
(-)-EGCG	Cancer, diabetic retinopathy, chronic inflammation	Suppression of angiogenesis by inhibiting growth factor triggered activation of receptors and PKC. Downregulation of VEGF production in tumour cells. Repression of AP-1, NF-κB and STAT-1 transcription factor pathways. Suppression of angiogenesis by inhibiting growth factor triggered activation of receptors and PKC. Downregulation of VEGF production in tumour cells. Repression of AP-1, NF-κB and STAT-1 transcription factor pathways. Inhibits capillary endothelial cell proliferation and blood vessel formation.	Wollin and Jones, 2001; Cao and Cao, 1999; Jung et al., 2001
Proanthocyanidin (GSPE)	Cardiovascular disorders	Inhibitory effects on proapoptotic and cardioregulatory genes. Modulating apoptotic regulatory bcl-X_L, p53 and c-myc genes.	Bagchi et al., 2003
Ferulic acid	Diabetes	Decrease lipid peroxidation and enhances the level of glutathione and antioxidant	Balsubashini et al., 2004

Some important phytochemicals and their health effects

Carotenoids: Found in vegetables, carrots, spinach and tomatoes. The primary antioxidant role of ß-carotene is to "quench" the highly reactive singlet oxygen species of free radicals. Converted into Vitamin A if needed. Carotenoids give color to vegetables and boosts immunity, alpha carotene protects against cancer, beta-carotene boosts immunity, lycopene reduces prostate cancer, lutein decreases cataracts (Russell, 1998; Hughes, 2001; Bendich, 2004; Chew and Park, 2004).

Lipoic Acid: Found in potatoes and spinach. Lipoic acid is a naturally occurring compound that is synthesized by plants and animals. Alpha-lipoic acid contains two sulphur molecules that can be oxidised or reduced. This feature allows alpha-lipoic acid to function as a cofactor for several enzymes as well as a potent antioxidant. It provides powerful protection against heart disease and stroke, cancer, adult-onset of diabetes and memory loss (Ames, 1998; Ziegler et al., 1999; Ruhe and McDonald, 2001; Sauer et al., 2004).

Flavonoids: Found in citrus fruits, apples, onions, red grapes, berries, pine bark. Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating properties (Korkina and Afanasev, 1997). They occur in fruits, vegetables, wines, tea and cocoa. Flavonoids have been reported to improve memory and concentration, boost the effectiveness of vitamin C, prevent blood clots better than aspirin, reduce LDL cholesterol rancidity, lower high blood pressure, bolster immune function, reduce inflammation (Robak and Gryglewski, 1996; Middleton et al., 2000; Horvathova et al., 2001; Crespy and Williamson, 2004). Epidemiological studies suggest an inverse relation between flavonoid intake and incidence of chronic diseases such as

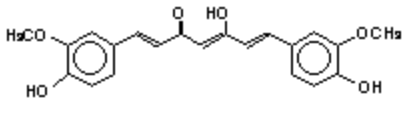
coronary heart disease (CHD) (Yao et al., 2004; Zern and Fernandez, 2005; Rasmussen et al., 2005).

Coenzyme Q-10: The major sources are soybean oil, nuts, wheat germ and some vegetables. Ubiqinones are lipid - soluble quinone derivatives with an isoprenoid tail. The predominant form of ubiqinone in humans is ubiquinone-10. Regenerates vitamin E, used to treat/prevent heart disease, rejuvenates brain cells, used to treat gum disease (Jones et al., 2002; Shults, 2003; Jones et al., 2004).

Resveratrol: Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), a common phytoalexin, found in grape skins, peanuts, and red wine has been purported to have health benefits. Resveratrol acts as an antioxidant, promoter of nitric oxide production, inhibitor of platelet aggregation, and increases high-density lipoprotein cholesterol and thereby serves as a cardioprotective agent (Delmas et al., 2005; Olas and Wachowicz, 2005). It has been demonstrated that resveratrol functions as a cancer chemopreventive agent (Le Corre, 2005; Signorelli and Ghidoni, 2005). It is suggested that resveratrol could act as a signaling molecule within tissues and cells to modulate the expression of genes and proteins. Stimulation of such proteins and enzymes could explain some of the intracellular antioxidative properties of reseveratrol (Dore, 2005; Shay and Banz, 2005). The modulation of genes by resveratrol could suffice as an explanation for cytoprotective actions, as well as its influence on blood flow, cell death, and inflammatory cascades (Pervaiz, 2004). Resveratrol stimulation of the expression of heme oxygenase is one example (Dore, 2005). Increased heme oxygenase activity has led to significant protection against models of *in vitro* and *in vivo* oxidative stress injury. In addition, resveratrol exhibits antiinflammatory, neuroprotective, and antiviral properties (Pervaiz,

2003; Kimura, 2003; Dong, 2003). Studies with resveratrol show protective effects on neuron cell death induced by oxidative agents (Wang et al., 2003; Kiziltepe, 2004).

Some important phytochemicals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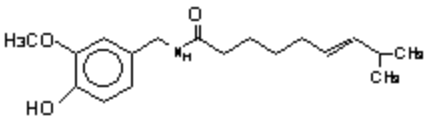
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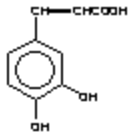
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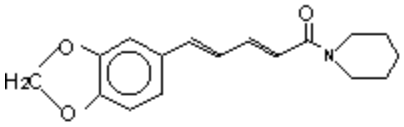
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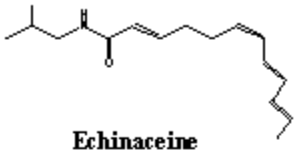
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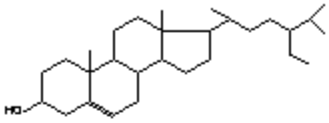
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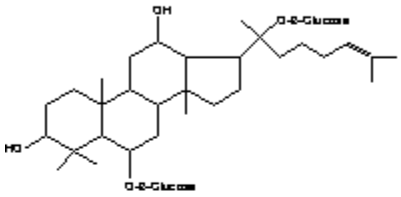
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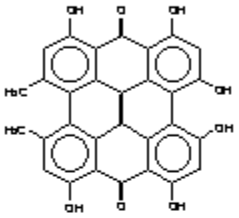
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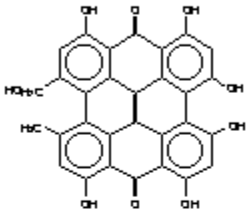
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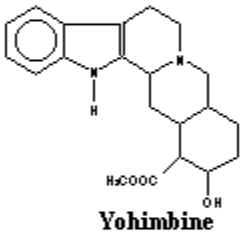
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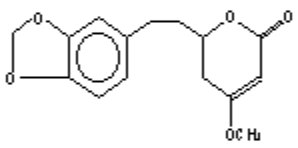
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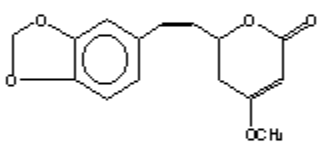
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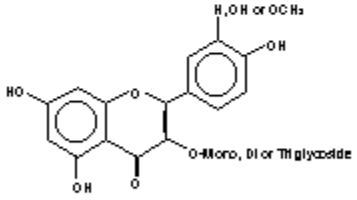
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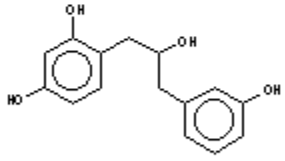
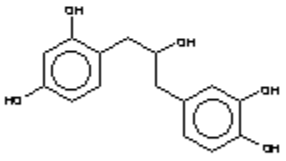

Quracol A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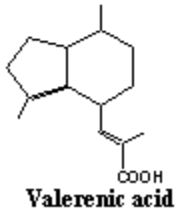
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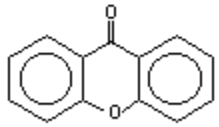
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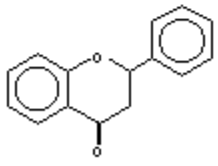
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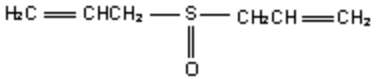
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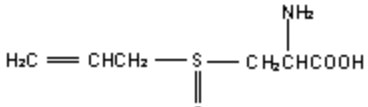

Alliin

Indian Medicinal Plants

Although the concept of nutraceuticals is gaining popularity recently, earliest reference to health promoting potential of plants is traceable to the ancient Indian system of medicine, '*Ayurveda*' (Tripathi et al., 2005). In Ayurveda, it is believed that the food, besides providing nutrition also helps to maintain the healthy state and plays preventive role in the occurrence of diseases (Chopra and Doiphode, 2002; Hankey, 2005). According to the classical texts of Ayurveda, *Charaka Samhita* and *Sushruta Samhita* (1000B.C.), the Ayurvedic Materia Medica includes about 600 medicinal plants along with therapeutics. The classical texts of *Ayurveda* are filled with scattered references of implication of food products in various disease states. The concept of '*Aajasrik Rasayana*' (general rejuvenation) deals with food products that can be consumed daily for improving quality of life by offering protection from external and internal stressors. Commonly used tonics (nutraceuticals) of *Ayurveda* include *Chyavanprash* (for general health and prevention of respiratory disorders); *Brahma Rasayana* (for protection from mental stress); *Phala Ghrita* (for reproductive health); *Arjuna Ksheerpaka* (for cardioprotection); *Shatavari Ghrita* (for general health of women during various physiological states) and *Rasona Ksheerpaka* (for cardioprotection) (Buhler, 2003; Hankey, 2001). Herbs like turmeric, fenugreek, ginger, garlic and holy basil are integral part of Ayurvedic formulations (Gogtay et al., 2002; Gupta et al., 2002; Chainani-Wu, 2003). The formulations incorporate single herb or more than two herbs (poly-herbal formulations) (Khan and Balick, 2001). For the last few decades herbal products are becoming popular (Bhatt, 2001). Medicinal herbs are significant source of synthetic and herbal drugs. Medicinal herb is considered to be a chemical factory as it contains multitude of chemical

compounds like alkaloids, glycosides, saponins, resins, oleoresins, sesquiterpene lactones and oils (essential and fixed). Today there is growing interest in chemical composition of plant based medicines. Several bioactive constituents have been isolated and studied for their pharmacological activity (Tripathi, 2000; Okamoto and Hino, 2000; Dhiman and Chawla, 2005; Kronenberg et al., 2005).

Among the different Indian plants, some of them have been specifically investigated for their well-demonstrated antioxidant activity (Table 1.5.)

Table 1.5.: Some well-known Indian medicinal plants with antioxidant activity.

Plant	Family	Parts used	Antioxidant constituents
Phyllanthus emblica L.	Euphorbiaceae	Dried fruit and fresh fruit, seed, leaves, root bark, flowers	Ascorbic acid, Emblicanin A and B, Punigluconino, Pedunclagin
Curcuma longa L.	Zingiberaceae	Rhizomes	Curcumin, TAP (turmeric antioxidant protein), Demethoxycurcumin, Bis-demethoxycurcumin
Mangifera indica L.	Anacardiaceae	Ripe and unripe fruits, root, bark, leaves, flowers, resin from bark	Mangiferin, Gallic acid, Quercetine
Momordica charantia L.	Cucurbitaceae	Unripe fruits, seeds, root, leaves	Acylglucosil sterols
Santalum album L.	Santalaceae	Bark and volatile oil	Santalol, Curcumone, Nuciferol
Swertia chirata Buch-Ham.	Gentianaceae	Whole plant	Swertinin, Mangiferin
Withania somnifera (L.) Dunal	Solanaceae	Root, leaf, seed	Chlorogenic acid, Steroidal lactones, Withanolides

(from: Scartezzini and Speroni, 2000)

Decalepis hamiltonii (Wight & Arn.) (Family: Asclepiadaceae)

Vernacular names of *D. hamiltonii*
Sanskrit: Sariba, Sveta sariva
Telugu: Maredu kommulu or Barre sugnadhi or Maredugaddalu
Kannada: Magali beru
Malayalam: Nannari
Tamil: Mahali kizhangu, Mavilinga kilangu, Peru nannari

Decalepis hamiltonii (Wight and Arn.) referred to as swallow root (family: Asclepiadaceae) is a monogenric climbing shrub native of the forests of deccan peninsula and western ghats of India. Its tubers are consumed as pickles and juice for its alleged health promoting properties. The roots of *D. hamiltonii* are used as a flavoring principle (Wealth of India, 1990), appetizer, "blood purifier" (Jacob, 1937) and preservative (Phadke et al., 1994). Similarly, the roots of this taxon as described by Nayar et al. (1978) are considered as "Sariva Bheda" in Ayurveda where these find use as an alternative or substitute to roots of *Hemidesmus indicus* in several herbal preparations like *Amrutamalaka taila* (hair tonic), *Drakshadi churna* (general vitalizer), *Shatavari rasayana* (adapatogenic) and *Yeshtimadhu taila* (mild analgesic, rheumatism). The tuberous roots are 3 to 4m or more long, transverse deep into the soil, emit a strong aromatic odor when fresh, gradually diminishing on drying; they are markedly fleshy and cylindrical, 3-6cm across, brownish outside, very pale yellow inside, the outer surface smooth when fresh, soon becomes wrinkled and longitudinally ridged on drying. The smooth transverse surface shows a thin cork and a hard white wood. The roots contain 92% fleshy matter and 8% woody core. Of late, the highly aromatic roots have been subjected to over exploitation by destructive harvesting that has endangered the survival

(a)

(b)

(c)

Plate 1.1. *Decalepis hamiltonii* : (a) plant with fruits, (b) the plant with flowers; inset: roots, and (c) roots with transverse and longitudinal sections.

of this plant. A method for rooting of *D. hamiltonii* for field transfer is reported (Obul Reddy et al., 2001). In earlier reports by George et al. (1999a) it was observed that the aromatic roots of *D. hamiltonii* possess bioinsecticide property on storage pests at lethaland sub-lethal levels. The extracts of these roots have also been shown to be potent antimicrobial agents (George et al., 1999b; Thangandurai et al., 2002).

We have reported for the first time that the roots of *D. hamiltonii* possess strong antioxidant activity (Shereen, 2001; Srivastava et al., 2005). Further, The root extract as well as root powder were not toxic at acute (upto 1g/ kg b.w.) and subchronic (upto 2.5% in basal diet for 90 days) doses to rats as assessed by the mammalian toxicity experiments (Shereen, 2005).

Earlier work has shown that the *D. hamiltonii* roots contain aldelydes, inositols, saponins, amyrins, lupeols (Murti and Sheshadri, 1940; 1941a; b). The volatile flavour compounds such as 2-hydroxy-4methoxybenzaldehyde, vanillin, 2-phenyl ethyl alcohol, benzaldehyde, and others (Nagarajan et al., 2001; Nagrajan and Rao, 2003). Thangandurai et al. (2002) have also reported the essential oil costitiuents of *D. hamiltonii* roots: 2-hydroxy-4-methoxy bezaldehyde, β-pinene, benzyl alcohol, γ-hexalactone, 2-hydroxy benzaldehyde, 4-o-methylresorcylaldehyde, α-atlantone, γ-terpinene, 2-phenylethanol, 4-methoxy benzaldehyde, geraniol etc. Recently, Harish et al. (2005) from our laboratory have reported the antioxidant compounds from the methanolic extract of *D. hamiltonii* roots: 2-hydroxy, 4-methoxy benzaldehyde, *p*-anisaldehyde, vanillin, borneol, salicylaldehyde, *bis*-2,3,4,6-galloyl α/βD-glucopyranoside (decalepin) (Harish et al., 2005).

OBJECTIVES OF THE PRESENT STUDY

Several of the plants used in ancient folk and traditional medicine are often not subjected to scientific scrutiny. The experimental proof for the claimed benefit or curative property is lacking. It is imperative that the beneficial or medicinal effects be subjected to scientific testing and the active principles isolated. *Decalepis hamiltonii* is one such plant whose beneficial effects have not been subjected to scientific investigations. CFTRI has undertaken study on several aspects of the health promoting potential and safety of the roots of *D. hamiltonii*. The present study is aimed at elucidation of the antioxidant activity, often associated with the health effects, and isolation and characterization of the active principles from the aqueous extract of the roots of *D. hamiltonii.* Further, evaluate their antioxidant properties, in *in vitro* and cell culture systems and assess the hepatoprotective & neuroprotective potential of the aqueous extract of *D. hamiltonii* roots *in vivo*. It is hoped that, the results obtained from this work will give a sound scientific basis for the health promoting potential of *D. hamiltonii*.

Chapter II

ANTIOXIDANT COMPOUNDS: ISOLATION, CHARACTERIZATION AND *IN VITRO* ANTIOXIDANT ACTIVITY

INTRODUCTION

The reactive oxygen species generated *in vivo* by various mechanisms attack biological molecules including DNA, proteins and lipids (Vinson and Howard, 1996; Halliwell and Gutteridge, 1999; Sevanian and Ursini, 2000; Spencer et al., 2000). Oxidative stress is implicated in the etiology of several diseases (Halliwell and Gutteridge, 1999). Antioxidants prevent the damage to macromolecules by interfering with the free radicals. Currently there is a great deal of interest in newer bioactive molecules from nature with health promoting potential. Natural products containing antioxidants from plants often called "nutraceuticals" are believed to modulate oxidative stress and prevent or delay degenerative disorders (Thatte et al., 2000). Several natural compounds that possess antioxidant activity are reported from plant sources and are commercially promoted as nutraceuticals (Schuler, 1990). Examples of common plant phenolic antioxidants include cinnamic acid derivatives, coumarins, flavonoids, polyfunctional organic acids and tocopherols (Pratt and Hudson, 1990). Phenolic antioxidants have also been found to function as free radical terminators or metal chelators (Shahidi et al., 1992). Many of the natural antioxidants have been shown to possess antimutagenic and anticancer properties, and inhibit LDL oxidation.

Decalepis hamiltonii (family: Asclepediaceae) grows wild in the forests of peninsular India as a climbing shrub. Its tubers are consumed as pickles and juice for its alleged health promoting properties. The roots are used in folk medicine and as a substitute for *Hemidesmus indicus* in ayurvedic preparations of ancient Indian medicine (Nayar et al., 1978). We have shown that the roots of *D. hamiltonii* possess potent antioxidant properties which may be associated with their alleged health benefits

(Srivastava et al., 2005). Earlier work has shown that the roots contain aldelydes, inositols, saponins, amyrins, lupeols (Murti and Sheshadri, 1940; 1941a; b) and volatile flavour compounds such as 2-hydroxy-4methoxybenzaldehyde, vanillin, 2-phenyl ethyl alcohol, benzaldehyde and others (Nagarajan et al., 2001; Nagarajan and Rao, 2003). We have shown that the extract of the roots of *D. hamiltonii* possess strong antioxidant activity that contributes to the health promoting potential of *D. hamiltonii* (Srivastava et al., 2005).

In this chapter, we describe the isolation and characterization of antioxidant constituents from the aqueous extract of *D. hamiltonii* roots and evaluation of their antioxidative properties.

MATERIALS AND METHODS

Chemicals: Human low density lipoprotein (LDL), butylated hydroxylanisole (BHA), nitro blue tetrazolium (NBT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulphate (PMS), thiobarbituric acid (TBA), bovine serum albumin (BSA), quercetin, tetraethoxy propane and ethylenediamine tetra-acetic acid (EDTA) were purchased from Sigma Chemicals Co. (St.Louis, MO). Nicotinamide adenine dinucleotide-reduced (NADH), trichloroacetic acid (TCA), deoxyribose, ascorbic acid and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All other reagents were of analytical grade.

Preparation of the root powder and extraction: Fresh tuberous roots of *D. hamiltonii* (10 kg) were procured from the local suppliers. The roots were washed with water followed by crushing with a roller to separate the inner woody core from the outer fleshy layer. The fleshy portion was collected, dried at 40^{o}C in a hot air oven and then fine powdered. The powder (1.9 kg) was used for extraction.

Sequential extraction of the root powder was done using different solvents with increasing polarity i.e. hexane, chloroform, ethyl acetate, acetone, methanol and water. 50g of the root powder was extracted in 0.5 liter of the solvent in a conical flask on a shaker for 24 h at room temperature. The extract was filtered with Whatman paper No.1 and dried by flash evaporation/lyophilization.

The aqueous extract was prepared by homogenizing the root powder (200g) in warm water (50^{o} C) and allowed to stand for 24 h, filtered with Whatman paper No. 1 and the filtrate was lyophilized and weighed (34.75g).

Isolation of the antioxidant compounds: The aqueous extract (34.75g) was re-extracted with methanol and the supernatant showing antioxidant activity was concentrated under reduced pressure, and subjected to further fractionation by column chromatography using a glass column (length: 43 cm, diameter: 3 cm) and silica gel (60-120 mesh) and eluted with chloroform followed by a stepwise gradient of chloroform, ethyl acetate and methanol. 18 fractions of 300ml each were collected, concentrated in a flash evaporator, and assayed for antioxidant activity by ROS and DPPH radical scavenging assays. Based on the activity and TLC profile the active fractions were pooled into two major fractions I and II.

Fraction I was loaded on to a silica gel column (length: 32 cm, diameter: 2 cm) and eluted with a stepwise gradient of chloroform and ethyl acetate. The active subfractions collected were further purified by preparative TLC and LH-20 column chromatography, purity confirmed by RP-HPLC. This yielded two pure compounds, designated as DHA I and II.

Fraction II was concentrated under reduced pressure and loaded on to a silica gel column (length: 32 cm, diameter: 2 cm) and eluted with a stepwise gradient of ethyl acetate and methanol. Twenty fractions of 50ml each were collected, concentrated and assayed for antioxidant activity (ROS/DPPH radical scavenging). Purity of the active fractions was checked by TLC. As none of the fractions were pure, the active fractions were concentrated and subjected to a further round of purification by silica gel column chromatography as described above. Three active fractions (A, B and C) were further subjected to preparative TLC, A (solvent system- chloroform, 80: methanol, 19: acetic acid, 1), B (solvent system- chloroform, 70: methanol, 29: acetic acid, 1), and C (solvent

system- chloroform, 60: methanol, 39: acetic acid, 1). Fraction A showed four spots of which one was active and pure, confirmed by RP-HPLC (designated as DHA III). Fraction B showed five spots of which two spots were active and pure, confirmed by RP-HPLC (designated as DHA IV and DHA V). Fraction C showed three spots of which one was active and but not pure, which was again loaded on a LH-20 column (length: 20 cm, diameter: 1.5 cm) and eluted with methanol. A pure active compound was isolated, as confirmed by RP-HPLC (designated as DHA VI). Protocol is shown in Scheme 1.

***High Performance Liquid Chromatography (HPLC)*:** Reverse phase HPLC was done using a Shimadzu-LC-8A system equipped with a rheodyne 7725i injection valve fitted with a 20µl sample port and a C_{18} column. The purified fractions were eluted with an isocratic solvent mixture comprising 0.1% TFA in water:methanol (70:30) with a flow rate of 1ml/min and monitored with UV detector at 216nm.

UV and Infrared Spectrometry: Sample dissolved in methanol (0.1mg/ml) was used for recording the UV-spectrum at 200-800nm in a Shimadzu UV-Vis spectrophotometer. IR spectra of the compounds dissolved in DMSO (10mg/ml) were recorded on a Perkin-Elmer FT-IR spectrometer (spectrum 2000) at 400-4000cm^{-1}.

Gas Chromatography-Mass Spectrometry (GC-MS):

GC- MS analysis of the isolated compounds was carried out in an Agilent 6890 GC equipped with a 5973N mass selective detector, Hp- 5 Ms capillary column (length 30m, internal diameter 0.25mm, film thickness (0.25µm). Helium was the carrier gas at 1.0ml/min constant flow rate; oven temperature was maintained at 75^0C for 5min and increased to 300^0C at the rate of 10^0C/min and held for 5min at 300^0C; interface temperature: 150^0C; ion source temperature: 230^0C and quadrupole temperature: 150^0C.

Liquid Chromatography-Mass Spectrometry (LC-MS):

The LC system consisted of a Hitachi L-6000 pump (Hitachi, Tokyo, Japan) for the mobile phase, a Rheodyne Model 7125 injector with a 25ml loop, and a 4.6 i.d. 3250mm Devosil C30 UG-5 column (Nomura Chemical, Seto, Japan). LC was performed using an aqueous solution containing 1% v/v acetonitrile, 20mM ammonium heptafluorobutyrate, and 10mM ammonium formate (pH 4.0) as the mobile phase at a flow rate of 0.4ml/min at 15^{o}C. The column was connected to the ion interface of the mass spectrometer through a fused-silica capillary without splitting. All LC-MS experiments were recorded on a TSQ 700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an ESI source with an ICIS II data system in the positive ion mode. ESI was effected by a spray voltage of 14.8 KV and the heated capillary temperature was maintained at 250^{o}C. Nitrogen served as the sheath gas at an operating pressure of 60 psi and as the auxiliary gas at a flow rate of 3L/min. The dwell time was set at 500 ms per Da.

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR):

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz ^{13}C). Proton and carbon 90^{0} pulse widths were 12.25 and 10.5 μs respectively. About 40mg of the sample dissolved in DMSO-d_6 and D_2O was used for recording the spectra at 25^{0}C. Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard. Two dimensional heteronulear multiple quantum coherence transfer spectra (2-D-HMQCT) were recorded in magnitude mode with sinusoidal shaped Z-gradients of strength 25.7, 15.42 and 20.56 G/cm with a gradient recovery delay of 100 μs to defocus unwanted coherences. The t_1 was incremented in 256 steps. Size of the computer memory used to accumulate the 2-D data

was 4K. The spectra were processed using unshifted and π/4 shifted bell window function in F_1 and F_2 dimensions respectively.

Quantification of the antioxidant compounds in the crude extract: The antioxidant compounds isolated were quantified in the crude extract by RP-HPLC. 20µl (1mg) crude extract was injected for RP-HPLC and the corresponding peaks (retention time) of the compounds were taken for calculating the quantity/g crude extract. Standard curves were made with the pure compounds for reference.

Antioxidant activity (*in vitro*):

The relative antioxidant activity of the isolated compounds was assayed and compared with that of the known antioxidants, quercetin and BHA (dissolved in DMSO). Antioxidant activity was assayed by the following methods:

DPPH radical scavenging assay: DPPH radical scavenging activity was done by the method of Shon et al., (1998). Briefly, 1 ml of DPPH solution (0.1 mM, in 95% ethanol) was mixed with different concentrations of the isolated compounds, shaken and incubated for 20 min at room temperature, and the absorbance was read at 517 nm against a blank. The radical scavenging activity was measured as the decrease in the absorbance of DPPH and calculated using the following equation:

Scavenging effect (%) = $[1- A_{\text{Sample (517nm)}} / A_{\text{Control (517nm)}}] \times 100$

Superoxide radical scavenging assay: The superoxide radical scavenging ability of the isolated compounds was measured by the method of Nishikimi et al. (1972). The reaction mixture containing varying concentrations of the compounds, PMS (0.1mM), NADH (1mM) and NBT (1mM) in phosphate buffer (0.1 M, pH 7.4), was incubated at room temperature for 5 min and the color was read at 560 nm against a blank. The scavenging

effect was calculated using the equation described as in the case of DPPH radical scavenging assay.

Hydroxyl radical scavenging assay: The reaction mixture containing the isolated compound was incubated with deoxyribose (10 mM), H_2O_2 (10 mM), $FeCl_3$ (5mM), EDTA (1mM) and ascorbic acid (5mM) in potassium phosphate buffer (50 mM, pH 7.4) for 60 min at 37 °C (Halliwell and Cross, 1987). The reaction was terminated by adding TCA (5% w/v) and the reaction product was measured by the reaction with TBA (0.2 % w/v) in boiling water bath for 15 min. The absorbance was measured at 535 nm against the reagent blank and inhibition of the oxidation of deoxyribose was calculated against the control.

Nitric oxide radical scavenging assay: Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside, in aqueous solution at physiological pH, spontaneously generates nitric oxide (Marcoci et al., 1994), which in turn reacts with oxygen to produce nitrite ions that can be estimated by the Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with the isolated compound and incubated at 25^{o}C for 150 min. The samples from the above were reacted with Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546nm and referred to the absorbance of standard solutions of potassium nitrite, treated in the same way with Griess reagent. The radical

scavenging activity was measured using the equation described for DPPH radical scavenging assay.

Microsomal lipid peroxidation: Liver excised from adult male Wistar rats was homogenized (20% w/v) in 0.02M, tris buffer (pH 7.4). Microsomes were isolated by the calcium aggregation method (Kamath and Rubin, 1972). 100µl (0.5mg protein) of the liver microsomal suspension was mixed with $FeSO_4$ (1mM) and ascorbic acid (1mM) with or without the isolated compounds in a total volume of 1ml of 0.1M phosphate buffer (pH 7.4) followed by incubation at 37 °C for 60 min. To the reaction mixture was added 1ml of each TCA (10%) and TBA (0.67%) and boiled in a water bath for 15 min. The absorbance of the supernatant was read at 535nm and TBARS (thiobarbituric acid reactive substances) value was calculated using tetraethoxy propane as the standard (Buege and Aust, 1978). TBARS value is taken as a measure of lipid peroxide generation.

Metal ion chelating assay: The Fe^{2+}-chelating ability was assayed by measuring the formation of ferrous iron-ferrozine complex. (Decker and Welch, 1990) The reaction mixture containing $FeCl_2$ (2 mM) and ferrozine (5 mM) and the compound/extract was adjusted to a total volume of 0.8 ml with methanol, shaken well and incubated for 10 min at room temperature. The absorbance of the resultant color was read at 562 nm against a blank. EDTA was used as the positive control. The metal chelating ability of the purified compounds was calculated using the equation as described above.

Reducing power: The reducing power of the extract was quantified by the method described earlier by Shon et al., (2003) with modifications. Briefly, 1 ml of reaction mixture, containing the compounds/extract in phosphate buffer (0.2 M, pH 6.6), was

incubated with potassium ferricyanide (1% w/v) at 50 °C for 20 min. The reaction was terminated by adding TCA solution (10% w/v) and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1% w/v) solution and the resulting color was read at 700 nm.

Protein carbonyls: Rat liver homogenate (10% w/v) was prepared in 20mM tris-HCl buffer (pH 7.4) containing 0.14 NaCl, centrifuged at 10,000g for 10 min at 4^{o}C. 1.0ml of the supernatant was incubated with CCl_4 along with the extract/compounds for 1h and was precipitated with an equal volume of 20% TCA and centrifuged. The pellet was resuspended in 1.0ml of DNPH (10mM in 2M HCl) and allowed to stand at room temperature for 60 min with occasional vortexing. 0.5ml of 20% TCA was added to the reaction mixture and centrifuged, the pellet obtained was washed 3 times with acetone and 1.0ml of 2% of SDS (in 20mM tris-HCl, 0.1M NaCl , pH 7.4) was added to solublise the pellet. The absorbance of the sample was read at 360nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} (Levine et al., 1990).

Inhibition of Human Low Density Lipoprotein (LDL) oxidation: Human LDL was diluted in phosphate buffered saline (PBS) to 200μg of protein/ml and dialyzed overnight against PBS at 4^{o}C to remove the EDTA. LDL (100 μg protein/ml in 10 mM PBS) was subjected to oxidation *in vitro* in 10mM PBS with 10μM $CuSO_4$ in the presence or absence of the isolated compounds, followed by the addition of 1mM EDTA to stop the reaction. After incubation, aliquots of the reaction mixture were used for measuring TBARS formation and relative electrophoretic mobility (REM). TBARS was measured by adding to 0.5ml aliquots, 1ml each of 2.5% TCA and 1% TBA, vortexed and kept in a boiling water bath for 30 min. After cooling to room temperature and centrifugation, the

fluorescence of the product formed was measured in a spectrofluorimeter at 515nm excitation and 553nm emission wavelengths (Schuh et al., 1978). The electrophoretic mobility of the native or oxidized LDL was detected by agarose gel electrophoresis. The samples were eletrophoresed in 0.7% agarose gel at 85V in running buffer (40mM tris, 40mM glacial acetic acid and 1mM EDTA) for 2h. After electrophoresis, the lipoprotein bands were stained with Coomassie Brilliant Blue. REM, defined as the ratio of the distances migrated from the origin by oxidized LDL versus native LDL, was measured using a centimeter scale (Jeong et al., 2004).

Protein content in the microsomes was estimated by the method of Lowry et al., (1951) using BSA as the standard.

Statistical analysis: Data were expressed as mean ± S.E. of three separate experiments done in duplicate and the significance was determined by the analysis of variance ($p<0.05$) using the computer programme, Excel and Statistica software (1999).

RESULTS

Antioxidant activity of the extracts

Antioxidant activity of the sequential extracts is presented in Table 1. Among the extracts, maximum antioxidant activity was shown by the aqueous extracts and therefore, was chosen for further study. The results indicate the choice of the solvent for obtaining the extract with high antioxidant activity.

Identification of the compounds

Six compounds showing antioxidant activity were isolated and characterized by spectroscopic analysis. The compounds were designated as DHA I-VI.

DHA I: 4-hydroxy isophthalic acid; DHA II: ellagic acid; DHA III: 14-aminotetradecanoic acid; DHA IV: 4-(1-hydroxy-1-methylethyl)-1-methyl-1, 2-cyclohexane diol; DHA V: 2-hydroxymethyl-3-methoxybenzaldehyde**;** DHA VI: 2,4,8 trihydroxybicyclo [3.2.1]octan-3-one.

Quantitative determination of the purified compounds in the crude extract

The isolated compounds were quantified in the crude extract of the roots by RP-HPLC. Among the compounds isolated DHA I was present in the highest concentration and DHA IV was the lowest in the extract (Table 2.2.).

Antioxidant properties of the isolated compounds

DPPH radical scavenging activity: DPPH radical scavenging property of the compounds from *D. hamiltonii* is shown in Table 2.3.. All the isolated compounds showed high radical scavenging activity. Among the compounds, DHA II was the most potent radical scavenger.

Superoxide radical scavenging activity: Table 2.3. shows the inhibition of superoxide radical generation by the isolated compounds. The IC_{50} values for the isolated compounds ranged from 1.5 – 508.24 nmole/ml. Among the compounds, DHA II showed highest scavenging activity and DHA III the lowest.

Hydroxyl radical scavenging activity: The hydroxyl radical scavenging potential of the isolated compounds is presented in Table 2.3.. The order of potency based on the IC_{50} values were DHA I > DHA VI > DHA V > DHA III > DHA IV > DHA II. Since water insoluble compounds cannot be tested by this method, BHA and quercetin activities were not checked for comparison.

Nitric oxide radical scavenging activity: Nitric oxide generated from sodium nitroprusside at physiological pH was inhibited by the isolated compounds with IC_{50} in the range of 159.56 – 659.45 nmole/ml (Table 2.3.). DHA III showed highest and DHA II the lowest scavenging activity.

Inhibition of lipid peroxidation: The LPO inhibiting potential of the isolated compounds was tested using $FeCl_2$-ascorbate induced peroxidation of the liver microsomes (Table 2.3.). IC_{50} values for the inhibition of LPO were in the following order: DHA II > DHA I > DHA V > DHA III > DHA VI > DHA IV.

Metal ion chelating activity: The isolated compounds showed metal chelating activity which was concentration dependent. The order of metal chelating activity was, DHA I > DHA IV > DHA VI > DHA III > DHA V > DHA II (Table 2.4.).

Reducing power: The reducing power of *D. hamiltonii* extract/compounds was concentration-dependent (Table 2.4.). Among the isolated compounds DHA II showed highest reducing power.

Inhibition of Protein carbonylation: All the compounds/extract inhibited protein carbonylation, among them DHA I showed highest and DHA IV lowest inhibition (Table 2.4.).

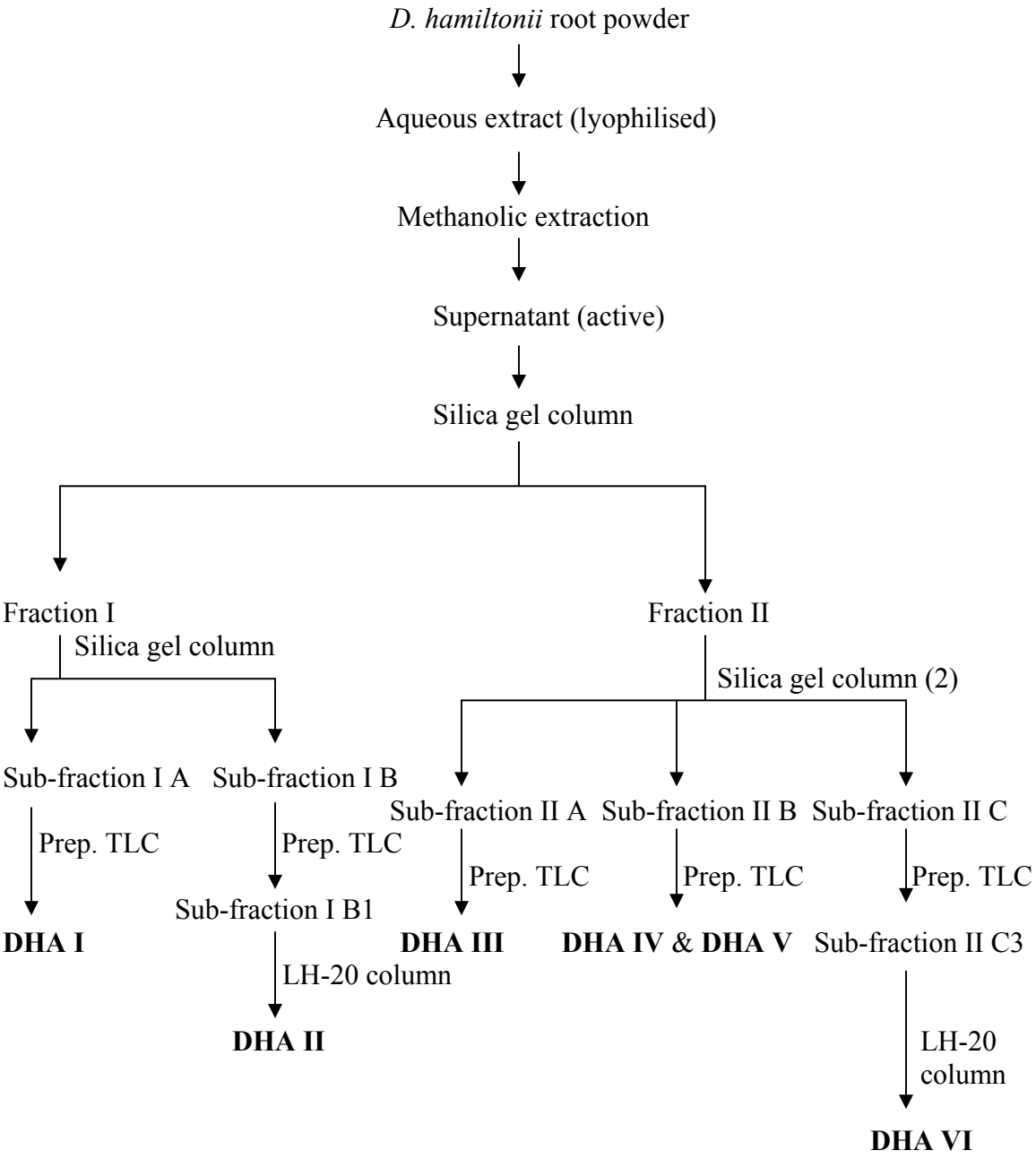
Inhibition of Human Low Density Lipoprotein (LDL) oxidation: All the isolated compounds from *D. hamiltonii* showed protection against copper-induced LDL oxidation *in vitro* as measured by REM and TBARS formation. The protective effect of the compounds at equimolar concentration was in the following order: DHA II > DHA I > DHA VI > DHA V > DHA III > DHA IV (Table 2.5.).

Overall, the isolated compounds from *D. hamiltonii* showed antioxidant activity in all the assays and their potency was comparable to that of the well-known antioxidants, quercetin and BHA.

Table 2.1.: Relative antioxidant activity* of the sequential extracts of *D. hamiltonii*.

Solvent	Extract yield (g)	DPPH radical scavenging (%)	Superoxide radical scavenging (%)	LPO (% inhibition)
Hexane	3.78	3.6 ± 0.4	0	43.3 ± 2.8
Chloroform	4.76	36.06 ± 2.6	0	64.7 ± 3.6
Ethyl acetate	1.0	47.51 ± 3.1	13.7 ± 2.1	71.4 ± 4.2
Acetone	1.19	55.47 ± 3.6	27.2 ± 3.4	76.5 ± 5.1
Methanol	12.95	69.77 ± 4.2	49.9 ± 4.6	68.8 ± 3.9
Water	8.36	73.54 ± 4.7	86.2 ± 4.8	60 ± 3.3

* Antioxidant activity was assayed at a concentration of 1mg/ml for all the extracts.

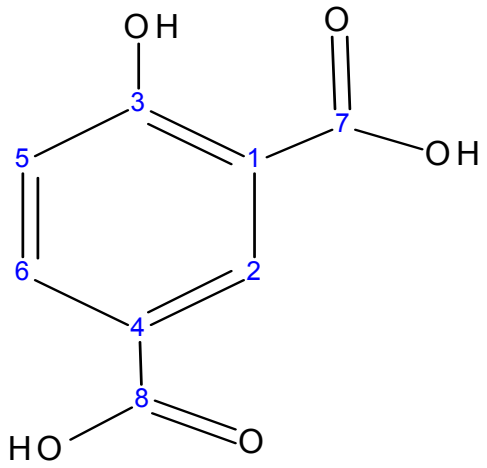


Scheme 2.1.: Purification scheme for the isolation of the antioxidant compounds from the aqueous extract of the roots of *D. hamiltonii.*

MOLECULAR CHARACTERIZATION OF DHA I

Spectral Characteristics of DHA I

Compound (Mass)	UV λmax (nm)	IR (Stretching)	^{1}H-NMR	^{13}C-NMR
4-hydroxy isophthalic acid (182.13)	221,253.5 & 291	3424 cm^{-1} (OH) 1645 cm^{-1} (C=O) 2253 cm^{-1} (CH) 1564 cm^{-1} (C=C)	δ 7.35 (s, 1H, H-2), 7.25 (d, 1H, J=7.70, H-6), 6.72 (d, 1H, J=7.95, H-5).	δ 171.58 (C-7), 166.83 (C-3), 164.88 (C-8), 135.91 (C-2), 131.72 (C-6), 125.66 (C-4), 119.85 (C-5), 111.19 (C-1).

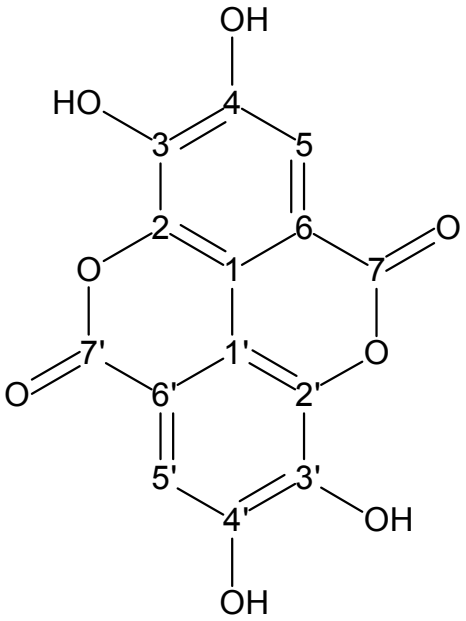


Structure of DHA I: 4-hydroxy isophthalic acid

MOLECULAR CHARACTERIZATION OF DHA II

Spectral Characteristics of DHA II

Compound (Mass)	**UV λmax (nm)**	**IR (Stretching)**	**^{1}H-NMR**	**^{13}C-NMR**
Ellagic acid (364.0)	214 & 273	1700 cm^{-1}(C=O) 3426, 3762 cm^{-1}(OH)	δ 12.20, 9.15, 8.80 (s, 4 X OH), 6.91 (s, 1H, CH-5, 5').	δ 170.1(C-7,7'), 144.4(C-2,2',4,4'), 138.0 (C-3,3'), 120.8(C-1,1'), 110.0 (C-5,6,5',6').

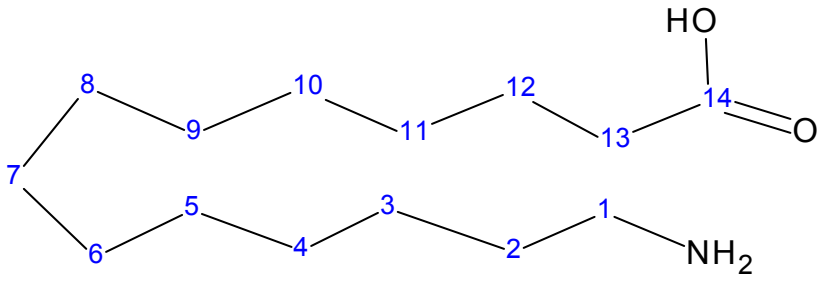


Structure of DHA II: Ellagic acid

MOLECULAR CHARACTERIZATION OF DHA III

Spectral Characteristics of DHA III

Compound (Mass)	UV λmax (nm)	IR (Stretching)	^{1}H-NMR	^{13}C-NMR
14-aminotetradecanoic acid (243.38)	212	3427 cm^{-1} (NH) 1658 cm^{-1} (C=O) 2995 cm^{-1} (CH)	δ 3.70 (m, 3H, NH_2, COOH), 2.50 (t, 2H, J=6.08, CH_2-14), 2.05 (t, 2H, J=6.6, CH_2-2), 1.51 (m, 2H, CH_2-3), 1.31 (m, 2H, CH_2-13), 1.06 -1.24 (m, 18H, CH_2-4, 5, 6, 7, 8, 9,10, 11, 12).	δ 180.66 (C-14), 42.60 (C-1), 34.55 (C-2), 34.22 (C-13), 29.99 (C-4), 29.80 (C-8, 9), 29.63 (C-5), 29.52 (C-10), 29.48 (C-6), 29.32 (C-7), 29.17 (C-11), 27.40 (C-3), 24.76 (C-12).

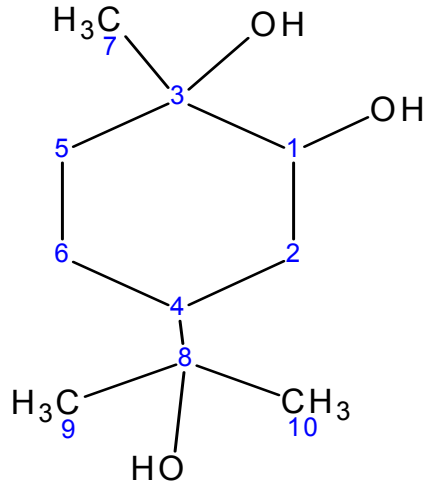


Structure of DHA III: 14- aminotetradecanoic acid

MOLECULAR CHARACTERIZATION OF DHA IV

Spectral Characteristics of DHA IV

Compound (Mass)	Optical rotation $[\alpha]^{D}_{25}$	UV λmax (nm)	IR (Stretching)	^{1}H-NMR	^{13}C-NMR
4-(1-hydroxy-1-methylethyl)-1-methyl-1, 2-cyclohexane diol (188.26)	-1.4	205.5	3624 cm^{-1} (OH) 2870 cm^{-1} (CH)	δ 4.61, 3.44, 3.32 (s, 3 X OH), 3.45 (m, 1H, CH-2), 1.60 (m, 2H, CH_2-6), 1.59 (m, 2H, CH_2-5), 1.58 (m, 1H, CH-4), 0.97 (m, 3H, CH_3), 0.81 (s, 3H, $C(CH_3)$), 0.77 (s, 3H, $C(CH_3)$).	δ 80.05 (C-1), 77.15 (C-8), 72.15 (C-3), 46.32 (C-4), 37.20 (C-5), 31.51 (C-2), 27.35 (C-6), 26.49 (C-9, 10), 23.49 (C-7).

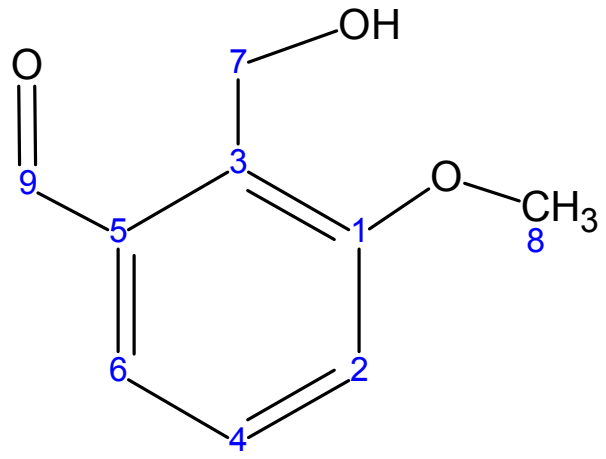


Structure of DHA IV: 4-(1-hydroxy-1-methylethyl)-1-methyl-1, 2-cyclohexane diol

MOLECULAR CHARACTERIZATION OF DHA V

Spectral Characteristics of DHA V

Compound (Mass)	UV λmax (nm)	IR (Stretching)	^{1}H-NMR	^{13}C-NMR
2-hydroxymethyl-3-methoxybenzaldehyde (166.17)	219.5 & 278.5	3479 cm^{-1} (OH) 1483 cm^{-1} (C=C) 1589 cm^{-1} (C=O) 3004 cm^{-1} (CH)	δ 8.47 (s, 1H, CHO), 6.92 (d, 1H, J=7.6, CH-6), 6.75 (m, 1H, J=7.6, CH-5), 6.73 (d, 1H, J=8.2, CH-4), 4.48 (s, 2H, CH_2OH), 3.77 (s, 3H, OCH_3).	δ 195.53 (C-8), 157.44 (C-1), 138.91 (C-5), 130.90 (C-4), 128.84 (C-6), 124.73 (C-3), 119.79 (C-2), 56.38 (C-9), 53.64 (C-7).

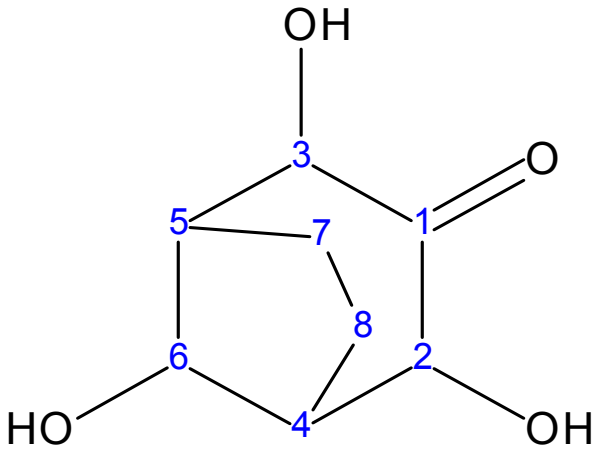


Structure of DHA V: 2-hydroxymethyl-3-methoxybenzaldehyde

MOLECULAR CHARACTERIZATION OF DHA VI

Spectral Characteristics of DHA VI

Compound (Mass)	Optical rotation $[\alpha]^{D}_{25}$	UV λmax (nm)	IR (Stretching)	^{1}H-NMR	^{13}C-NMR
2,4,8 trihydroxybicyclo [3.2.1]octan-3-one (172.17)	-30.8	214 & 273	3435 cm^{-1} (OH) 2996 cm^{-1} (CH) 1724 cm (C=O)	δ 3.92 (m, 1H, CH-2), 3.75 (m, 1H, CH-8), 3.25 (s, 3 X OH), 1.92 (m, 2H, CH-1, CH-5), 1.89 (t, 4H, CH_2-6, CH_2-7).	δ 207.60 (C-1), 76.70 (C-6), 70.02, (C-2, 3), 43.88 (C-4, 5), 24.21 (C-7,8).



Structural of DHA VI: 2,4,8 trihydroxybicyclo [3.2.1]octan-3-one

Table 2.2.: Relative concentration of the antioxidant compounds in the aqueous extract of the roots of *D. hamiltonii*.

Compound	Retention time (min)	Quantity (mg/g extract)
DHA I	7.61	4.63
DHA II	7.66	2.27
DHA III	7.88	2.16
DHA IV	1.58	1.29
DHA V	6.19	1.89
DHA VI	1.23	1.34

Quantitation of compounds was performed by RP-HPLC using C-18 column, eluted with methanol and water with 0.1% TFA (30:70), detected at 216nm.

Table 2.3.: Free radical scavenging activity of the compounds isolated from the aqueous extract of *D. hamiltonii.* [expressed as IC_{50} (nmole/ml)]

Compounds	$LOO^{\cdot}$	DPPH	$^{\cdot}OH$	$O_2^{\cdot-}$	$^{\cdot}NO$
D.h. aq.	510±40.42 µg/ml	290±20.24 µg/ml	1840±211.34 µg/ml	530±45.89 µg/ml	971.3±80.56 µg/ml
DHA I	2.15±0.19	21.36±26	27.56±2.45	11.74±1.23	187.73±16.59
DHA II	1.008±0.89	9.34±0.12	856.55±74.23	1.5±0.14	659.45±54.27
DHA III	36.78±2.98	1042±9.98	323.15±30.8	135.89±12.69	159.56±13.89
DHA IV	56.48±4.25	2715±29.17	565.12±49.34	508.24±49.21	582.31±51.34
DHA V	5.14±62	151±14.7	214.53±26.34	126.34±13.58	174.23±18.13
DHA VI	42.65±3.81	1104.98±99.49	192.3±20.64	281.43±29.67	291.43±30.56
BHA	1.45±1.29	34.4±4.12	-	0	0
Quercetin	1.86±0.92	8.61±0.79	-	335.58±31.24	415.41±39.73

Table 2.4.: Metal chelating activity, reducing power and protein carbonylation inhibiting activity of the compounds isolated from *D. hamiltonii*. [expressed as IC_{50} (nmole/ml)]

Compounds	Metal chelating activity	Reducing power*	Inhibition of protein carbonylation
D.h. aq.	5500±621.32 µg/ml	2940±312.48 µg/ml	1113±108.27 µg/ml
DHA I	20.6±1.96	21.79±2.12	75.97±6.54
DHA II	1037.8±98.36	14.02±1.23	122.55±13.10
DHA III	304.6±28.65	41.39±3.45	206.13±18.74
DHA IV	198.7±17.34	1965.11±173.21	551.93±53.21
DHA V	987.5±71.64	18.43±1.46	171.68±18.45
DHA VI	213.7±18.99	50.81±3.68	255.11±21.49
BHA	2115.6±164.35	45.72±3.87	0
Quercetin	557.2±43.28	34.25±2.98	811.93±79.89

*Concentration required to get an absorbance of appx. 0.5 at 700nm

Table 2.5.: Inhibition of LDL oxidation by the compounds isolated from the aqueous extract of *D. hamiltonii*.

Treatment*	TBARS (nmoles MDA/mg protein)	REM
LDL	3.56±0.29	1.0
LDL + $CuSO_4$	38.24±2.98	2.48±0.13
LDL + $CuSO_4$ + DMSO	37.65±3.02	2.46±0.12
LDL + $CuSO_4$ + DHA (0.5mg/ml)	11.04±1.21	1.25±0.14
LDL + $CuSO_4$ + DHA I	11.52±1.23	1.21±0.06
LDL + $CuSO_4$ + DHA II	9.54±0.10	1.14±0.04
LDL + $CuSO_4$ + DHA III	19.28±1.86	1.53±0.08
LDL + $CuSO_4$ + DHA IV	29.87±3.14	1.98±0.1
LDL + $CuSO_4$ + DHA V	17.53±1.67	1.41±0.7
LDL + $CuSO_4$ + DHA VI	12.86±1.31	1.3±0.04
LDL + $CuSO_4$ + BHA	26.94±2.73	1.61±0.06
LDL + $CuSO_4$ + Quercetin	16.27±1.63	1.4±0.05

* The antioxidant compounds (DHA I-VI) and the standard antioxidants were used at equimolar concentrations (5μM)

DISCUSSION

Natural antioxidants are characterized by their ability to scavenge free radicals. Proton-radical scavenging action is an important attribute of antioxidants, which is measured by the DPPH radical scavenging assay. DPPH, a protonated radical, has a characteristic absorbance maxima at 517nm which decreases in the presence of antioxidant due to the scavenging of the proton radical (Yamaguchi et al., 1998). Hydrogen-donating ability of the antioxidant molecule contributes to its free radical scavenging potential (Chen and Ho, 1995). Among the compounds isolated from the roots of *D. hamiltonii*, DHA I (4-hydroxy isophthalic acid) and DHA II (ellagic acid) were the most potent in their ability to scavenge DPPH radical.

The superoxide anion is produced in cells during the course of normal metabolism which is removed by enzymatic detoxication. Excessive production of superoxide in cells does occur due to both endogenous and exogenous factors. Although superoxide anion is, by itself, a weak oxidant but it gives rise to the powerful and dangerous hydroxyl radicals as well as the singlet oxygen both of which contribute to the oxidative stress (Dahl and Richardson, 1978; Meyer et al., 2005). Therefore superoxide radical scavenging by antioxidants has physiological implications. Our results show that the *D. hamiltonii* root extracts exhibit potent superoxide radical scavenging properties (Srivastava et al., 2005). The active principles responsible for the superoxide radical scavenging activity have now been reported in this study. Among the antioxidant compounds isolated from the roots of *D. hamiltonii*, DHA I, II, III, V and VI showed higher activity compared to those of standard antioxidants used.

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging biomolecules found in living cells (Hochestein and Atallah, 1988; Gordon, 1990; Halliwell, 1991). Hydroxyl radical has the capacity to cause DNA strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, this radical species is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991). In this study the compounds isolated from *D. hamiltonii* roots showed high hydroxyl radical scavenging activity of which DHA I (4-hydroxy isophthalic acid) was found to be the most potent.

Under physiological conditions, nitric oxide (NO) plays important role as a vasodilator, neurotransmitter and in the immunological system as a defense against tumor cells and infectious agents (Nakagawa and Yokozawa, 2002). During inflammatory reactions, NO produced by the inducible enzyme NO synthase (iNOS) in cells such as macrophages, hepatocytes and renal cells after the stimulation with lipopolysaccharide (LPS), tumor necrosis factor (TNF-α), interleukin (IL-1) or interferon (IFN-γ) acts as a defense and regulatory signal molecule (Kuo and Schroeder, 1995). However, NO is also pathogenic when excessively produced. NO *per se* as a reactive radical directly damages normal tissues (Moncada et al., 1991), further, nitric oxide can also react with superoxide anion radical to form the even stronger oxidant peroxynitrite (DeRojas-Walker et al., 1995; Szabo at al., 1996). The isolated antioxidant compounds from *D. hamiltonii* scavenged nitric oxide radical and DHA II (14- aminotetradecanoic acid) was most active among them.

Lipid peroxidation (LPO) has been broadly defined as the oxidative deterioration of polyunsaturated lipids. Initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid is due to abstraction of a hydrogen atom from the double bond in the fatty acid. The free radical tends to stabilize by a molecular rearrangement to produce a conjugated diene, which then readily reacts with oxygen molecule to give a peroxy radical (Jadhav et al., 1996). Peroxy radicals can abstract a hydrogen atom from another molecule to give lipid hydroperoxide, R-OOH. A probable alternative fate of peroxy radicals is to form cyclic peroxides; these cyclic peroxides, lipid peroxides and cyclic endoperoxides fragment to aldehydes including MDA and polymerization products. Malondialdehyde and 4-hydroxy nonenal are the major break down products of LPO. MDA is usually taken as a marker of LPO as well as oxidative stress (Janero, 1990). All the isolated antioxidant compounds from *D. hamiltonii*, inhibited microsomal membrane LPO. The natural compounds which exhibit high LPO inhibiting potential can replace the synthetic antioxidants which are known to be toxic. Among the antioxidant compounds of *D. hamiltonii*, DHA I and II showed comparable activity with those of standard antioxidants used.

Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction (Halliwell and Gutteridge, 1990). Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al., 1999). It is reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the

oxidized form of the metal ion (Gordon, 1990). By virtue of both metal chelating properties and radical scavenging ability, phytochemicals such as the antioxidants from *D. hamiltonii* may have a role in the prevention of free radical formation.

It is believed that antioxidant activity and reducing power are related (Duh et al., 1999). Reductones donate a hydrogen atom and inhibit LPO by donating a hydrogen atom and thereby terminating the free radical chain reaction (Huang et al., 2005). Among the compounds isolated from the aqueous extract of the roots of *D. hamiltonii,* DHA II exhibited highest reducing power.

Oxidative stress leads to irreversible protein modification, such as generation of carbonyls or loss of thiol residues (Berlett and Stadman, 2001). These oxidative modifications alter the biological properties of proteins, leading to their fragmentation, increased aggregation and enzyme dysfunction. Evidence suggests that irreversible oxidative modifications of proteins are crucial in the pathophysiology of several degenerative diseases (Beal, 2002). The isolated antioxidant compounds from *D. hamiltonii* prevented the carbonylation of proteins.

Oxidation of low density lipoprotein (LDL) is considered to be an essential step in the pathogenesis of atherosclerosis (Chisolm and Steinberg, 2000; Weber and Erl, 2000). Low density lipoprotein (LDL) oxidation is characterized by alterations in structure and biological properties not only of the lipids but also on apolipoprotein B (apoB), including early fragmentation of the protein which contains sensitive cystein residues (Guy et al., 2001), followed by cross-linking of reactive aldehydes formed as end products of the oxidative chain. Although data concerning the mechanism by which oxidation of LDL occurs *in vivo* are scarce, several lines of evidence suggest that some endogenous and

exogenous compounds with antioxidant activity could have some beneficial effects in the prevention of atherosclerosis (Craig, 1999; Giugliano, 2000; Fuhrman and Aviram, 2001). Transition-metal induced oxidation of LDL is one of the classical models of oxidation employed in research (Lamb et al., 1995; Corti et al., 1997). It has been suggested that the protection of LDL by antioxidants in a copper-induced system could be due to both metal-chelating and radical scavenging action (Nardini et al., 1995). Some possible explanations for the protective effect on LDL oxidation by the antioxidants suggested are: a) scavenging of various radical species in the aqueous phase, b) interaction with peroxyl radicals at the LDL surface, c) partitioning into the LDL particle and terminating chain-reactions of lipid peroxidation by scavenging lipid radicals and d) regenerating endogenous α-tocopherol back to its active antioxidant form (Laranjinha et al., 1994). A close relation was observed between REM and TBARS value of LDL oxidation. Metal chelating activity of the antioxidant compounds might be attributed for the prevention of LDL oxidation along with the free radical scavenging activity. These nonnutrient phytochemicals may have a protective effect on the susceptibility of LDL to oxidative modification and therefore in the prevention of atherosclerosis.

The alleged beneficial health effects of novel antioxidants could be attributed to their ability to scavenge reactive oxygen/nitrogen/chlorine species (Halliwell and Gutteridge, 1999; Halliwell, 1999). Herbal extracts, including traditional medicines and nutraceuticals, would be expected to possess antioxidant properties since they are rich sources of phenolic compounds (Zheng and Wang, 2001). The present study along with our earlier work shows that *D. hamiltonii* roots contain atleast a dozen potent antioxidant constituents which scavenge free radicals, chelate metal ions and prevent

LDL oxidation (Harish et al., 2005). Isolation of the antioxidant compounds confirms our earlier observation that *D. hamiltonii* crude extract could be a source of new bioactive nutraceuticals with high antioxidant activity (Shereen et al., 2001; Srivastava et al., 2005). Our study opens up avenues for exploiting food sources such as *D. hamiltonii* for applications of novel bioactive molecules with health implications in both prevention and amelioration of degenerative diseases and general well being.

Chapter III

CYTOPROTECTIVE ACTIVITY

INTRODUCTION

ROS, including the superoxide radical, hydrogen peroxide and the hydroxyl radical, arise as by-products of normal cellular metabolism or as a consequence of exposure to certain toxicants (McConkey, 1998). When the cellular antioxidant defenses are unable to counteract the ROS, oxidative stress occurs resulting in damage or injury (Halliwell, 1999; Sun et al., 2001). Oxidative stress is associated with inflammation, cardiovascular diseases, aging and cancer (Hogg, 1998). Cellular injury occurs due to exposure to toxic substances when the antioxidant defense system is overwhelmed (Halliwell, 1996; Sougioltzis et al., 2005). ROS readily interact with cellular macromolecules and structures resulting in changes in membrane permeability, activation of proteases, nucleases and altered gene expression (Halliwell, 1996; Saikumar et al., 1998). ROS is also known to induce DNA damage in a variety of cell types (Collins, 1999; Termini, 2000).

Antioxidants prevent the damage to macromolecules and cells by interfering with the free radicals. Natural products containing antioxidants from plants are believed to modulate oxidative stress and prevent or delay degenerative disorders (Thatte et al., 2000; Meyer et al., 2005). Examples of natural antioxidants include carotenoids, flavonoids, cinnamic acid derivatives, coumarins, polyfunctional organic acids and tocopherols (Pratt & Hudson, 1990).

In vitro studies using cell cultures offer a good model system to understand the mechanism of xenobiotic-induced cell injury/death and its amelioration by phytochemicals (Robertson, & Orrenius, 2000). Ehrlich Ascites tumor (EAT) cells derived originally from the mice mammary tumor, which can be conveniently cultured in

the mice peritoneal cavity, offer a good model to study oxidative stress mediated cytotoxicity involving LPO and ROS induction by xenobiotics (Srivastava and Shivanandappa, 2006). Isolated hepatocytes are also a useful *in vitro* cell model for pharmacological and toxicological studies of xenobiotics (Skettand Bayliss, 1996). Several phytochemicals have been evaluated for their ameliorative potential against xenobiotic induced toxicity in experimental models *in vitro* and *in vivo*. Xenobiotics such as hexachlorocyclohexane (HCH), carbon tetrachloride (CCl_4), ethanol and cumene hydroperoxide (CHP) are well known inducers of oxidative stress in cell systems (Tseng et al., 1996; Ahmed et al., 2000; Lee et al., 2001; Lee et al., 2004; Lee, 2004; Srivastava and Shivanandappa, 2006). Since cytotoxic injury is believed to be integral to toxicological manifestation(s) agents that ameliorate cytotoxic injury are considered to possess health promoting potential.

In the present work, we have examined the ameliorative potential of the antioxidant compounds isolated from the aqueous extract of the roots of *D. hamiltonii* against xenobiotic-induced oxidative injury in (a) EAT cells and (b) rat primary hepatocytes, and the possible mechanism(s) underlying their protective effects.

MATERIALS AND METHODS

Chemicals: Thiobarbituric acid (TBA), glutathione (GSH), cumene hydroperoxide (CHP), bovine serum albumin (BSA), tetraethoxypropane, trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), 5,5'dithiobis(2-nitrobenzoic acid) (DTNB), Nitroblue tetrazolium (NBT), dimethyl sulphoxide (DMSO) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

EAT cells: Ehrlich Ascites Tumor cells were cultured in the peritoneum of male Swiss albino mice (Estrela et al., 1992). After harvesting, cells were suspended in Hanks Balanced Salt Solution (HBSS) with 0.1% dextrose and 0.4% bovine serum albumin which were found to be essential for retaining good viability of cells.

Hepatocyte isolation: Hepatocytes were isolated from male Wistar rats (200-240g) by collagenase perfusion of the liver as described by Moldeus et al. (1978). The pre-perfusion and perfusion solutions were buffered at pH 7.4 with 0.01 M HEPES. The total length of the perfusion was approximately 15-20min. Aliquots of freshly isolated hepatocytes were immediately counted with a haemocytometer in 0.4% trypan blue solution containing 0.9% NaCl. The viability of the cells isolated by this method was always more than 90%.

Cell viability: EAT cells/hepatocytes ($10x10^6$) suspended in 1.0ml of HBSS were treated with xenobiotics (dissolved in DMSO) at LC_{50} concentration with/without the antioxidant compounds and incubated for 60min in a shaking water bath at 37^oC. At the end of

incubation, an aliquot of cells was taken for viability assay by trypan blue exclusion method (Frandsen & Schousboe, 1987).

Lactate dehydrogenase leakage: After incubation of cells in the presence of xenobiotics with/without the antioxidant compounds, cells were centrifuged and the supernatant was assayed for LDH with sodium lactate as the substrate (Bergmeyer & Bernt, 1974). The reaction mixture consisted of NADH (0.02M), sodium pyruvate (0.01M), sodium phosphate buffer (0.1M, pH 7.4) in a total volume of 3ml. The changes in the absorbance were recorded at 340nm at 30 sec interval for 3 min.

Lipid peroxidation: After incubation, as above, the cells were centrifuged and the cell pellet was washed in saline. The cell pellet was boiled in TCA (5.5%) and TBA (0.34%) for 15min, cooled and centrifuged. Fluorescence of the supernatant was measured in a fluorescence spectrophotometer at excitation and emission wavelengths of 532nm and 553nm respectively (Cereser et al., 2001). LPO i.e. thiobarbituric acid reactive substances (TBARS) value, was calculated using tetraethoxypropane standard curve.

Reactive Oxygen Species (Superoxide anion): The cells ($10x\ 10^6$) suspended in 1.0ml HBSS were incubated with NBT (0.2mM), xenobiotics (in DMSO) and with/without antioxidant compounds in a shaking water bath at 37^oC. The generation of ROS by cells (respiratory burst) was measured by the formation of colored formazan due to reduction of NBT (Pompeia et al., 2003).

Glutathione: EAT cells/hepatocytes ($10x10^6$) suspended in 1.0ml HBSS were treated with xenobiotics (dissolved in DMSO) at LC_{50} concentration with/without the antioxidant compounds and incubated for 60min in a shaking water bath at 37^oC. At the end of incubation, cells were homogenized in 1.0ml of 5% (w/v) trichloroacetic acid,

centrifuged at 2,000g for 5min and glutathione (GSH) in the deproteinized supernatant was estimated by Ellman's reagent with a standard curve (Ellman, 1959).

Alkaline phosphatase: After incubation of cells in the presence of xenobiotics with/without the antioxidant compounds, cells were centrifuged and the supernatant was assayed for alkaline phosphatase (ALP) activity by the method of Walter and Schutt (Walter and Schutt, 1974). p-Nitrophenol phosphate (1.25mM) and supernatant were incubated in tris buffer (0.1M, pH 8.5) at 37^{o}C for 30 min. The reaction was stopped by adding NaOH (0.02M) and the absorbance was read at 405nm. The enzyme activity was calculated using the extinction coefficient 1.85 X 10^{-3} $M^{-1}cm^{-1}$ for p-nitrophenol.

Alanine and Aspartate transaminases: After incubation of cells in the presence of xenobiotics with/without the antioxidant compounds, cells were centrifuged and the supernatant was assayed for alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) activities by the method of Reitman and Frankel (Reitman and Frankel, 1957). The reaction mixture in 0.1M phosphate buffer (pH 7.4) containing the substrate and enzyme was incubated for 30min and 60 min for ALT and AST, respectively; followed by the addition of DNPH (1mM) incubated for another 30 min at room temperature. The color developed by the addition of NaOH (0.4N) was read at 505nm.

Protein estimation was done by the method of Lowry et al. (1951) using BSA as the standard.

Statistical analysis: Data were expressed as mean ± S.E. of three separate experiments and the significance was determined by the analysis of variance ($p<0.05$) using the computer programme Excel and Statistica software.

RESULTS

Cell viability

The antioxidant compounds isolated from *D. hamiltonii* ameliorated xenobiotic-induced cell injury/death as measured by trypan blue method and lactate dehydrogenase leakage. Relative cytoprotective action of the antioxidant compounds **in EAT cells** was in the following order:

(a) *HCH-induced cytotoxicity* - DHA-II > DHA-III > DHA-V > DHA-I > DHA-VI > DHA-IV;

(b) *CCl_4-induced cytotoxicity* - DHA-I > DHA-III > DHA-II > DHA-V > DHA-VI > DHA-IV;

(c) *CHP-induced cytotoxicity* - DHA-VI > DHA-III > DHA-II > DHA-I > DHA-V > DHA-IV.

Amelioration of cytotoxicity **in hepatocytes** by the isolated compounds was in the following order: DHA-II > DHA-I > DHA-V > DHA-III > DHA-VI > DHA-IV for both ethanol and CCl_4 induced cytotoxicity.

All the antioxidant compounds isolated from *D. hamiltonii* were not toxic to the cells at the highest concentration used (Fig. 3.1.-3.18.).

Lipid peroxidation

Xenobiotic-induced LPO was inhibited by all the antioxidant compounds isolated from *D. hamiltonii* in both EAT cells and hepatocytes (Fig. 3.1.-3.18.). DHA-I and II were the most potent among all the antioxidant compounds, whereas, DHA-IV was the least potent in both EAT cells and hepatocytes.

Reactive Oxygen Species (Superoxide anion)

Xenobiotic-induced reactive oxygen species (ROS) production i.e. "respiratory burst" in the cells was inhibited by all the antioxidant compounds isolated from *D. hamiltonii* to different degrees (Fig. 3.1.-3.18.). Among the compounds, DHA-VI was found to be the most potent inhibitor/scavenger of ROS produced in EAT cells. ROS inhibition activity of the antioxidant compounds was in the following order: DHA-VI > DHA-III > DHA-II > DHA-I > DHA-V > DHA-IV in EAT cells. Highest inhibition of ROS production/ scavenging in hepatocytes was shown by DHA-II; the order of protection by the antioxidant compounds was in the following order: DHA II > DHA-I > DHA-V > DHA-III > DHA-VI > DHA-IV.

Glutathione content

Depletion of glutathione (GSH) content in the cells induced by exposure to the xenobiotics was restored by the prior exposure to the antioxidant compounds (Fig. 3.1.-3.18.); the effect was in the following order in EAT cells: DHA-III > DHA-VI > DHA-II > DHA-I > DHA-V > DHA-IV. In hepatocytes, GSH depletion by xenobiotics was inhibited by the isolated compounds in following order: DHA-II > DHA-I > DHA-V > DHA-III > DHA-VI > DHA-IV.

Alkaline phosphatase, Alanine and Aspartate transaminases

Leaching of alkaline phophatase, alanine transaminase and asparate transaminase by exposure to xenobiotics was prevented by the isolated compounds in hepatocytes. The order of protection was: DHA-II > DHA-I > DHA-V > DHA-III > DHA-VI > DHA-IV. (Fig. 3.2.-3.18.)

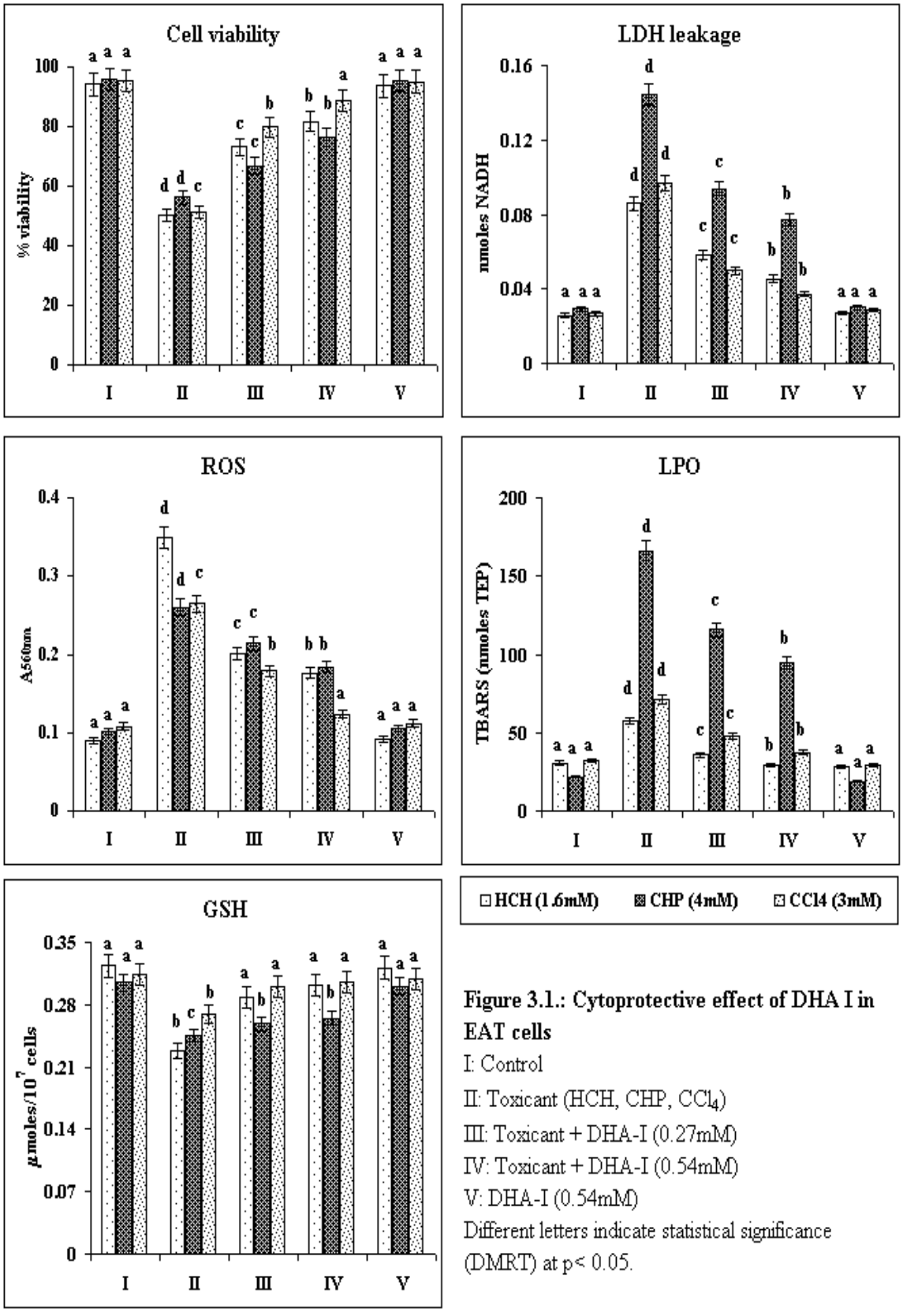


Figure 3.1.: Cytoprotective effect of DHA I in EAT cells

I: Control

II: Toxicant (HCH, CHP, CCl_4)

III: Toxicant + DHA-I (0.27mM)

IV: Toxicant + DHA-I (0.54mM)

V: DHA-I (0.54mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

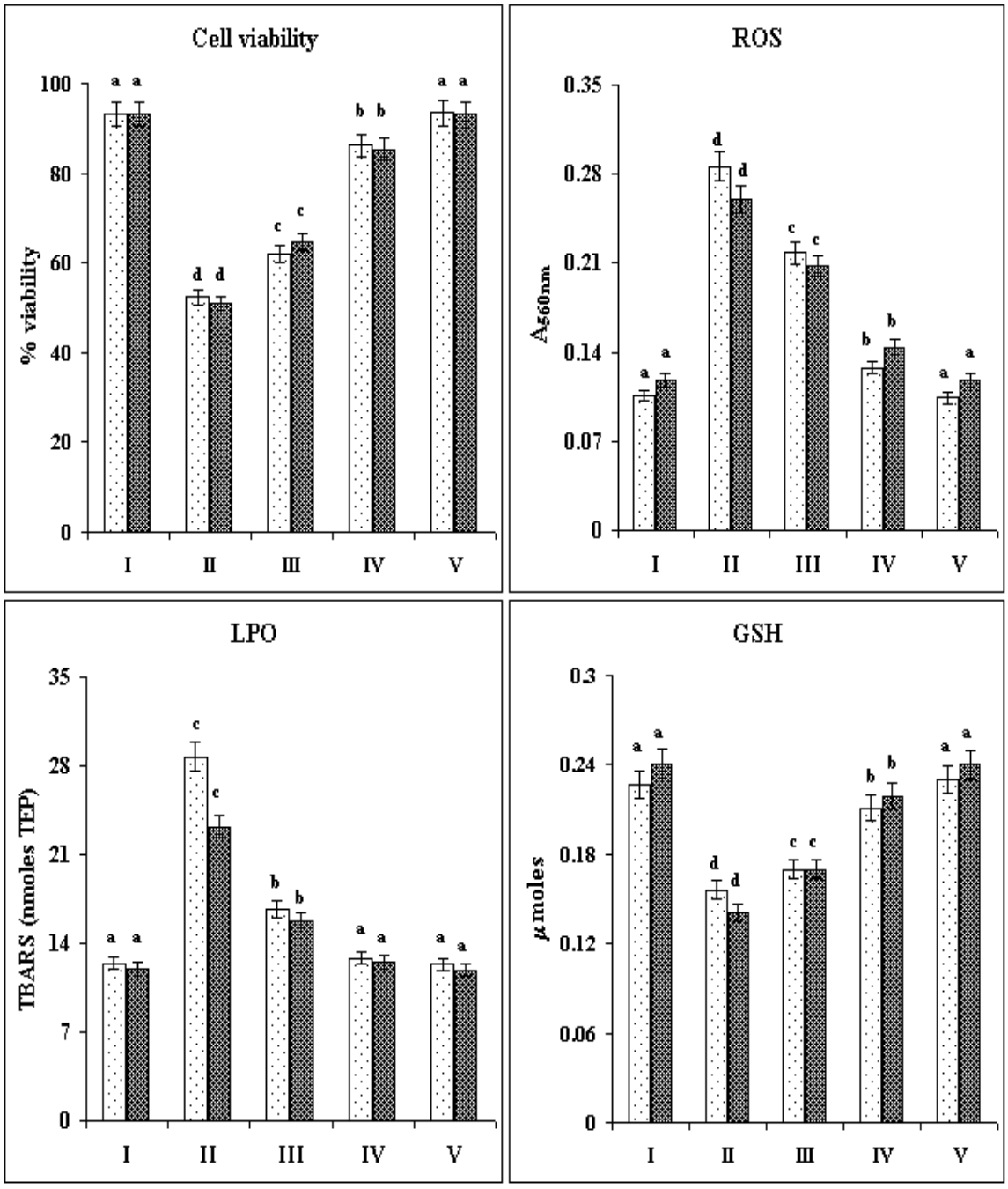


Figure 3.2.: Cytoprotective effect of DHA I in primary hepatocytes.

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.054mM), IV: Toxicant + DHA-I (0.216mM), V: DHA-I (0.216mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$.

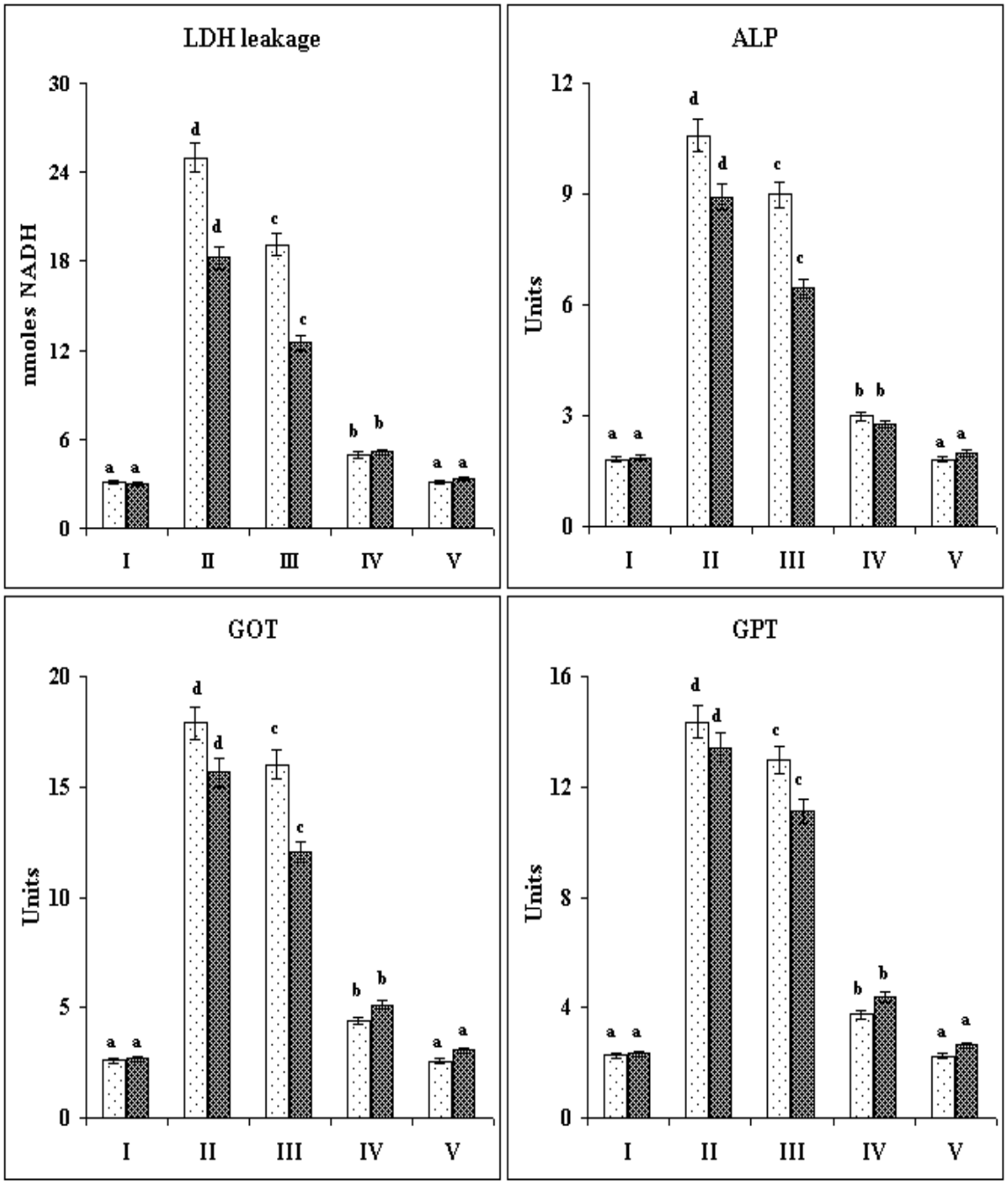


Figure 3.3.: Cytoprotective effect of DHA I in primary hepatocytes: Hepatic marker enzymes

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.054mM), IV: Toxicant + DHA-I (0.216mM), V: DHA-I (0.216mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$. GOT: Glutamate oxaloacetate transaminase, GPT: Glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

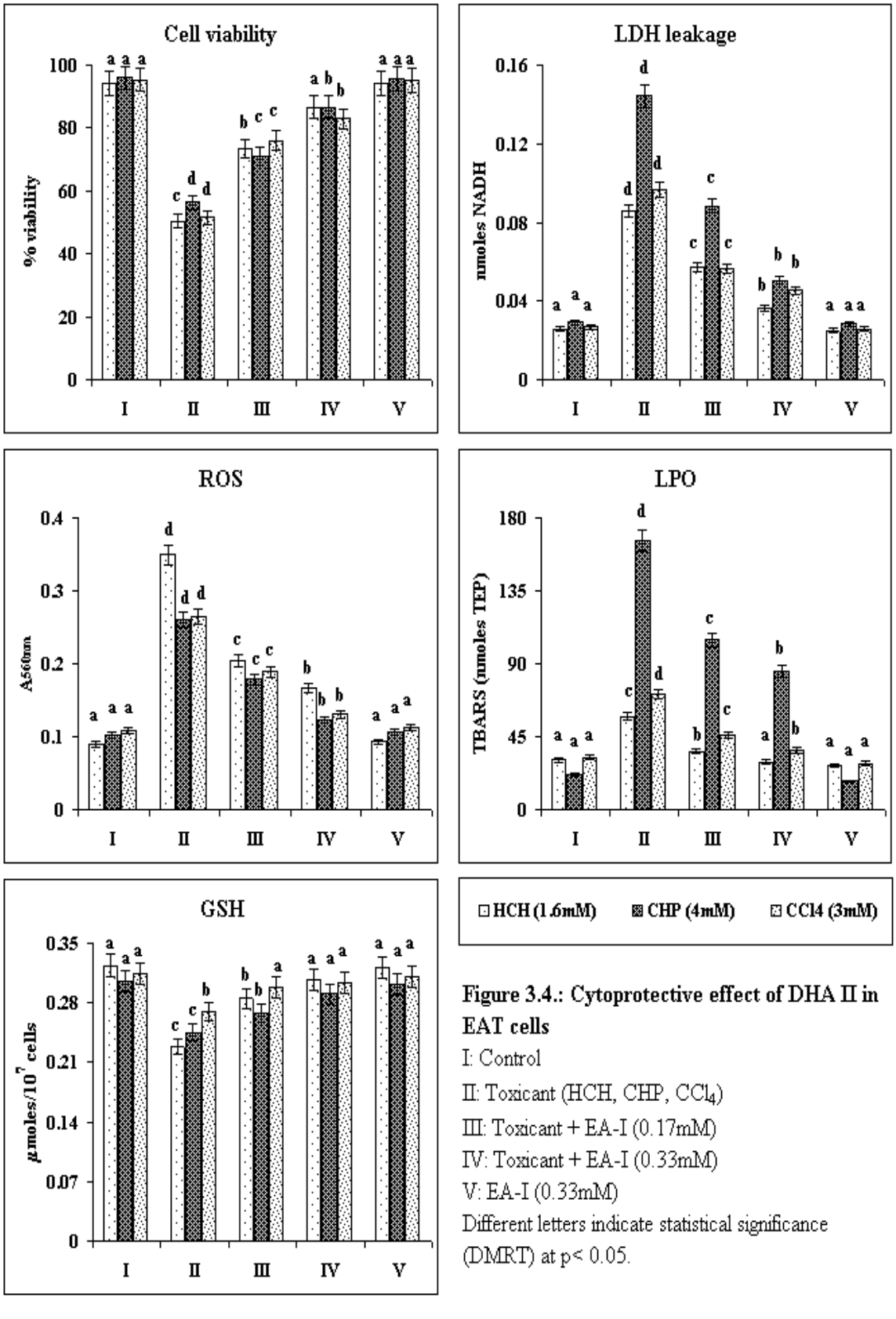


Figure 3.4.: Cytoprotective effect of DHA II in EAT cells

I: Control

II: Toxicant (HCH, CHP, CCl_4)

III: Toxicant + EA-I (0.17mM)

IV: Toxicant + EA-I (0.33mM)

V: EA-I (0.33mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

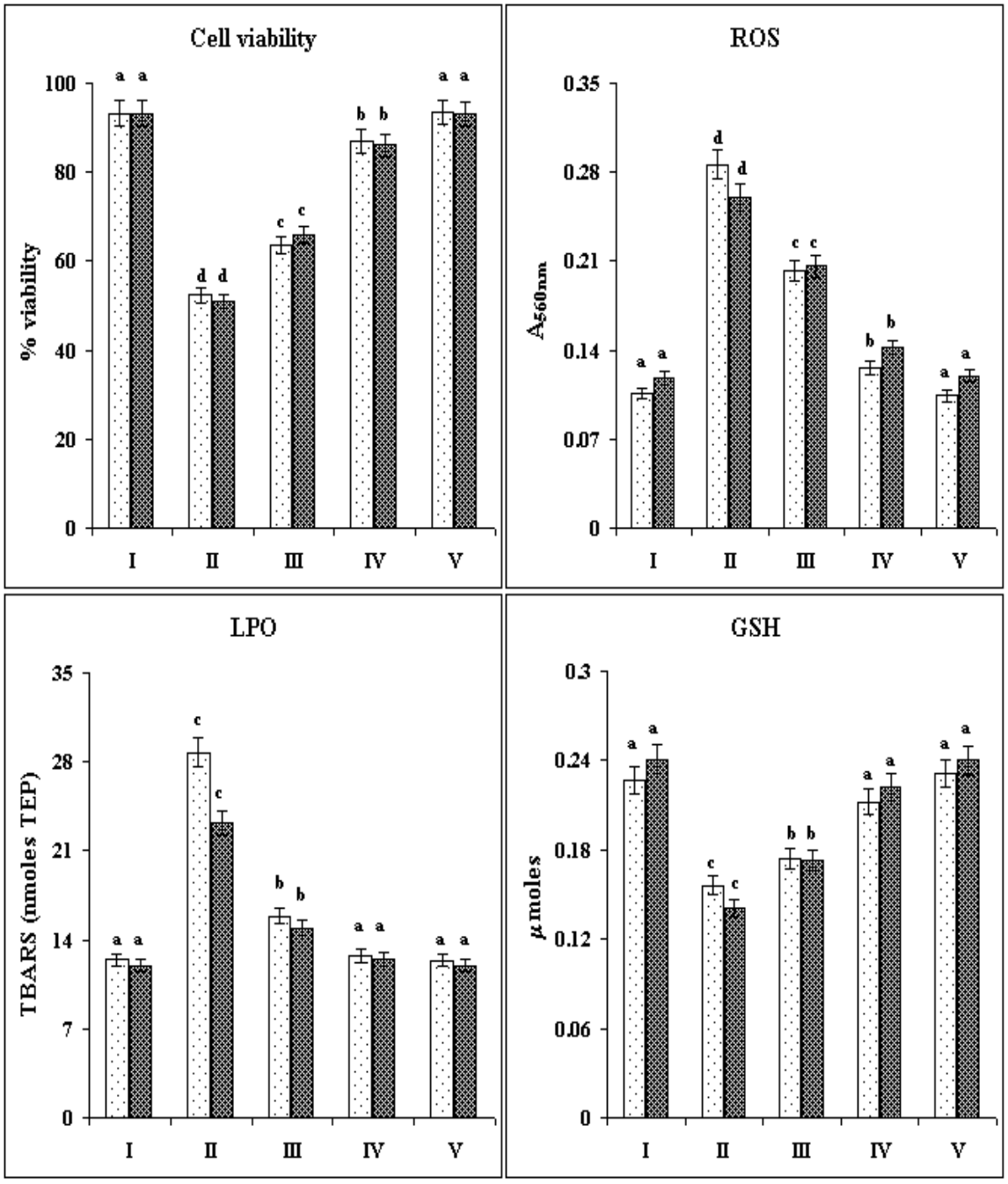


Figure 3.5.: Cytoprotective effect of DHA II in primary hepatocytes.

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.033mM), IV: Toxicant + DHA-I (0.132mM), V: DHA-I (0.132mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$.

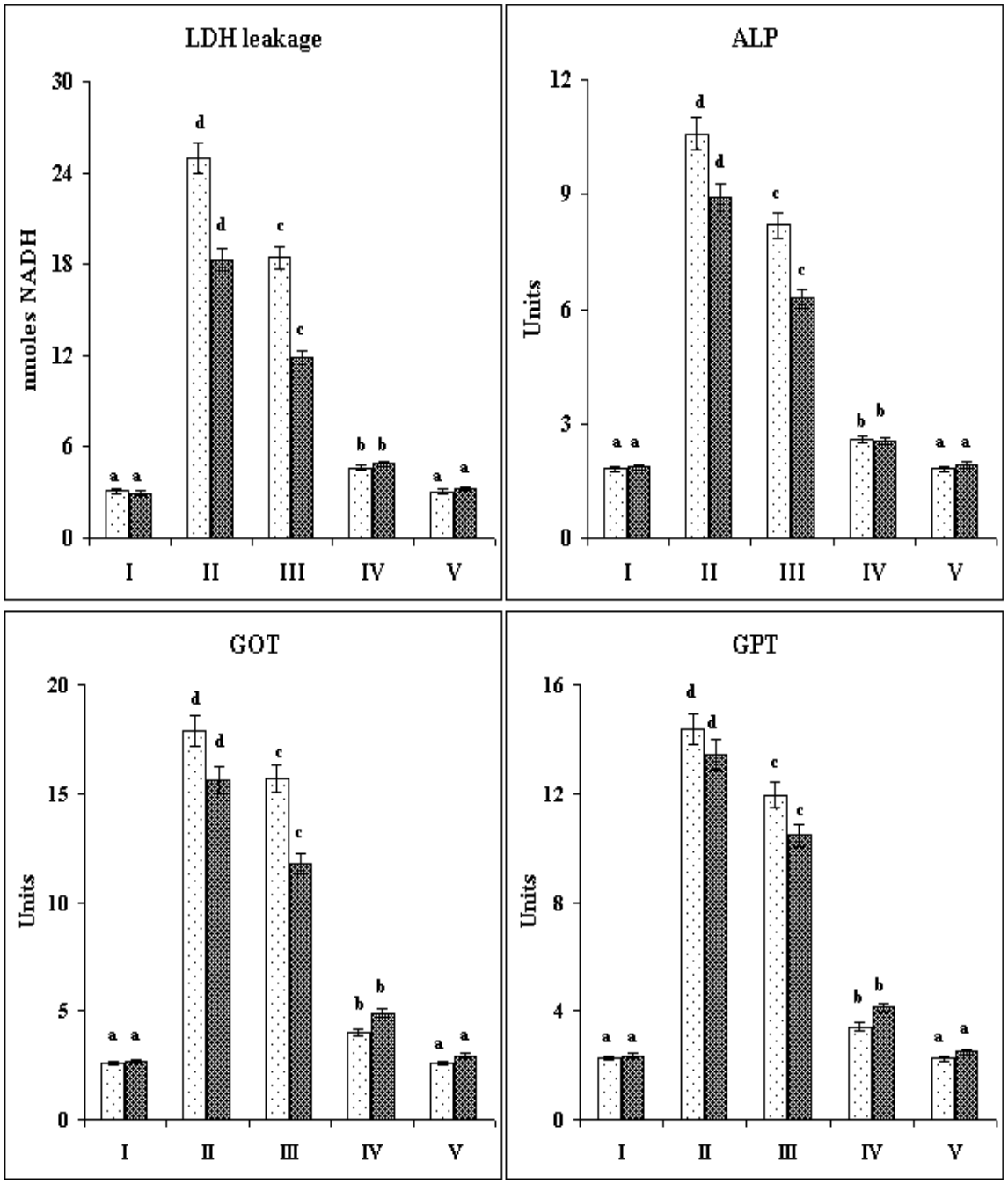


Figure 3.6.: Cytoprotective effect of DHA II in primary hepatocytes: Hepatic marker enzymes

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.033mM), IV: Toxicant + DHA-I (0.132mM), V: DHA-I (0.132mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$. GOT: Glutamate oxaloacetate transaminase, GPT: Glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

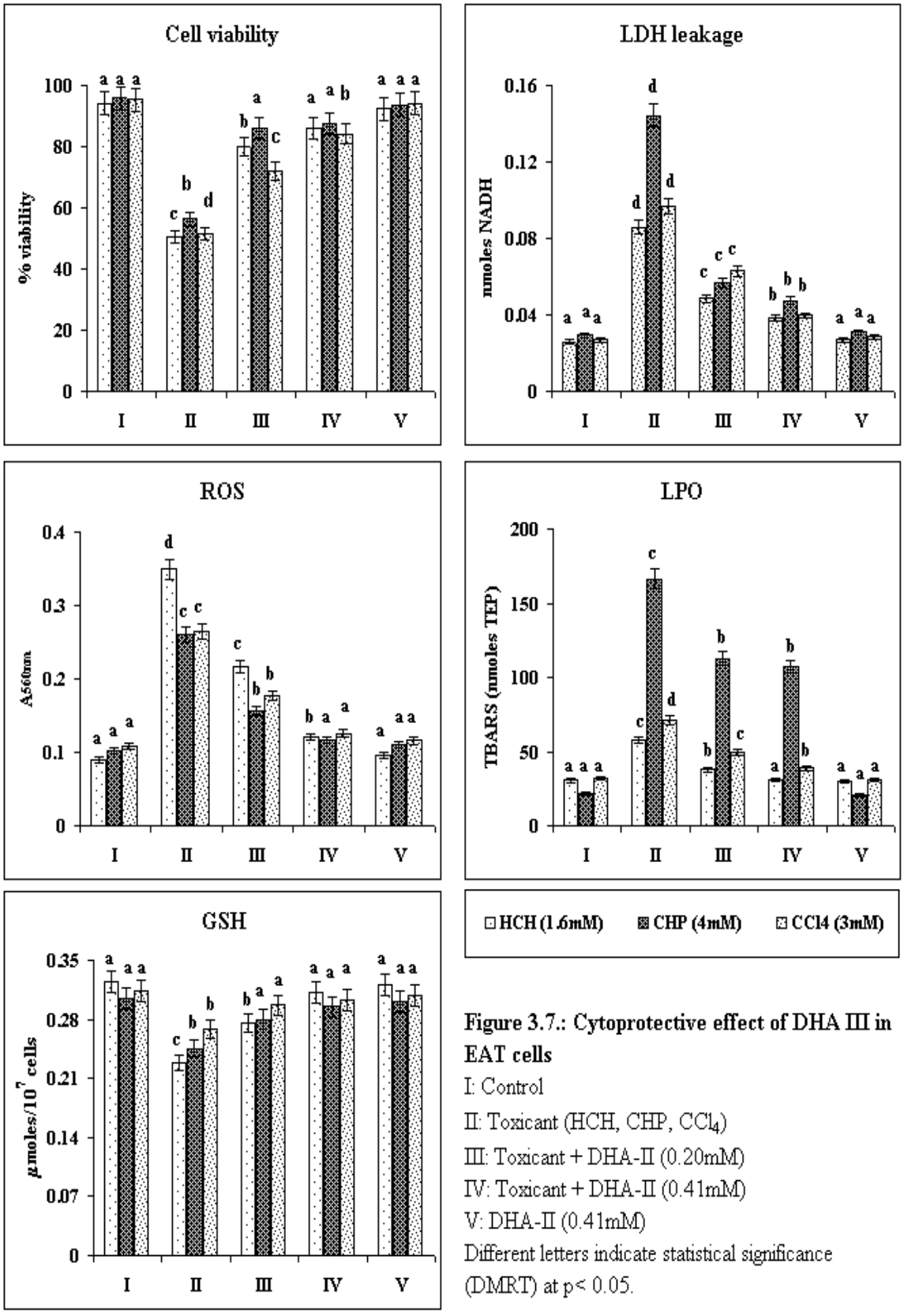


Figure 3.7.: Cytoprotective effect of DHA III in EAT cells

I: Control

II: Toxicant (HCH, CHP, CCl_4)

III: Toxicant + DHA-II (0.20mM)

IV: Toxicant + DHA-II (0.41mM)

V: DHA-II (0.41mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

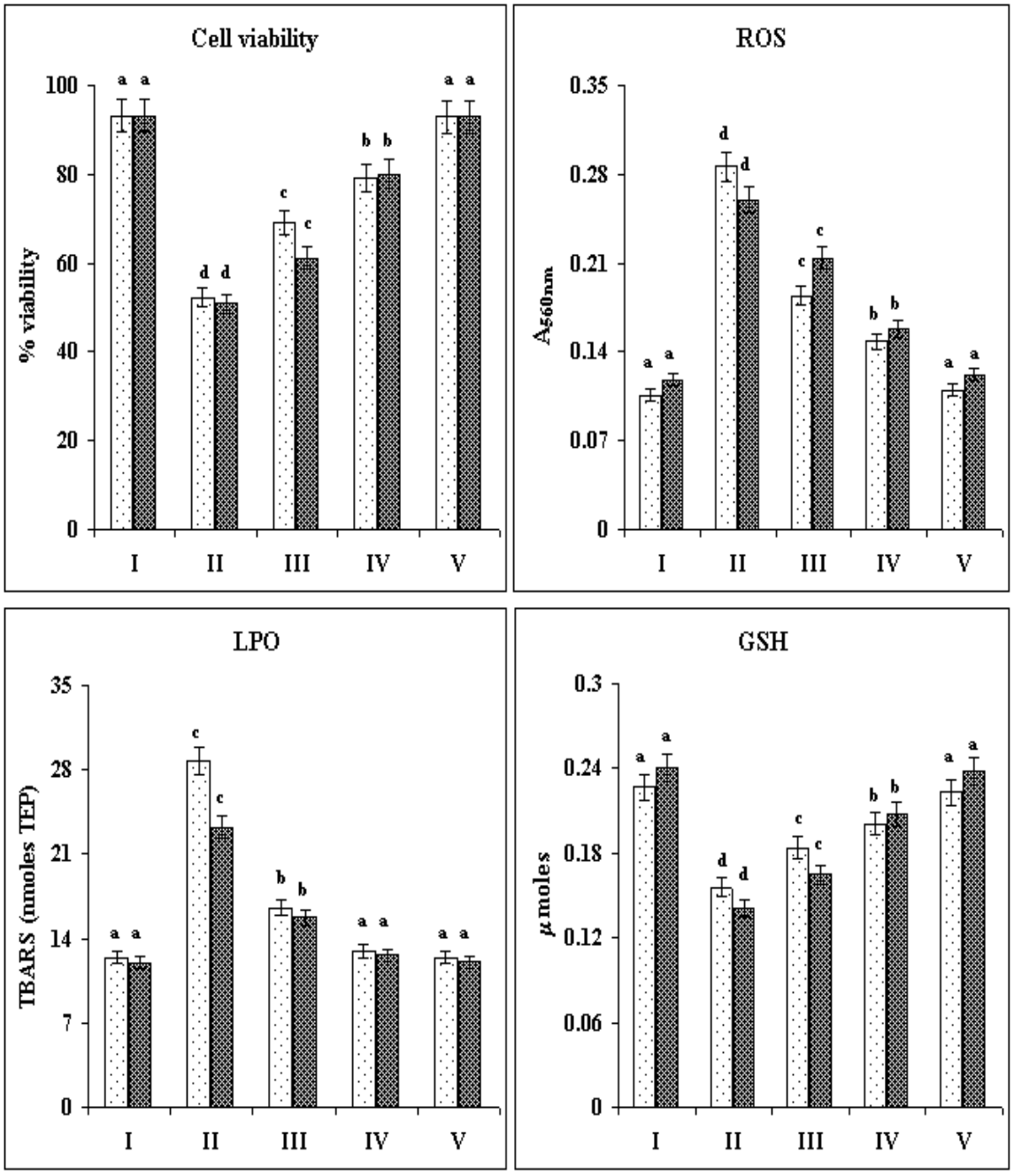


Figure 3.8.: Cytoprotective effect of DHA III in primary hepatocytes.

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.041mM), IV: Toxicant + DHA-I (0.164mM), V: DHA-I (0.164mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

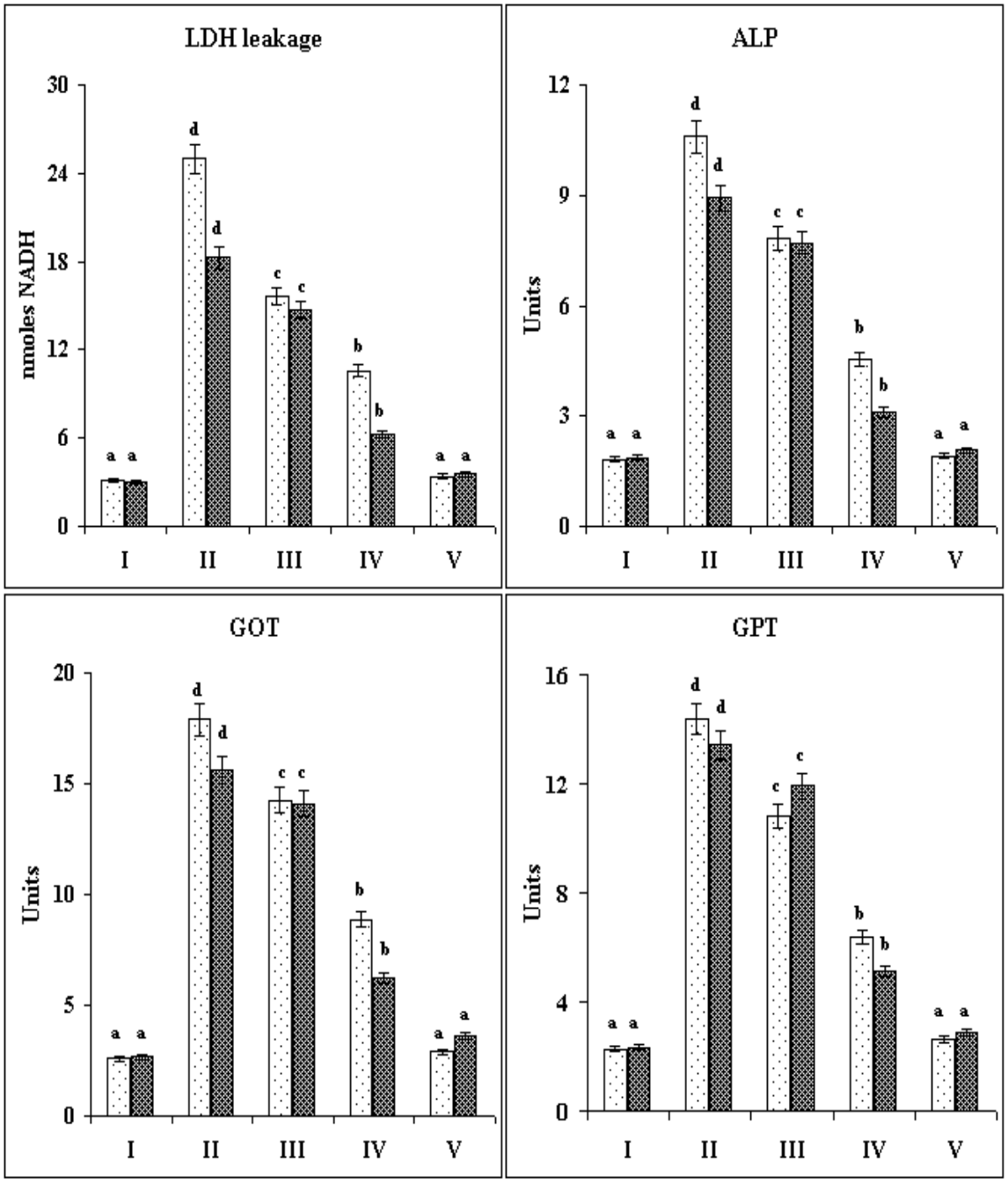


Figure 3.9.: Cytoprotective effect of DHA III in primary hepatocytes: Hepatic marker enzymes

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.041mM), IV: Toxicant + DHA-I (0.164mM), V: DHA-I (0.164mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$. GOT: Glutamate oxaloacetate transaminase, GPT: Glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

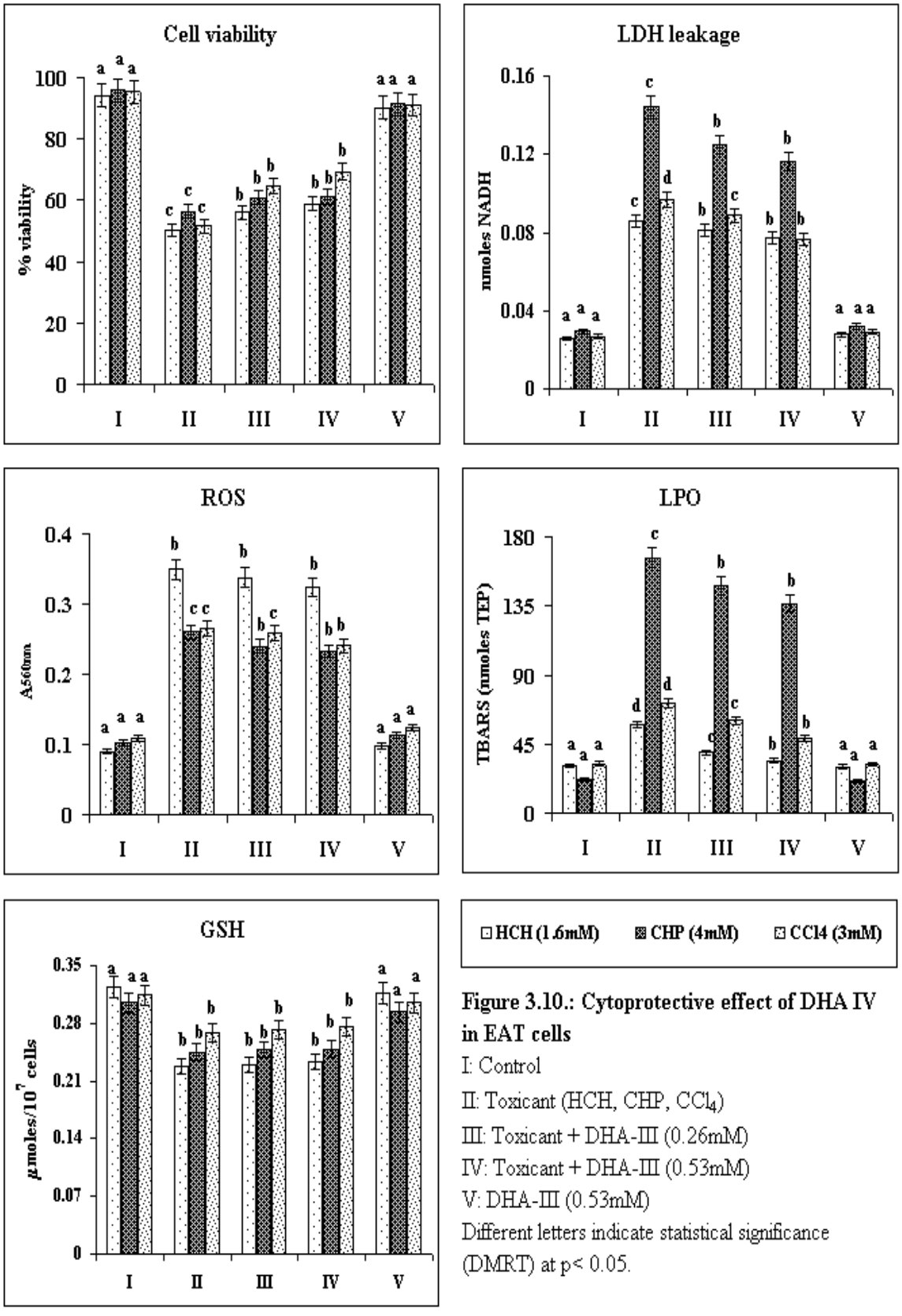


Figure 3.10.: Cytoprotective effect of DHA IV in EAT cells

I: Control

II: Toxicant (HCH, CHP, CCl_4)

III: Toxicant + DHA-III (0.26mM)

IV: Toxicant + DHA-III (0.53mM)

V: DHA-III (0.53mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

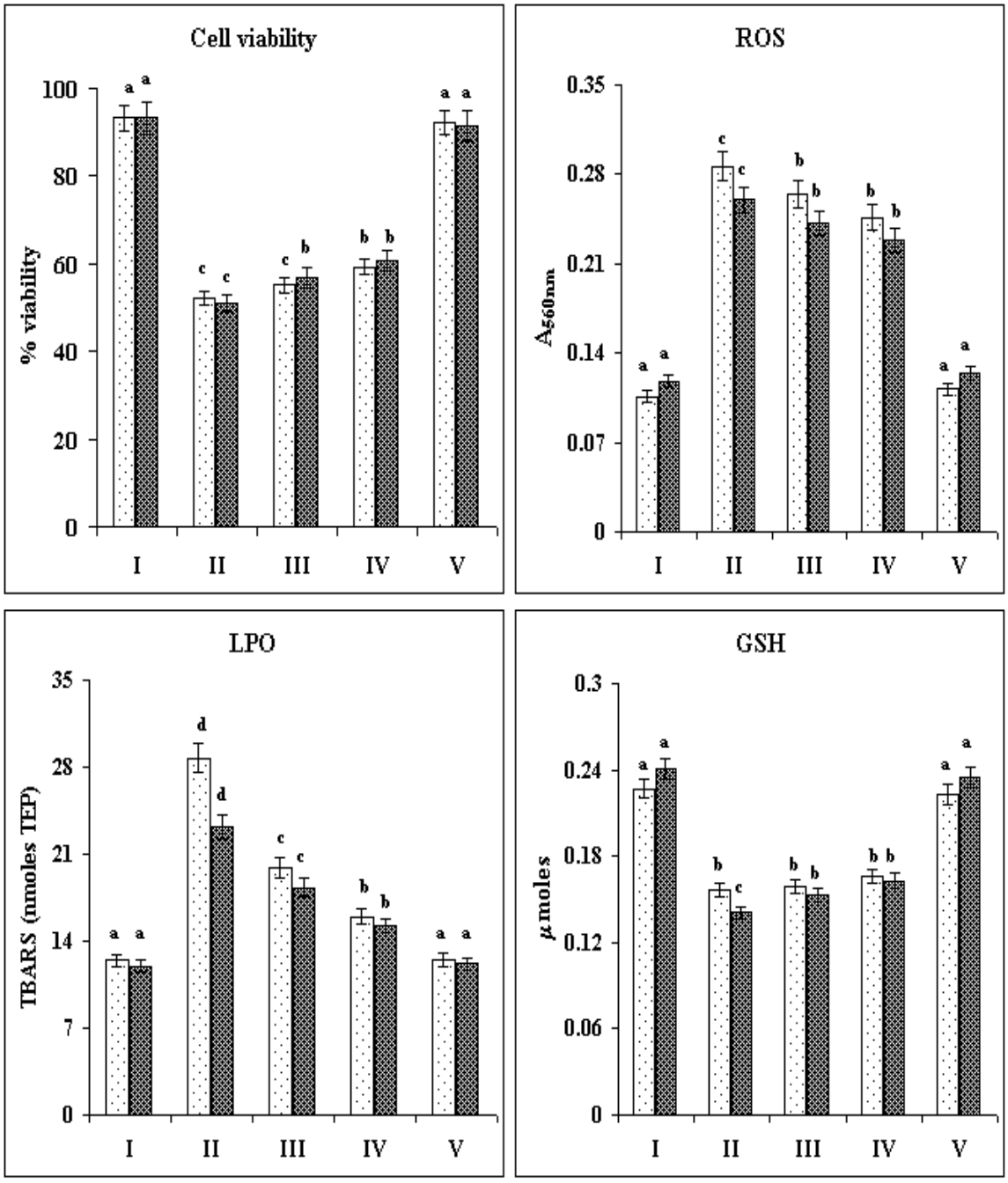


Figure 3.11.: Cytoprotective effect of DHA IV in primary hepatocytes.

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.053mM), IV: Toxicant + DHA-I (0.212mM), V: DHA-I (0.212mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

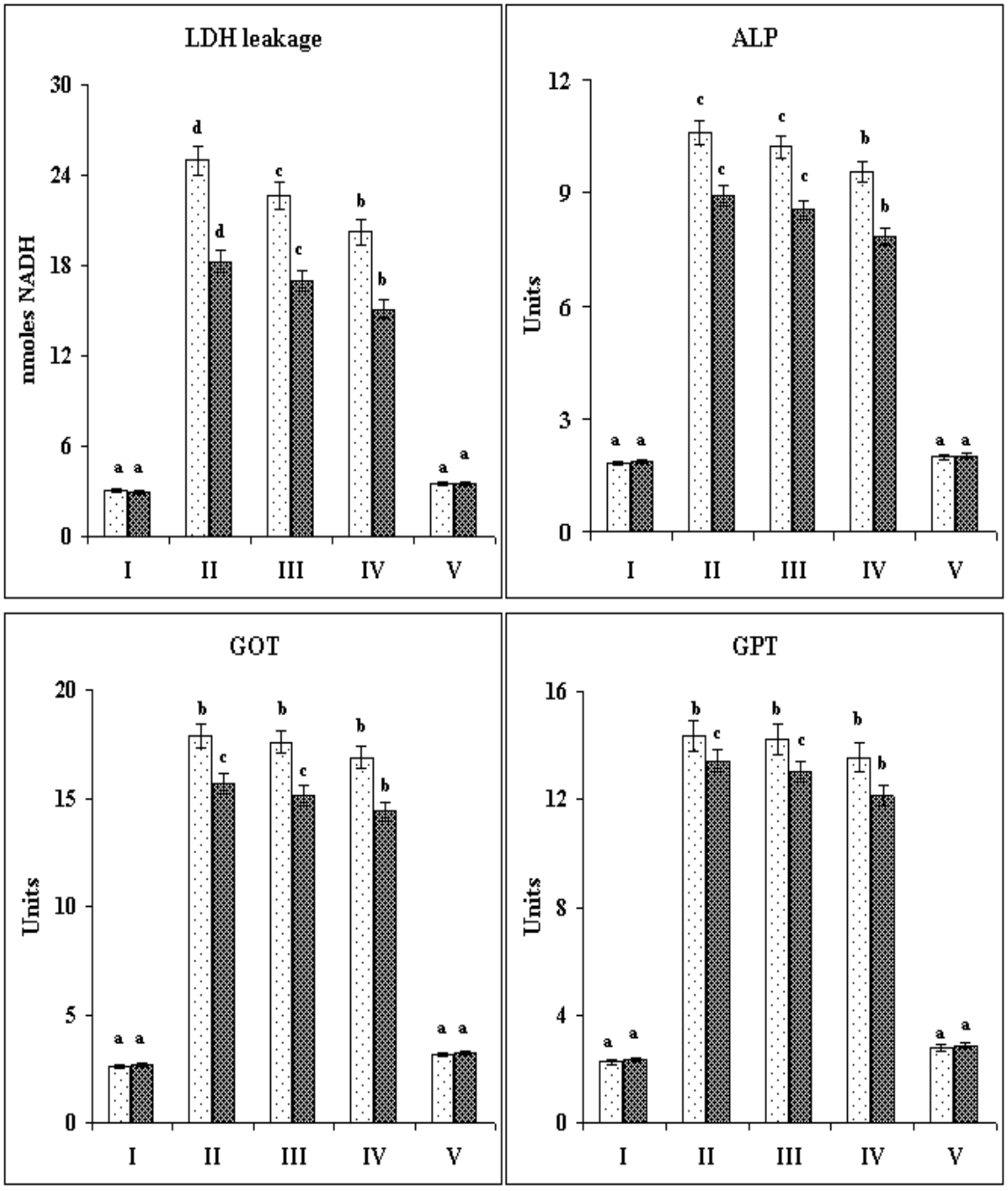


Figure 3.12.: Cytoprotective effect of DHA IV in primary hepatocytes: Hepatic marker enzymes

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.053mM), IV: Toxicant + DHA-I (0.212mM), V: DHA-I (0.212mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$. GOT: Glutamate oxaloacetate transaminase, GPT: Glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

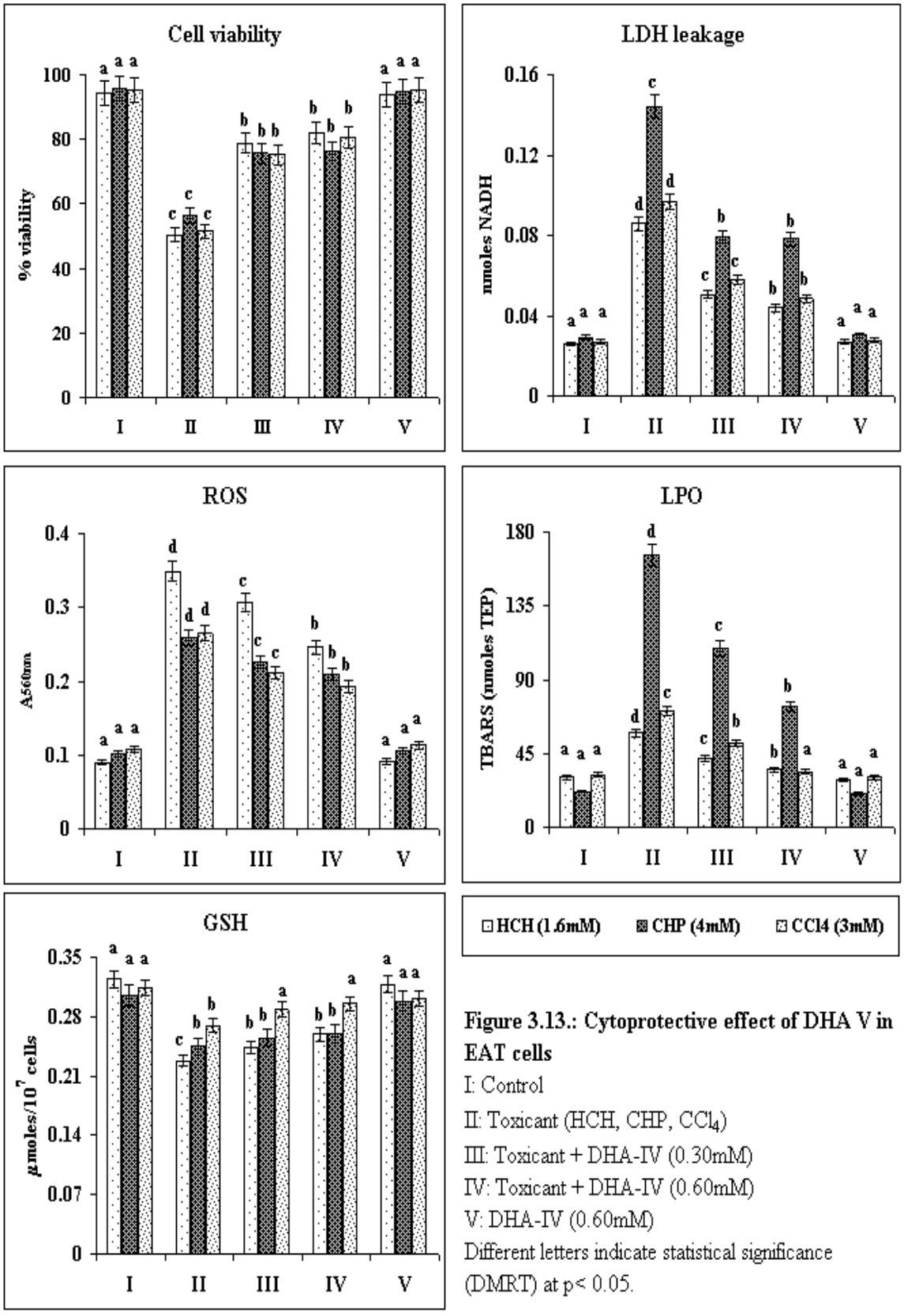


Figure 3.13.: Cytoprotective effect of DHA V in EAT cells

I: Control

II: Toxicant (HCH, CHP, CCl_4)

III: Toxicant + DHA-IV (0.30mM)

IV: Toxicant + DHA-IV (0.60mM)

V: DHA-IV (0.60mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

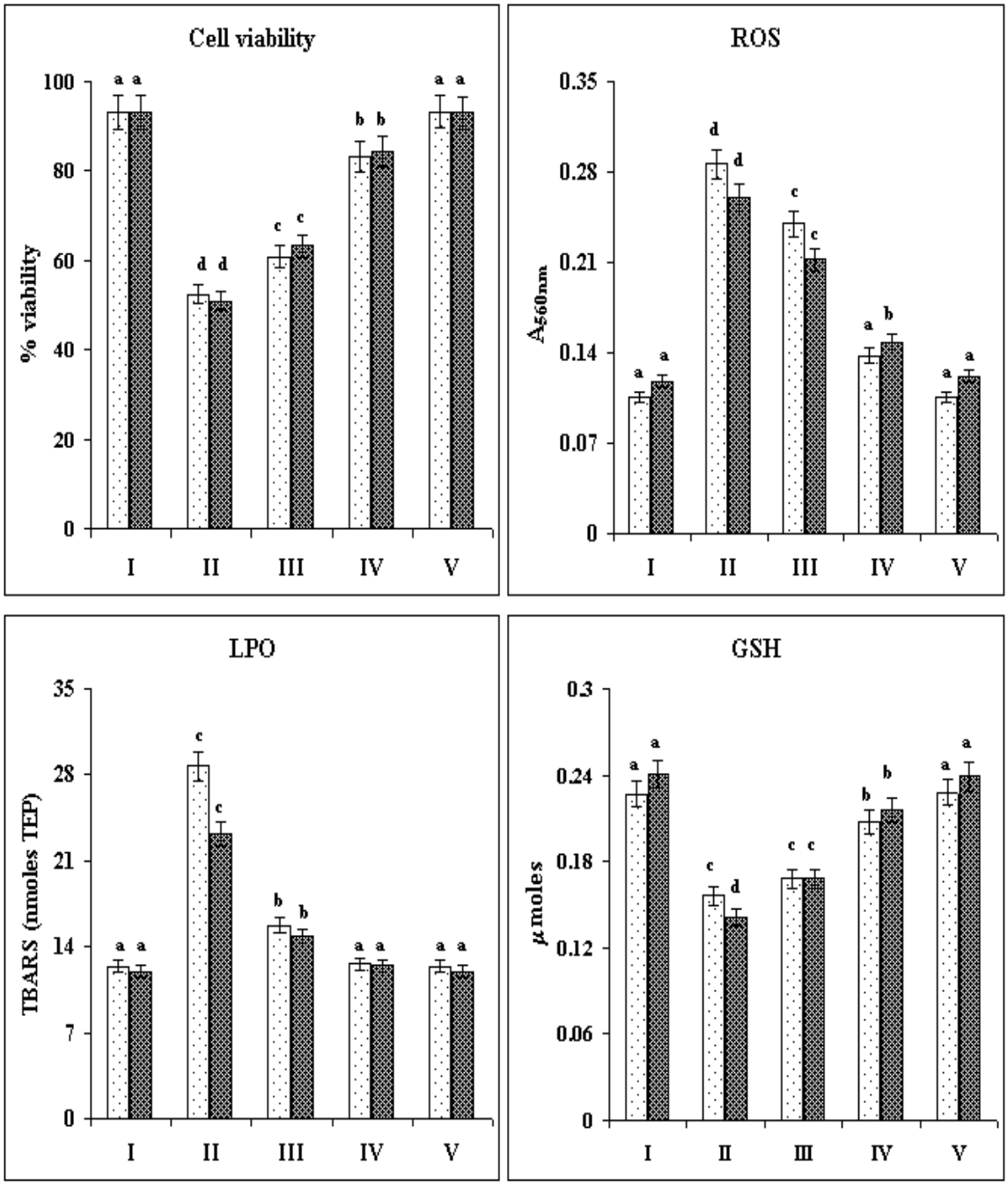


Figure 3.14.: Cytoprotective effect of DHA V in primary hepatocytes.

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.060mM), IV: Toxicant + DHA-I (0.240mM), V: DHA-I (0.240mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

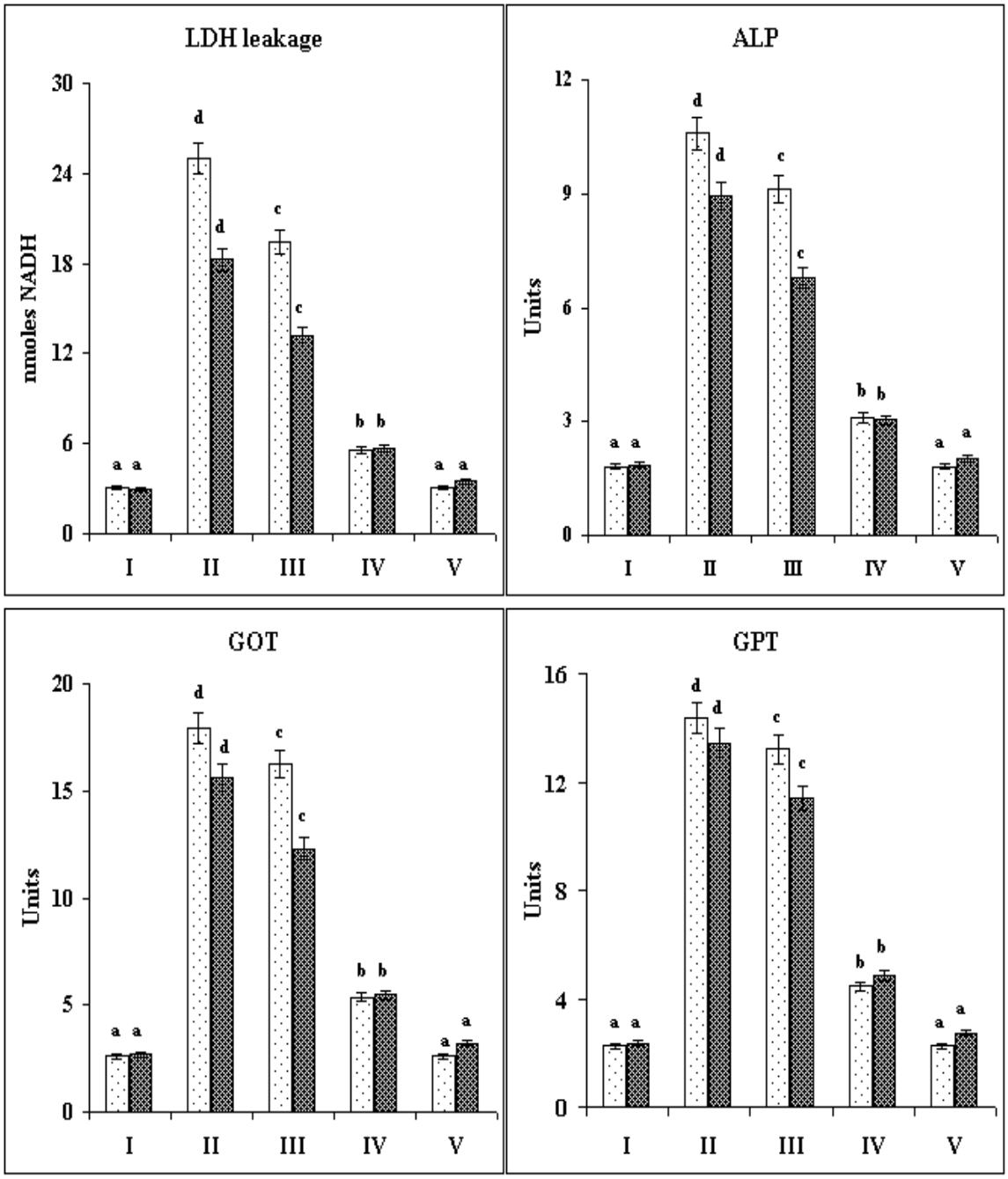


Figure 3.15.: Cytoprotective effect of DHA V in primary hepatocytes: Hepatic marker enzymes

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.060mM), IV: Toxicant + DHA-I (0.240mM), V: DHA-I (0.240mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$. GOT: Glutamate oxaloacetate transaminase, GPT: Glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

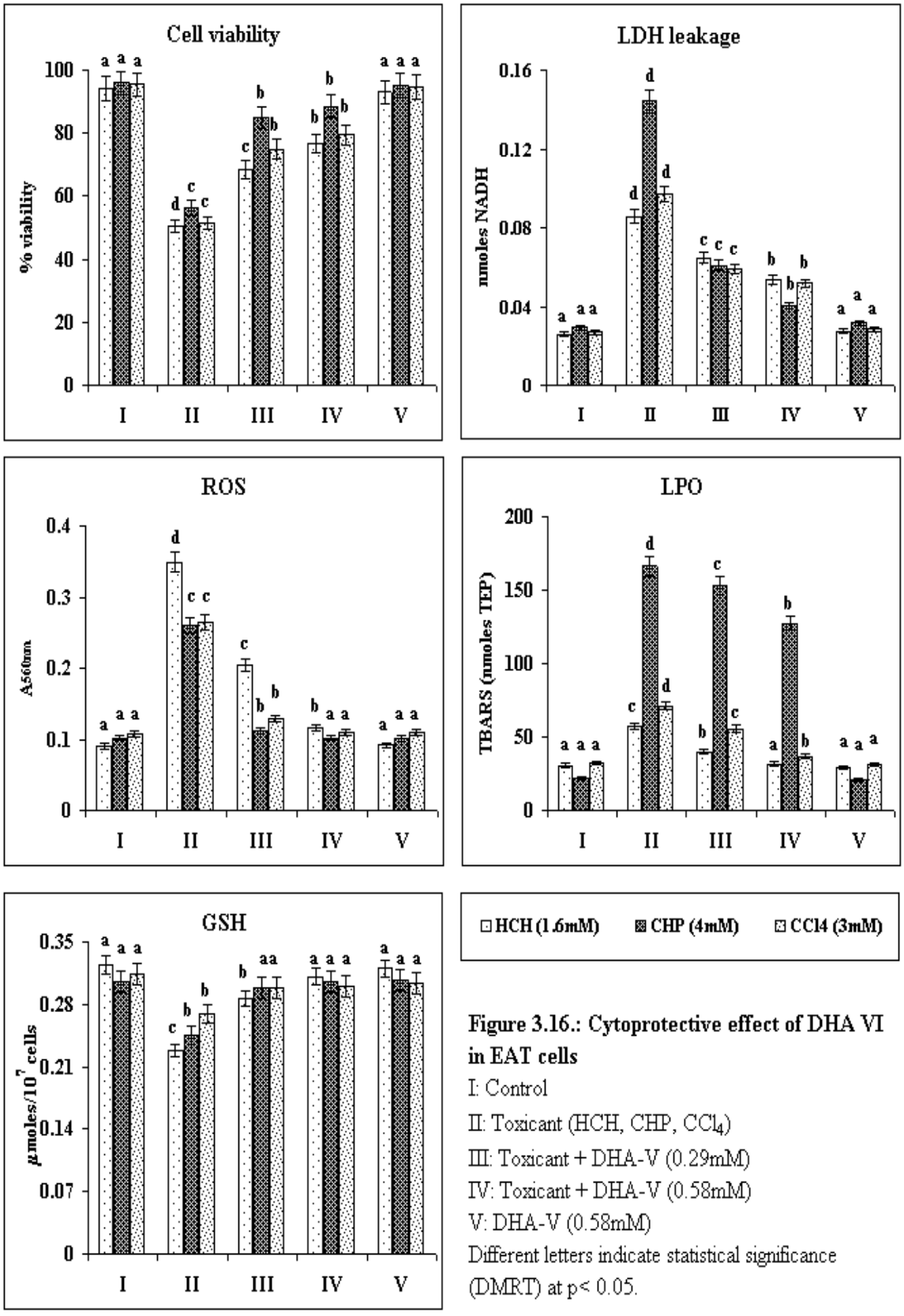


Figure 3.16.: Cytoprotective effect of DHA VI in EAT cells

I: Control

II: Toxicant (HCH, CHP, CCl_4)

III: Toxicant + DHA-V (0.29mM)

IV: Toxicant + DHA-V (0.58mM)

V: DHA-V (0.58mM)

Different letters indicate statistical significance (DMRT) at $p < 0.05$.

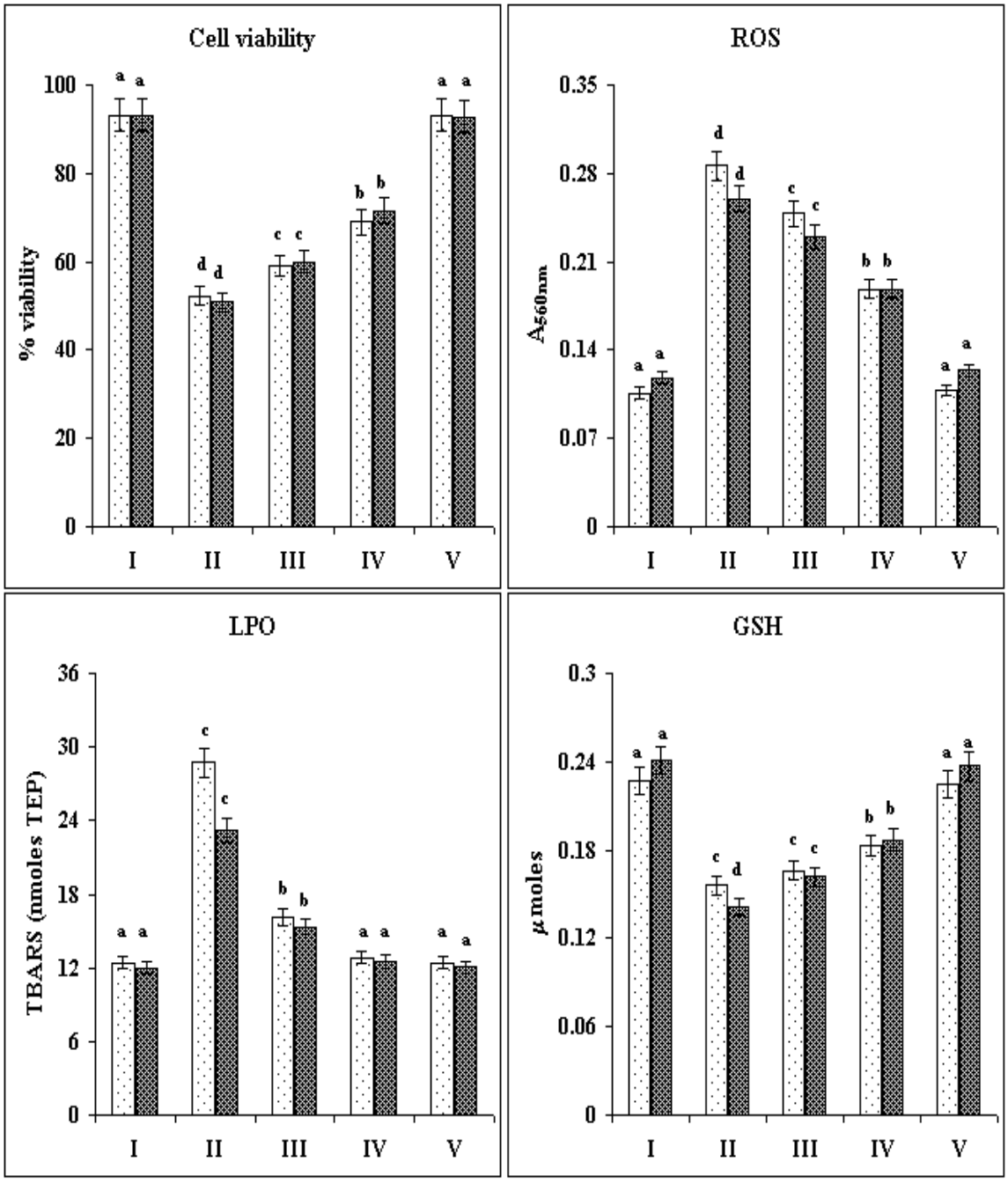


Figure 3.17.: Cytoprotective effect of DHA VI in primary hepatocytes.

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.058mM), IV: Toxicant + DHA-I (0.232mM), V: DHA-I (0.232mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$.

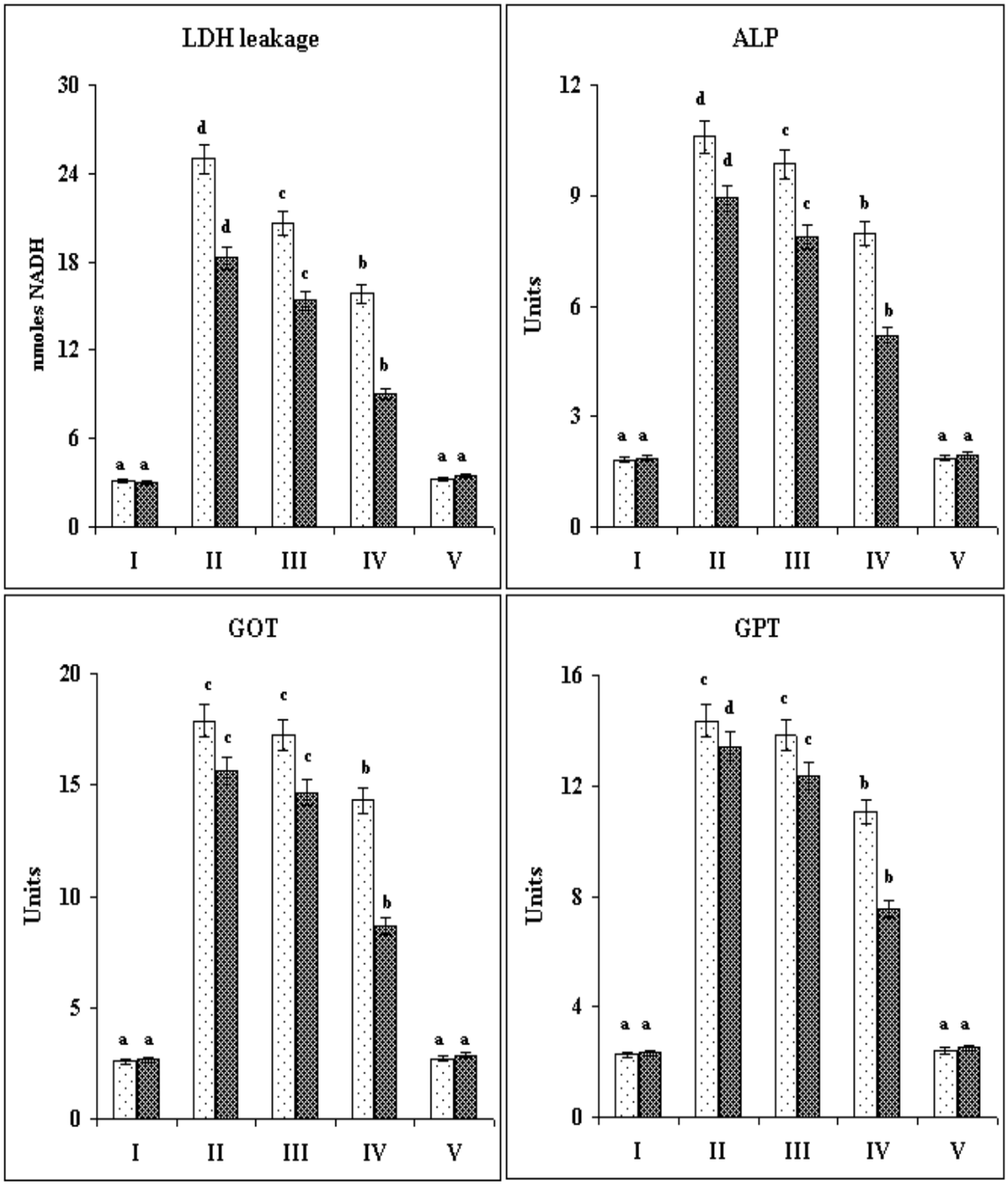


Figure 3.18.: Cytoprotective effect of DHA VI in primary hepatocytes: Hepatic marker enzymes

I: Control, II: Toxicant (CCl_4, Ethanol), III: Toxicant + DHA-I (0.058mM), IV: Toxicant + DHA-I (0.232mM), V: DHA-I (0.232mM)

Different letters indicate statistical significance (DMRT) at $p< 0.05$. GOT: Glutamate oxaloacetate transaminase, GPT: Glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDH: Lactate dehydrogenase

DISCUSSION

Several studies have shown that plant-derived polyphenolic antioxidants exhibit various biological activities such as anti-inflammatory, anticancer and antiatherosclerotic activities. The beneficial health effects of antioxidants have been attributed to their ability to scavenge free radicals (Halliwell, 1999). Herbal extracts, including traditional medicines possess antioxidant properties (Zheng & Wang, 2001). There is an overwhelming evidence that phytochemicals could be used as effective antioxidants for improving human health by preventing or delaying degenerative diseases (Cui et al., 2000).

In vitro studies on cell cultures are often used as a model system to study the mechanism of xenobiotic-induced cell injury/death and its amelioration by phytochemicals (Robertson, & Orrenius, 2000, Srivastava and Shivanandappa, 2006). Several phytochemicals have been evaluated for their protective activity against xenobiotic induced toxicity in experimental models *in vitro* and *in vivo* conditions (Saraswat et al., 2000; Iverson, 2003; Zhang, 2004).

A number of prooxidant drugs and other chemicals have been implicated in the oxidative stress and cell injury resulting from the intracellular production of injurious ROS (Halliwell and Gutteridge, 1999; Zima and Kalousova, 2005; Srivastava and Shivanandappa, 2006). Hexachlorocyclohexane, carbon tetrachloride, ethanol and cumene hydroperoxide are well known inducers of oxidative stress in cell systems (Tseng et al., 1996; Ahmed et al., 2000). CHP, a short-chain analog of lipid hydroperoxide, has often been used as a model to investigate the mechanism of cell injury initiated by acute oxidative stress (Lee et al., 2004). CHP is metabolized to free radical intermediates by

cytochrome P450 in hepatocytes or by hemoglobin in erythrocytes, which in turn initiates lipid peroxidation and GSH depletion, affecting cell integrity and results in cell injury in hepatocytes (Lin et al., 2000). CHP also causes mitochondrial depolarization within intact hepatocytes and mediates DNA damage in cultured mammalian cells (Altman et al., 1994). Alternatively, CHP could also be rapidly converted by glutathione peroxidase to *t*-butyl alcohol with concomitant oxidation of glutathione (GSH) to glutathione disulfide (GSSG). GSSG is then converted to GSH by GSSG reductase, resulting in pyridine nucleotide oxidation. The loss of GSH and the oxidation of pyridine nucleotides are associated with altered Ca^{2+} homeostasis, which is considered to be a critical event in CHP-induced toxicity (Shimizu et al., 1998).

Ethanol, another well known hepatotoxicant, induces the formation of reactive oxygen species (ROS) and enhances peroxidation of lipids, protein and DNA (Cederbaum, 2001). The exact mechanism (s) by which ethanol causes cell injury are still not certain. Some of the leading hypotheses suggested include redox state changes (decrease in the NAD^+/ NADH redox ratio) produced as a result of ethanol oxidation by alcohol and acetaldehyde dehydrogenases; production of the reactive product acetaldehyde as a consequence of ethanol oxidation; induction of CYP2E1; mobilization of iron which results in enhanced levels of low molecular weight non heme iron; effects on antioxidant enzymes and molecules; one electron oxidation of ethanol to the 1-hydroxy ethyl radical; conversion of xanthine dehydrogenase to the ‘oxidase’ form (Bondy, 1992; Nordmann, 1994; Tsukamoto and Lu, 2001). Many of these pathways are not exclusive of one another and it is likely that several systems contribute to ethanol induced oxidative stress.

Hexachlorocyclohexane (HCH) is an organochlorine insecticide comprising of at least six stereoisomers. HCH is known to induce cell injury or cell death in neuroactive (PC-12) cells, cerebellar granule neurons and alveolar macrophages (Rosa et al., 1997; Kang et al., 1998). *In vitro* studies using cell cultures have shown that HCH induces cell death that is mediated by rise in intracellular Ca^{2+} which triggers ROS production (Rosa et al., 1997; Kang et al., 1998). Induction of ROS and LPO in cells exposed to HCH has been reported. HCH induced cytotoxicity involves increased production of ROS. (Bagchi et al., 1995; Srivastava and Shivanandappa, 2006). In mammalian cells, ROS is generated in the presence of NADPH by the action of NADPH oxidase (Hurst and Barrette, 1989). It is believed that ROS generation is due to NADPH oxidase which is activated by protein kinase C as a consequence of raised intracellular Ca^{2+} (Lambeth, 1988). HCH-induced cytotoxicity could be ameliorated by antioxidants which act by suppressing the induction of ROS (Srivastava and Shivanandappa, 2006).

In this study, antioxidant compounds isolated from *D. hamiltonii* inhibited xenobiotic-induced LPO in both EAT cells and hepatocytes. Further, the results show that the antioxidant compounds either suppressed ROS production or scavenged the ROS produced, which is responsible for the amelioration of cytotoxic cell death. Our results are consistent with earlier reports wherein antioxidants have been shown to suppress xenobiotic induced ROS production in other cell types (Festa et al., 2001; Fedeli et al., 2004). Our results support the view that induction of ROS in cells by xenobiotics is correlated with oxidative stress-mediated cell death (Sreekumar et al., 2005)

It is well known that cells have evolved effective antioxidant defense systems against ROS. Of the cellular antioxidants, the best known endogenous antioxidant is

GSH, an abundant and ubiquitous low-molecular weight thiol which is involved in many cellular processes such as, amino acid transport, synthesis of proteins and metabolism of xenobiotics, carcinogens and ROS (Pompella et al., 2003). Depletion of GSH leads to severe oxidative stress and alters the redox status of cells (Cao & Li, 2002). In this study, we have demonstrated that xenobiotic-induced GSH depletion in EAT cells and hepatocytes was restored by the antioxidant compounds from *D. hamiltonii*. Our results show that the isolated compounds protect the cells from xenobiotic-induced cell injury/death by relieving oxidative stress and, atleast in part, by restoring the redox potential.

Antioxidant compounds react directly with radicals relieving oxidative stress and exerting positive effects against cellular damage (Gaetke and Chow, 2003). Our study has demonstrated that the antioxidant compounds isolated from the roots of *D. hamiltonii* protect against xenobiotic cytotoxicity. However, the relative cytoprotection by the antioxidant compounds could be explained by their different modes of action as evident from the data. Our results are consistent with the observations of Ishige et al. (2001) that the protective action of antioxidants in mammalian cells may involve distinct pathways. The antioxidant compounds isolated from *D. hamiltonii* are novel cytoprotective compounds reported for the first time in this study.

In conclusion, the antioxidant compounds from the aqueous extract of the roots of *D. hamiltonii* ameliorated xenobiotic-induced cell injury/death. The mechanism of cytoprotective action appears to involve maintaining the intracellular GSH, suppression of ROS induction and inhibition of LPO. These novel antioxidant compounds could be

potential candidates as therapeutic agents for preventing or delaying degenerative diseases.

Chapter IV

HEPATOPROTECTIVE ACTIVITY

INTRODUCTION

Chronic liver damage is a widespread pathology characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. Among the xenobiotics known to cause hepatotoxicity carbon tetrachloride (CCl_4) is a well characterized hepatotoxic agent (Kodavanti et al., 1989). The hepatotoxic effect of CCl_4 is attributed to the generation of $\cdot CCl_3$ radical formed by reductive dehalogenation by the P450 enzyme system. The highly reactive trichloromethyl radical rapidly reacts with molecular oxygen to form trichloromethyl peroxy radical. The peroxyl radical initiates lipid peroxidation which is considered to be the most important mechanism in the pathogenesis of liver damage induced by CCl_4 (Demirdag et al., 2004). CCl_4-induced hepatocellular damage leads to leakage of soluble enzymes as seen by the elevated levels of hepatic enzymes such as AST and ALT in the serum considered as diagnostic markers for hepatic damage (Wang et al., 2004). CCl_4-induced damage causes characteristic changes in the histological appreance of the liver showing massive necrotic areas caused by cell death and fatty degeneration (Teocharis et al., 2001). CCl_4 also alters the antioxidant profile of the liver including the antioxidant enzymes and glutathione level (Sheweita et al., 2001).

Ethanol is also a well known hepatotoxic chemical. Ethanol induces the production of reactive oxygen species (ROS) and enhances peroxidation of lipids, protein and DNA in a variety of experimental systems including humans (Cederbaum, 2001). The exact mechanism (s) by which ethanol causes cell injury are still not certain. Some of the leading hypotheses suggested to play a role in ethanol-induced cell injury include redox state changes (decrease in the NAD^+/ NADH redox ratio) produced as a result of

ethanol oxidation by alcohol and acetaldehyde dehydrogenases; production of the reactive product acetaldehyde as a consequence of ethanol oxidation; ethanol induction of CYP2E1; ethanol mobilization of iron which results in enhanced levels of low molecular weight non heme iron; effects on antioxidant enzymes and molecules; one electron oxidation of ethanol to the 1-hydroxy ethyl radical; conversion of xanthine dehydrogenase to the oxidase form (Bondy, 1992; Nordmann, 1994; Tsukamoto and Lu, 2001). Many of these pathways are not exclusive of one another and it is likely that several systems contribute to the ability of ethanol to induce a state of oxidative stress.

Oxidative damage to biological molecules is an important event in the development of a variety of degenerative diseases that result from overwhelming the biological defense system against oxidative stress, drugs and carcinogens. Higher intake in the human diet of antioxidant compounds can prevent and in some cases help in the treatment of some oxidative-related disorders and carcinogenic events (Havsteen, 2002). Natural products have been used for health benefits since ancient times, and there is greater demand for their use today. As the oxidative stress plays a central role in liver pathologies and progression, the use of antioxidants have been proposed as therapeutic agents, as well as drug coadjuvants, to counteract liver damage (Vitaglione et al., 2004). A number of plants have been reported to possess antioxidant properties like *Bacopa monnieri* (Bhattacharya et al., 2000), *Salvia officinalis* (Lima et al., 2005), *Phyllanthus niruri* (Harish and Shivanandappa, 2006) and *Acanthopanax senticosus* (Lin and Huang, 2000). A number of investigators have previously demonstrated that antioxidants prevent CCl_4 and ethanol hepatotoxicity by inhibiting lipid peroxidation, suppressing hepatocellular damage and enhancing the antioxidant enzyme activity (Jeon et al., 2003;

Naik et al., 2004; Wang et al., 2004). Several Indian medicinal plants have been reported to show hepatoprotective properties such as *Curcuma longa*, *Phyllanthus niruri*, *Swertia chirata, Cichorium intybus* (Scartezzini and Speroni, 2000; Harish and Shivanandappa, 2006).

Tubers of *D. hamiltonii* (Wight and Arn.)(family: Asclepiadaceae) are consumed as pickles and juice for its alleged health promoting properties. The roots are used in folk medicine and as a substitute for *Hemidesmus indicus* in ayurvedic preparations (Nayar et al., 1978). We have earlier shown that the roots of *D. hamiltonii* possess potent antioxidant properties which could be associated with their alleged health benefits (Srivastava et al., 2005). Earlier work suggested that the roots of *D. hamiltonii* contain aldelydes, inositols, saponins, amyrins, lupeols and volatile flavour compounds such as 2-hydroxy-4methoxybenzaldehyde, vanillin, 2-phenyl ethyl alcohol, benzaldehyde, and others (Nagarajan et al., 2001). Our recent work has shown that the aqueous extract of the roots of *D. hamiltonii* is a cocktail of novel antioxidants viz., 4-hydroxy isophthalic acid, 14-aminotetradecanoic acid, 4-(1-hydroxy-1-methylethyl)-1-methyl-1, 2-cyclohexane diol, 2-hydroxymethyl-3-methoxybenzaldehyde, 2,4,8 trihydroxybicyclo [3.2.1]octan-3-one (Srivastava et al., 2006). Since the root extract are rich in antioxidants, it is hypothesized that they possess hepatoprotective potential. The objective of the present study is to investigate (a) the hepatoprotective potential of *D. hamiltonii* aqueous extract (DHA) pretreatment against CCl_4 and ethanol induced hepatotoxicity in rats, and (b) antioxidant potential of DHA on the liver of rats.

MATERIALS AND METHODS

Chemicals: Nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), cumene hydroperoxide (CHP), pryogallol, bovine serum albumin (BSA), 2,4-dinitrophenyl hydrazine (DNPH), tetraethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

Preparation of the root powder and extraction: Tuberous roots of D.hamiltonii (10 kg) were procured from the local suppliers. The roots were washed with water, followed by crushing with a roller to separate the inner woody core from the outer fleshy layer. The fleshy portion was collected, dried at 40^{o}C in a hot air oven and fine powdered. The powder (1.9 kg) was used for extraction.

We have earlier reported that aqueous extract of *D.hamiltonii* shows high antioxidant activity among the different solvent extracts (Srivastava et al., 2005). The aqueous extract was prepared by homogenizing the root powder (200g) in warm water (50^{o} C) and allowed to stand for 24 hours, filtered with Whatman paper No. 1 and the filtrate was lyophilized and weighed (34.75g).

Animals and treatments: Sixty day old adult male Wistar rats (180-200g) were divided into different groups, eight in each. The prevailing Indian Animal Ethics Committee guidelines were followed for the use of experimental animals. In single dose experiment, administration of aqueous extract of the roots of *D. hamiltonii* at 50, 100 and 200 mg/kg

b.w. was followed, after 1h, by CCl_4 (1ml/kg b.w.) administration. In multiple dose experiment, aqueous extract of the roots of *D. hamiltonii* was administered for 7 consecutive days at 50 and 100 mg/kg b.w. followed by a single dose of CCl_4 (1ml/kg b.w.) administration on 7^{th} day, 1h after administering the extract. Animals were sacrificed by ether anesthesia after 16h; the livers perfused with saline, were dissected out and processed immediately for biochemical assays.

Experimental design

A. CCl_4 hepatotoxicity:

Single dose: Group I- Control; Group II- DHA (200mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group IV- DHA (100mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group V- DHA (200mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group VI- CCl_4 (1ml/ kg b.w.) (sunflower oil was used as the vehicle).

Multiple dose: Group I- Control; Group II- DHA (100mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group IV- DHA (100mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group V- CCl_4 (1ml/ kg b.w.).

B. Ethanol hepatotoxicity:

Single dose: Group I- Control; Group II- DHA (200mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + Ethanol (5g/kg b.w.); Group IV- DHA (100mg/kg b.w.) + Ethanol (5g/kg b.w.); Group V- DHA (200mg/kg b.w.) + Ethanol (5g/kg b.w.); Group VI- Ethanol (5g/kg b.w.) (physiological saline was used as the vehicle).

Multiple dose: Group I- Control; Group II- DHA (100mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + Ethanol (5g/kg b.w.); Group IV- DHA (100mg/kg b.w.) + Ethanol (5g/kg b.w.); Group V- Ethanol (5g/kg b.w.).

C. *In vivo* antioxidant potential of DHA:

Multiple doses for 5, 15 and 30 days: Group I- Control; Group II- DHA (50mg/kg b.w.); Group III- DHA (100mg/kg b.w.).

Serum enzymes: Blood samples were collected in tubes, allowed to clot and the serum was removed by centrifugation at 2000g for 10 min. The serum samples were stored at 4^0C until used for biochemical assays. Serum alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) were determined by the method of Reitman and Frankel (1957) as described earlier in Chapter III. Lactate dehydrogenase (LDH) activity was estimated by the method of Kornberg (1955). The reaction mixture consisted of NADH (0.02M), sodium pyruvate (0.01M), sodium phosphate buffer (0.1M, pH 7.4) in a total volume of 3ml. The changes in the absorbance were recorded at 340nm at 30 sec interval for 3 min. Alkaline phosphatase (ALP) activity was assayed by the method of Walter and Schutt (1974). p-nitrophenol phosphate (1.25mM) and serum were incubated in 1.0ml tris buffer (0.1M, pH 8.5) at 37^oC for 30 min. The reaction was stopped by adding NaOH (0.02M) and the absorbance was read at 405nm. The enzyme activity was calculated using the extinction coefficient 1.85×10^{-3} $M^{-1}cm^{-1}$ for p-nitrophenol.

Lipid peroxidation: Lipid peroxide content in the tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). Tissue homogenate (10% w/v in 50 mM phosphate buffer, pH 7.4) was boiled in TCA (10 %) and TBA (0.34 %) for 15min, cooled and centrifuged. Absorbance of the supernatant was read at 535 nm. TBARS was calculated using tetraethoxypropane as the standard.

Antioxidant enzymes: Liver tissue was homogenized (10% w/v) in ice-cold 50 mM phosphate buffer (pH 7.4), centrifuged at 10,000g for 20min at 4^{o}C and the supernatant was used to assay the enzyme activities. Superoxide dismutase (SOD) activity was measured using pyrogallol (2mM) autoxidation in tris buffer (Marklund and Marklund, 1974). Catalase (CAT) activity was measured using H_2O_2 (3 %) as the substrate in 0.1 M phosphate buffer (pH 7.4) (Aebi, 1974). Glutathione peroxidase (GPx) activity was measured by the indirect assay method using glutathione reductase. Cumene hydroperoxide (1mM) and glutathione (0.25mM) were used as substrates and oxidation of NADPH by glutathione reductase (0.25 U) in tris buffer was monitored at 340nm (Mannervik, 1985). Glutathione reductase (GR) activity was estimated using oxidized glutathione (20mM) and NADPH (2mM) in 0.1 M potassium phosphate buffer (pH 7.4) (Calberg and Mannervik, 1985). Glutathione transferase (GST) activity was estimate by the method of Warholm, et al. (Warholm et al., 1985) using glutathione (20mM) and CDNB (30mM) as the substrates in 0.05 M phosphate buffer (pH 7.4), change in absorbance at 344nm was monitored in a UV-Visible Spectrophotometer.

Glutathione: GSH assay was done as described previously (see Chapter III).

Protein carbonyls: Tissue homogenates (10% w/v) were prepared in 20mM tris-HCl buffer (pH 7.4) containing 0.14M NaCl, centrifuged at 10,000g for 10 min at 4^{o}C. 1.0ml of the supernatant was precipitated with an equal volume of 20% TCA and centrifuged. The pellet was resuspended in 1.0ml of DNPH (10mM in 2M HCl) and allowed to stand at room temperature for 60 min with occasional vortexing. 0.5ml of 20% TCA was added to the reaction mixture and centrifuged; the pellet obtained was washed 3 times with acetone and 1.0ml of 2% of SDS (in 20mM tris-HCl, 0.1M NaCl , pH 7.4) was added to

dissolve the pellet. The absorbance of the solution was read at 360nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} (Levine et al., 1990).

Protein estimation was done as described previously (see Chapter III).

Histopathological examination: Pieces of liver tissue were excised from the major lobe of the liver and fixed in Bouin's fluid for 24h. The tissue was further processed for paraffin embedding. 6μm sections were stained with hematoxylin and eosin for microscopic observation.

Statistics: The data were expressed as means ± S.E. of eight observations (n = 8) and significant difference between each of the groups was statistically analyzed by Duncan's multiple range test (Statistica Software, 1999). A difference was considered significant at $p<0.05$.

RESULTS

Serum enzymes

Levels of the serum marker enzymes AST, ALT, LDH and ALP, were raised significantly in CCl_4 and ethanol treated rats compared to the control group. In single dose experiments, administration of DHA at 100 and 200 mg/kg b.w. prevented the liver damage induced by CCl_4 and ethanol as evident by the restored serum marker enzymes (Figure 4.1. and 4.3.). Pretreatment of DHA with multiple dose (7 days) at a lower dose (50mg/kg b.w.) was more effective in hepatoprotective effect than a single higher dose (Figure 4.2. and 4.4.).

Lipid peroxidation

The effects of DHA on CCl_4 and ethanol induced lipid peroxidation measured as TBARS in liver are shown in Table 4.1.- 4.4.. CCl_4 and ethanol significantly increased the TBARS which was inhibited by DHA pretreatment. In the single dose experiment, DHA at 100 and 200 mg/kg b.w. inhibited LPO, whereas in multiple dose (7 days) experiment even a lower dose of 50mg/kg b.w.. DHA pretreatment was effective in lowering the basal TBARS level (Fig. 4.6.).

Antioxidant enzymes

The hepatic antioxidant enzyme activities were decreased more in rats after administration of CCl_4 compared to ethanol. The activities of the enzymes, SOD, CAT, GPx, GR and GST were restored by DHA pretreatment. Multiple dose pretreatment with DHA was more effective than single dose in enhancing the antioxidant enzymes. Further, DHA multiple dose pretreatment itself enhanced the antioxidant enzyme activities (Table 4.1.- 4.4.).

Glutathione

Administration of CCl_4 and ethanol decreased the hepatic GSH level, which was more in case of ethanol. GSH was restored to normal level by DHA pretreatment (Table 4.1.- 4.4.). Pretreatment of DHA increased the hepatic GSH levels, which was significant after one week pretreatment (Fig. 4.6.).

Protein carbonyls

CCl_4 and ethanol treatment increased the protein carbonyl content significantly in the rat liver. DHA pretreatment prevented the xenobiotic-induced protein carbonyl formation which was dose dependent (Table 4.1.- 4.4.).

Histopathology

In the CCl_4 administered rats, massive centrilobular necrosis, ballooning (vacuolar) degeneration and cellular infiltration of the liver was observed. Ethanol administered rats showed centrilobular degeneration and fatty changes in the liver. Liver histopathological changes were less severe in the case of ethanol compared to that of CCl_4. Pretreatment of DHA reduced the severity of the hepatic damage which was dose-dependent. Multiple dose pretreatment with a lower dose (50mg/kg x 7 days) of DHA was more effective than a single higher dose (200mg/kg b.w.) in hepatoprotective effect (Plate 4.1.- 4.4.).

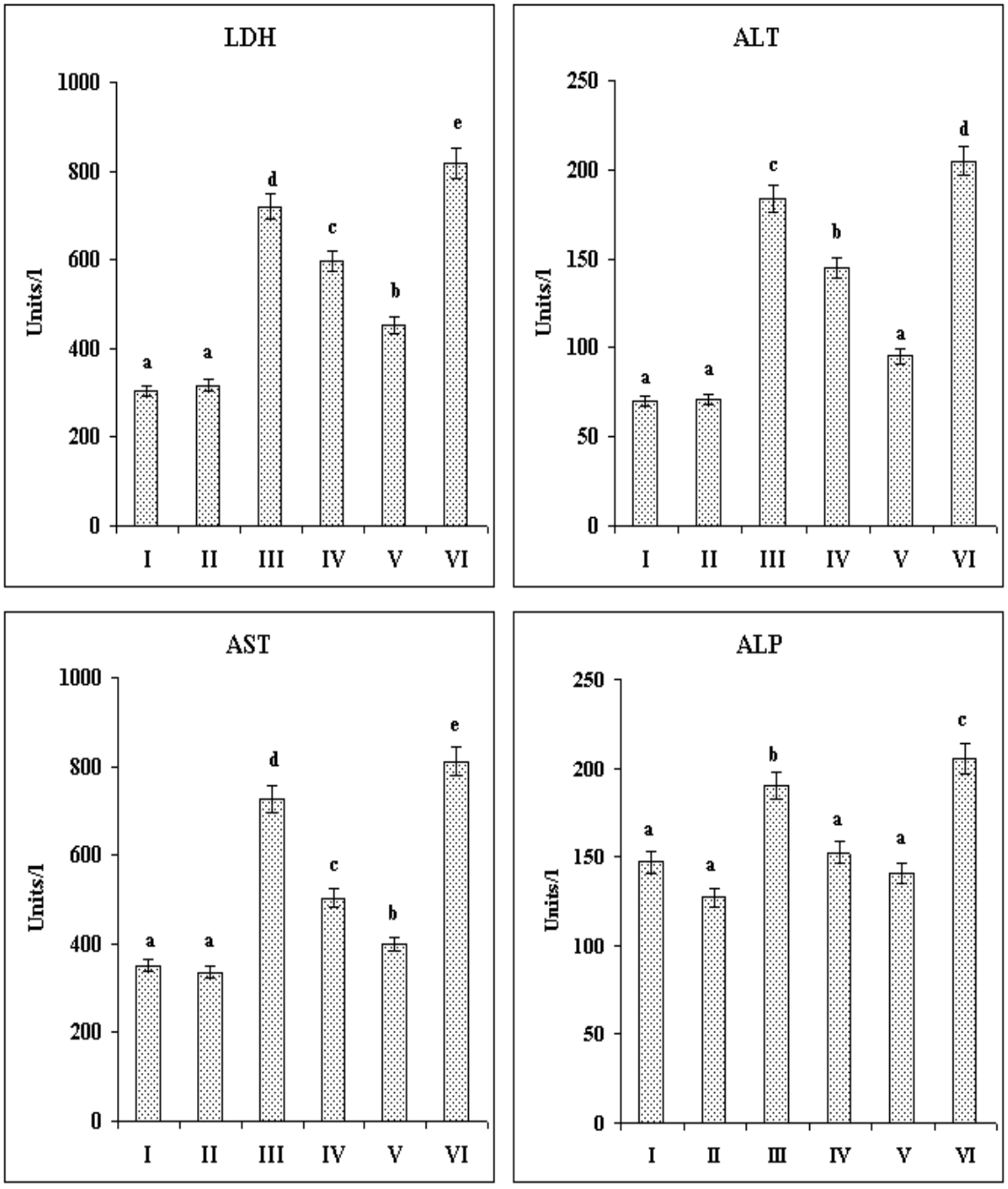


Figure 4.1.: Protective effect of the aqueous extract of the roots of *D. hamiltonii* (pretreatment- **single dose**) on CCl_4 hepatotoxicity: serum enzymes.

Treatments- I: Control; II: DHA (200mg/kg bw); III: DHA (50mg/kg bw) + CCl4 (1ml/kg bw); IV: DHA (100mg/kg bw) + CCl4 (1ml/kg bw); V: DHA (200mg/kg bw) + CCl4 (1ml/kg bw); VI: CCl4 (1ml/kg bw).

LDH: Lactate dehydrogenase, **ALT:** Alanine aminotransferase, **AST:** Aspartate aminotransferase, **ALP:** Alkaline phosphatase.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at $p < 0.05$ level (DMRT).

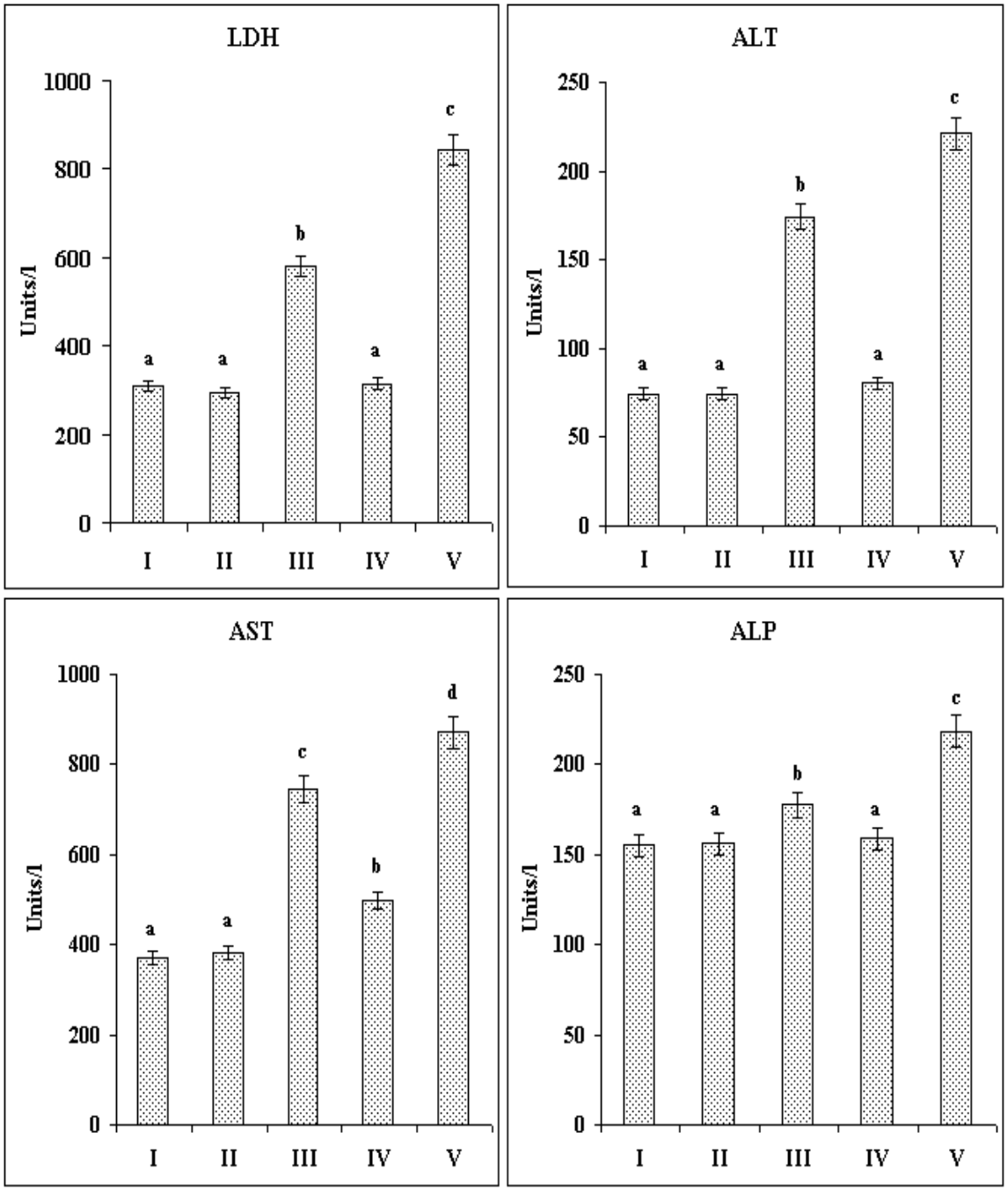


Figure 4.2.: Protective effect of the aqueous extract of the roots of *D. hamiltonii* (pretreatment- **multiple dose**) on CCl_4 hepatotoxicity: serum marker enzymes

Treatments- I: Control, II: D.h.aq. (100mg/kg bw); III: D.h.aq. (50mg/kg bw) + CCl_4 (1ml/kg bw); IV: D.h.aq. (100mg/kg bw) + CCl_4 (1ml/kg bw); V: CCl_4 (1ml/kg bw).

LDH: Lactate dehydrogenase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at $p < 0.05$ level (DMRT).

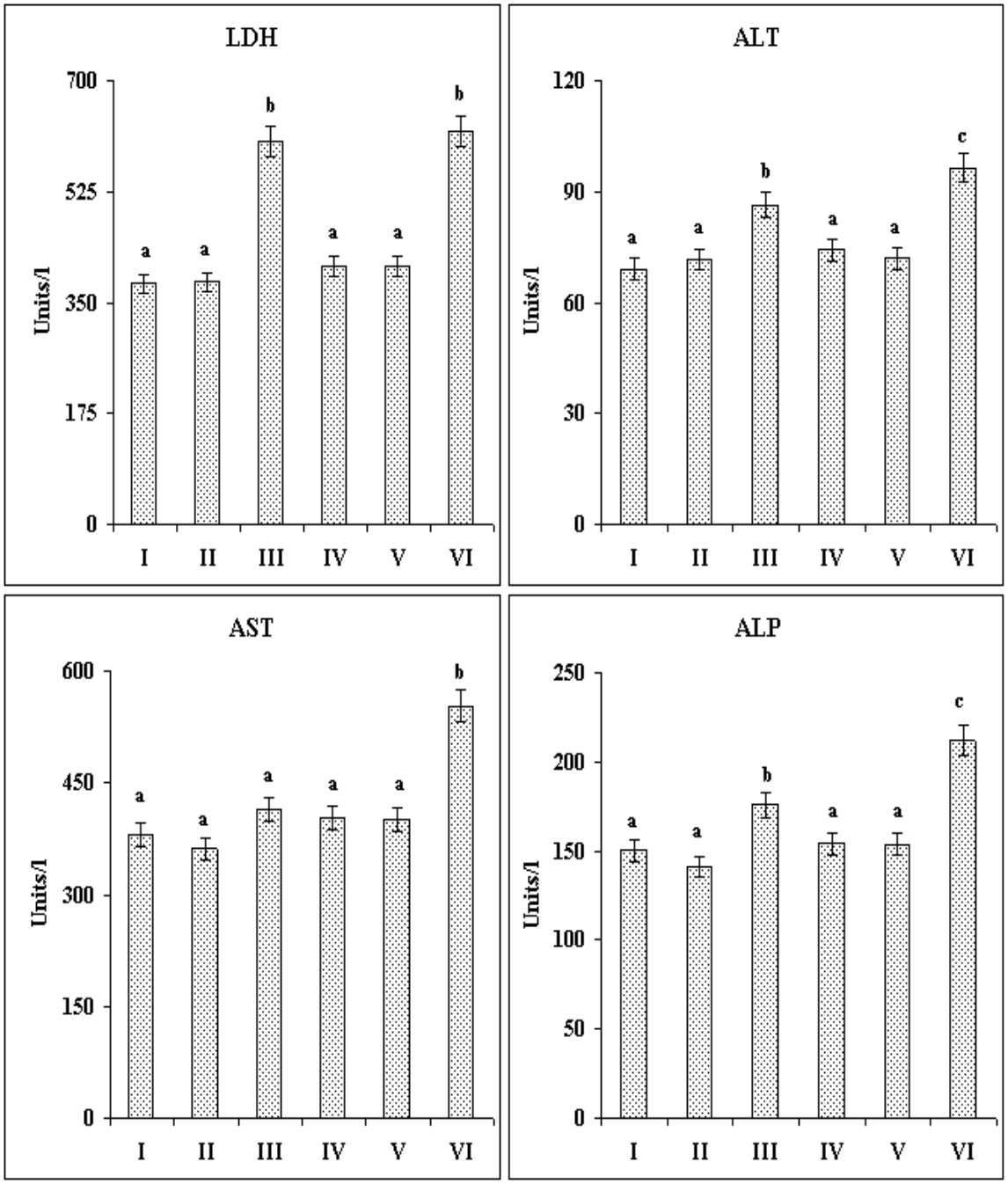


Figure 4.3.: Protective effect of the aqueous extract of the roots of *D. hamiltonii* (pretreatment- **single dose**) on ethanol hepatotoxicity: serum enzymes.

Treatments- I: Control; II: DHA (200mg/kg bw); III: DHA (50mg/kg bw) + ethanol (5g/kg bw); IV: DHA (100mg/kg bw) + ethanol (5g/kg bw); V: DHA (200mg/kg bw) + ethanol (5g/kg bw); VI: ethanol (5g/kg bw).

LDH: Lactate dehydrogenase, **ALT:** Alanine aminotransferase, **AST:** Aspartate aminotransferase, **ALP:** Alkaline phosphatase.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at $p < 0.05$ level (DMRT).

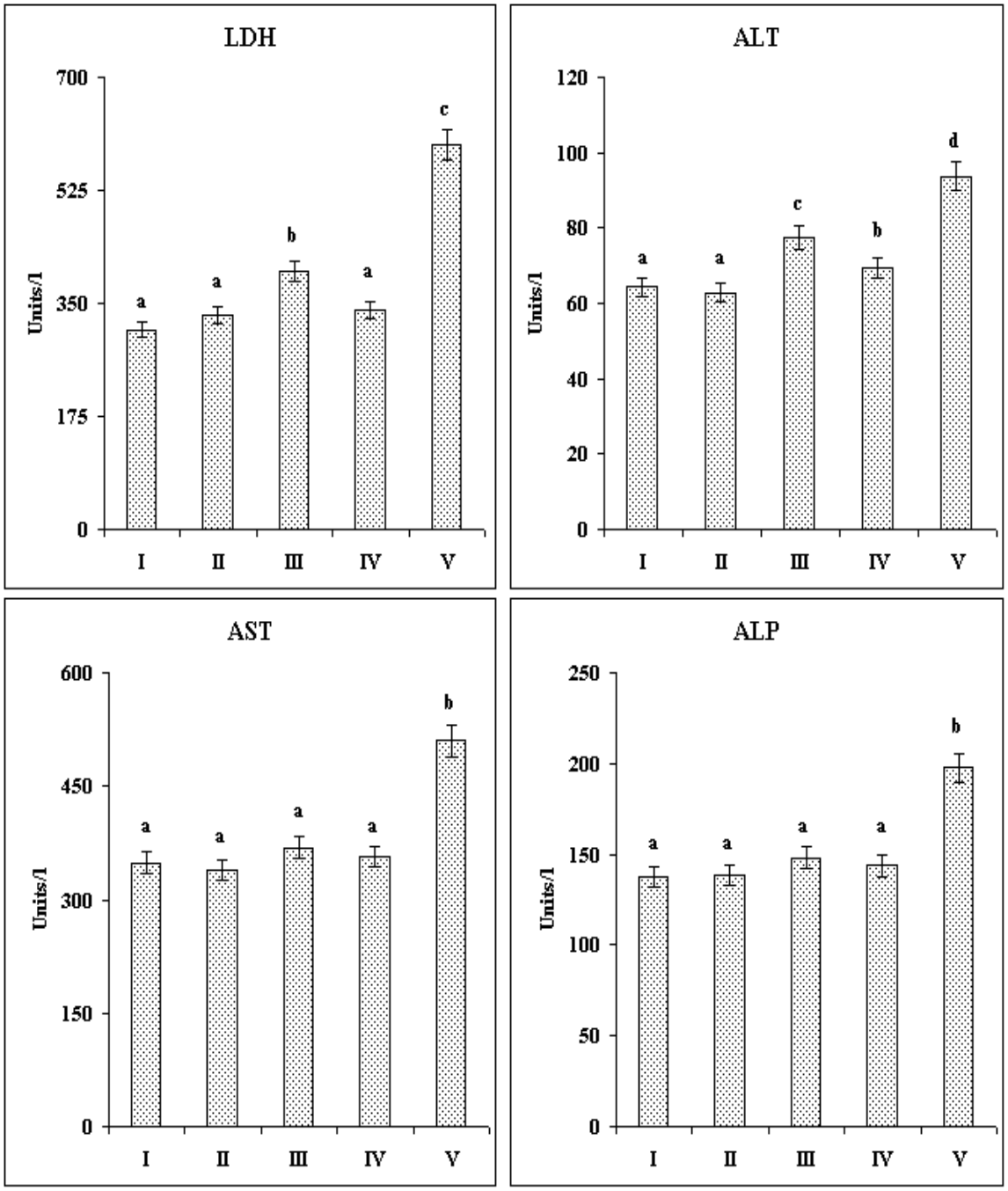


Figure 4.4.: Protective effect of the aqueous extract of the roots of *D. hamiltonii* (pretreatment- **multiple dose**) on ethanol hepatotoxicity: serum enzymes.

Treatments- I: Control; II: DHA (200mg/kg bw); III: DHA (50mg/kg bw) + ethanol (5g/kg bw); IV: DHA (100mg/kg bw) + ethanol (5g/kg bw); V: DHA (200mg/kg bw) + ethanol (5g/kg bw); VI: ethanol (5g/kg bw).

LDH: Lactate dehydrogenase, **ALT:** Alanine aminotransferase, **AST:** Aspartate aminotransferase, **ALP:** Alkaline phosphatase.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at $p < 0.05$ level (DMRT).

Table 4.1.: Effect of *D. hamiltonii* aqueous root extract (pretreatment-**single dose**) on hepatic lipid peroxidation, antioxidant profile and protein carbonylation of rats intoxicated with CCl_4.

Group	LPO[1]	SOD[2]	CAT[3]	GPx.[4]	GR[4]	GST[5]	GSH[6]	PC[7]
I	3.79^{a} ±0.28	2.48^{a} ±0.21	5.08^{c} ±0.46	107.87^{b} ±9.95	234.47^{d} ±21.23	134.82^{b} ±12.44	17.82^{c} ±1.61	30.73^{a} ±2.62
II	3.51^{a} ±0.31	2.58^{a} ±0.19	5.39^{c} ±0.52	109.66^{b} ±9.92	246.75^{d} ±22.78	159.01^{c} ±13.49	18.50^{c} ±1.73	30.93^{a} ±2.46
III	5.21^{c} ±0.046	0.48^{c} ±0.03	3.35^{a} ±0.31	83.76^{a} ±7.82	167.48^{b} ±15.49	135.28^{b} ±12.38	15.43^{b} ±1.32	50.42^{b} ±4.51
IV	4.43^{b} ±0.39	1.28^{b} ±0.11	4.17^{b} ±0.38	79.83^{a} ±7.33	208.79^{c} ±18.37	143.27^{bc} ±13.28	17.32^{bc} ±1.64	33.68^{a} ±3.24
V	3.68^{a} ±0.27	2.15^{a} ±0.19	4.90^{c} ±0.35	105.91^{b} ±9.56	230.00^{d} ±21.26	152.18^{bc} ±11.56	18.06^{c} ±1.75	32.32^{a} ±3.11
VI	5.45^{c} ±0.44	0.28^{c} ±0.02	3.00^{a} ±0.27	80.73^{a} ±7.87	128.40^{a} ±11.24	97.09^{a} ±8.41	13.09^{a} ±1.21	49.16^{b} ±3.56

Treatments- I: Control; II: DHA (200mg/kg b.w.); III: DHA (50mg/kg b.w.) + CCl_4 (1ml/kg b.w.); IV: DHA (100mg/kg b.w.) + CCl_4 (1ml/kg b.w.); V: DHA (200mg/kg b.w.) + CCl_4 (1ml/kg b.w.); VI: CCl_4 (1ml/kg b.w.).

[1]nmoles MDA/mg protein, [2]Units/mg proteins, [3]µmole H_2O_2/min/mg protein, [4]nmoles NADPH/min/mg protein, [5]µmole CDNB conjugate/min/mg protein, [6]µg/ mg protein, [7]µmole/mg protein

Means with different superscript letters differ significantly ($p<0.05$).

Table 4.2.: Effect of *D. hamiltonii* aqueous root extract (pretreatment-**multiple dose**) on hepatic lipid peroxidation, antioxidant profile and protein carbonylation of rats intoxicated with CCl_4.

Group	LPO[1]	SOD[2]	CAT[3]	GPx.[4]	GR[4]	GST[5]	GSH[6]	PC[7]
I	3.92^{b} ±0.21	2.35^{a} ±0.22	4.34^{d} ±0.39	105.55^{d} ±10.17	227.77^{c} ±21.36	170.93^{c} ±16.20	16.17^{c} ±1.38	33.20^{a} ±2.91
II	3.47^{a} ±0.29	2.69^{a} ±0.21	4.65^{e} ±0.42	117.70^{e} ±10.26	278.01^{d} ±22.19	189.34^{d} ±17.55	17.41^{d} ±1.59	33.38^{a} ±3.27
III	4.99^{c} ±0.36	0.76^{c} ±0.052	3.49^{b} ±0.31	89.12^{b} ±7.99	196.50^{b} ±17.48	120.35^{a} ±11.45	14.36^{b} ±1.24	38.45^{b} ±3.43
IV	4.08^{b} ±0.37	1.69^{b} ±0.13	3.92^{c} ±0.33	99.84^{c} ±8.46	226.65^{c} ±20.34	150.21^{b} ±12.38	14.50^{c} ±1.32	33.68^{a} ±3.24
V	5.80^{d} ±0.49	0.30^{d} ±0.02	2.67^{a} ±0.24	78.76^{a} ±7.21	118.35^{a} ±10.18	115.96^{a} ±10.38	11.72^{a} ±1.07	50.42^{c} ±4.12

Treatments- I: Control; II: DHA (100mg/kg b.w.); III: DHA (50mg/kg b.w.) + CCl_4 (1ml/kg b.w.); IV: DHA (100mg/kg b.w.) + CCl_4 (1ml/kg b.w.); V: CCl_4 (1ml/kg b.w.).

[1]nmoles MDA/mg protein, [2]Units/mg proteins, [3]µmole H_2O_2/min/mg protein, [4]nmoles NADPH/min/mg protein, [5]µmole CDNB conjugate/min/mg protein, [6]µg/ mg protein, [7]µmole/mg protein

Means with different superscript letters differ significantly ($p<0.05$).

Table 4.3.: Effect of *D. hamiltonii* aqueous root extract (pretreatment-**single dose**) on hepatic lipid peroxidation, antioxidant profile and protein carbonylation of rats intoxicated with ethanol.

Group	LPO[1]	SOD[2]	CAT[3]	GPx.[4]	GR[4]	GST[5]	GSH[6]	PC[7]
I	3.97^{a} ±0.22	2.36^{a} ±0.21	5.92^{e} ±0.47	103.41^{e} ±9.21	276.89^{d} ±23.55	157.73^{c} ±13.47	16.24^{c} ±1.83	36.28^{a} ±3.07
II	3.82^{a} ±0.31	2.52^{a} ±0.16	6.01^{e} ±0.56	106.80^{e} ±9.83	292.52^{d} ±27.64	180.42^{d} ±14.26	16.79^{c} ±1.39	36.33^{a} ±3.38
III	4.60^{b} ±0.37	0.61^{d} ±0.04	4.71^{b} ±0.38	77.87^{b} ±6.43	211.02^{b} ±20.34	133.32^{a} ±12.43	12.87^{b} ±1.17	47.34^{b} ±4.24
IV	4.05^{a} ±0.36	1.41^{c} ±0.11	5.25^{c} ±0.43	86.44^{c} ±7.48	248.98^{c} ±22.38	148.13^{b} ±12.38	14.96^{c} ±12.62	42.21^{ab} ±3.81
V	3.94^{a} ±0.34	2.04^{b} ±0.14	5.60^{d} ±0.46	98.94^{d} ±8.13	276.89^{d} ±24.17	156.58^{c} ±13.84	16.10^{c} ±1.43	37.47^{a} ±3.26
VI	5.43^{c} ±0.49	0.33^{d} ±0.24	4.15^{a} ±0.37	68.58^{a} ±5.39	170.82^{a} ±15.43	127.88^{a} ±11.38	10.04^{a} ±1.01	47.82^{b} ±3.77

Treatments- I: Control; II: DHA (200mg/kg b.w.); III: DHA (50mg/kg b.w.) + ethanol (5g/kg b.w.); IV: DHA (100mg/kg b.w.) + ethanol (5g/kg b.w.); V: DHA (200mg/kg b.w.) + ethanol (5g/kg b.w.); VI: ethanol (5g/kg b.w.).

[1]nmoles MDA/mg protein, [2]Units/mg proteins, [3]µmole H_2O_2/min/mg protein, [4]nmoles NADPH/min/mg protein, [5]µmole CDNB conjugate/min/mg protein, [6]µg/ mg protein, [7]µmole/mg protein

Means with different superscript letters differ significantly ($p<0.05$).

Table 4.4.: Effect of *D. hamiltonii* aqueous root extract (pretreatment-**multiple dose**) on hepatic lipid peroxidation, antioxidant profile and protein carbonylation of rats intoxicated with ethanol.

Group	LPO[1]	SOD[2]	CAT[3]	GPx.[4]	GR[4]	GST[5]	GSH[6]	PC[7]
I	3.72^{b} ±0.28	2.30^{a} ±0.19	5.17^{c} ±0.47	110.55^{d} ±10.98	290.29^{c} ±28.61	143.50^{c} ±12.35	16.62^{cd} ±1.46	31.16^{a} ±2.88
II	3.30^{a} ±0.27	2.59^{a} ±0.22	5.49^{d} ±0.46	112.88^{d} ±10.64	332.72^{d} ±31.45	155.88^{d} ±12.63	17.85^{d} ±1.58	31.28^{a} ±2.63
III	4.62^{b} ±0.43	1.77^{b} ±0.15	4.13^{b} ±0.37	93.05^{b} ±8.48	234.47^{b} ±21.34	132.27^{b} ±11.48	14.03^{b} ±1.21	36.99^{ab} ±3.41
IV	4.09^{b} ±0.36	2.26^{a} ±0.21	5.03^{c} ±0.48	105.20^{c} ±9.53	289.17^{c} ±27.77	140.72^{c} ±12.52	16.26^{c} ±1.23	33.94^{a} ±3.07
V	5.31^{c} ±0.44	0.84^{c} ±0.08	3.58^{a} ±0.31	73.94^{a} ±68.18	177.52^{a} ±15.44	121.97^{a} ±11.73	10.50^{a} ±0.92	41.41^{b} ±3.82

Treatments- I: Control; II: DHA (200mg/kg b.w.); III: DHA (50mg/kg b.w.) + ethanol (5g/kg b.w.); IV: DHA (100mg/kg b.w.) + ethanol (5g/kg b.w.); V: DHA (200mg/kg b.w.) + ethanol (5g/kg b.w.); VI: ethanol (5g/kg b.w.).

[1]nmoles MDA/mg protein, [2]Units/mg proteins, [3]μmole H_2O_2/min/mg protein, [4]nmoles NADPH/min/mg protein, [5]μmole CDNB conjugate/min/mg protein, [6]μg/ mg protein, [7]μmole/mg protein

Means with different superscript letters differ significantly ($p<0.05$).

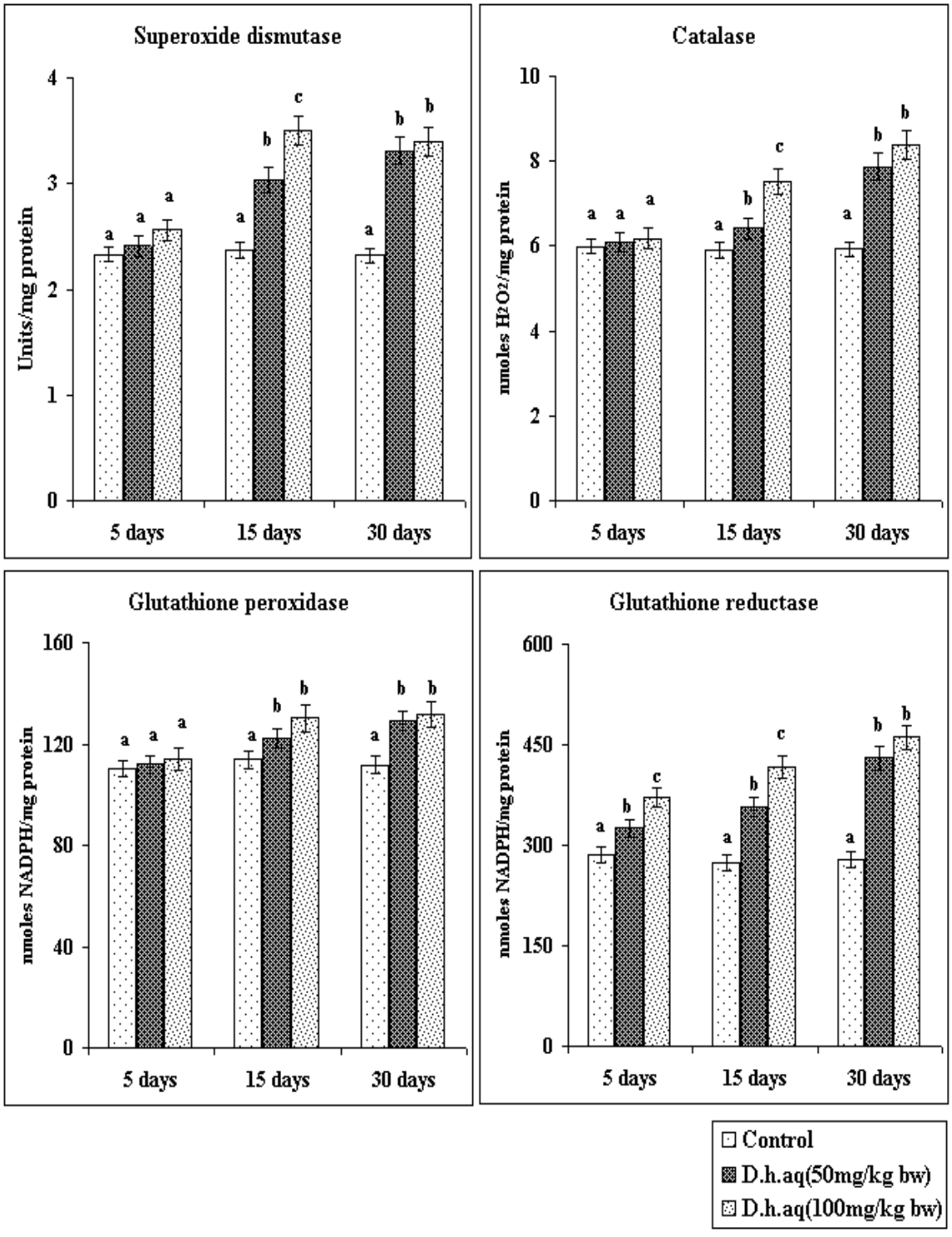


Figure 4.5.: Influence of DHA (**multiple dose**) on the hepatic antioxidant profile.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at p< 0.05 level (DMRT).

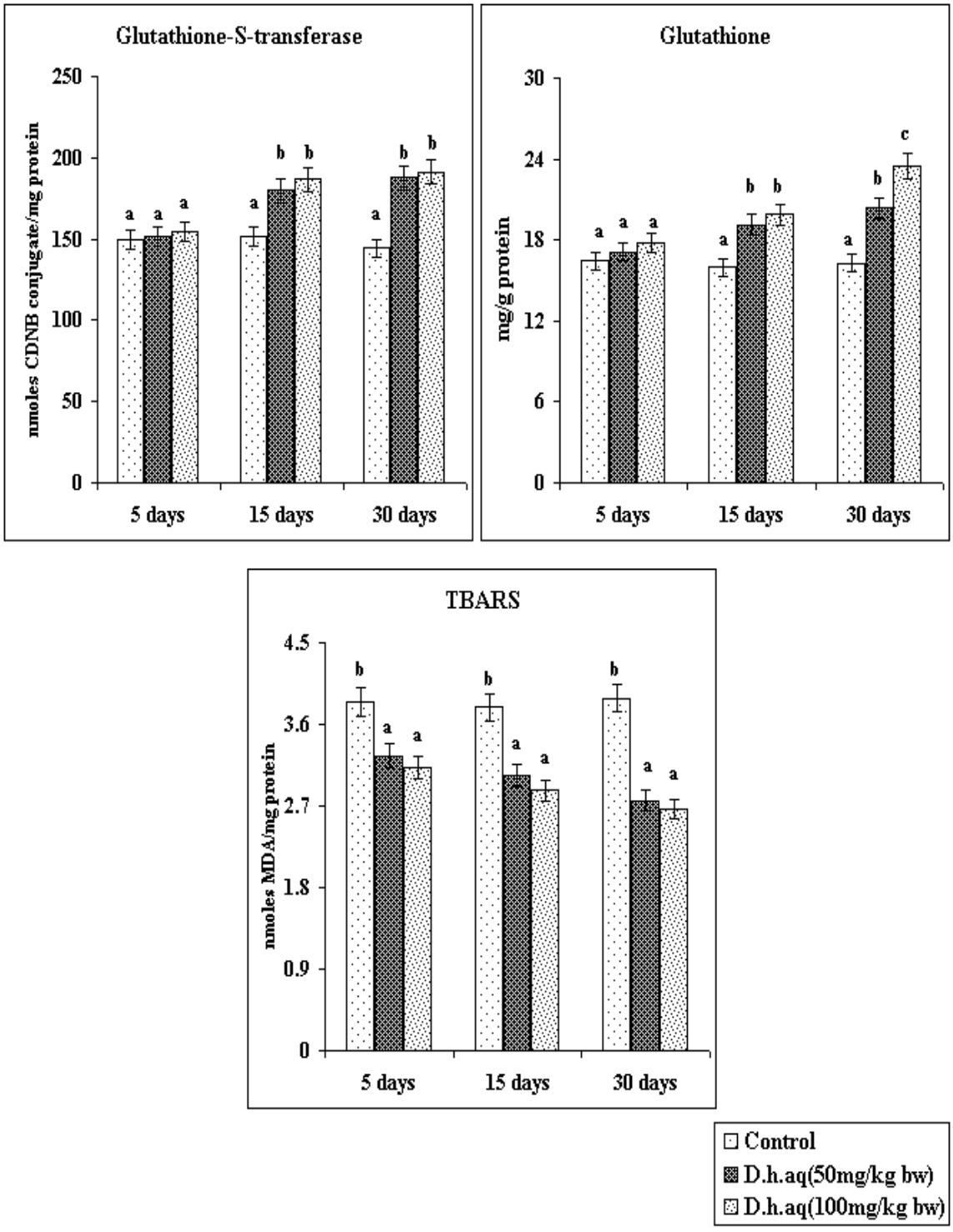


Figure 4.6.: Influence of DHA (**multiple dose**) on the hepatic antioxidant profile.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at $p < 0.05$ level (DMRT).

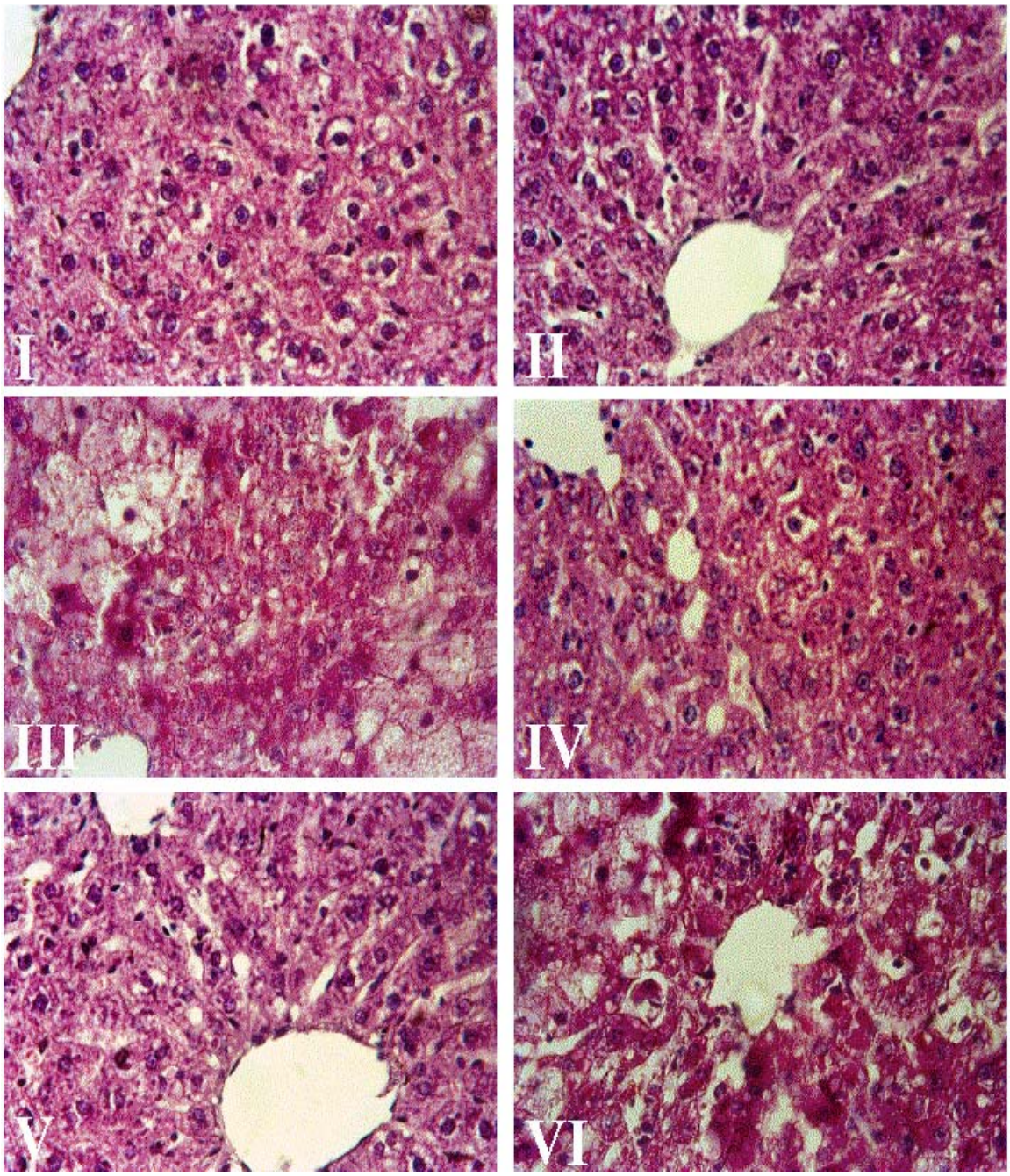


Plate 4.1.: Effects of the aqueous extract of the roots of *D. hamiltonii* pretreatment (single dose) on CCl_4-induced liver damage.

H & E staining, magnification, x 400.

Group I- Control; Group II- DHA (200mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group IV- DHA (100mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group V- DHA (200mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group VI- CCl_4 (1ml/ kg b.w.).

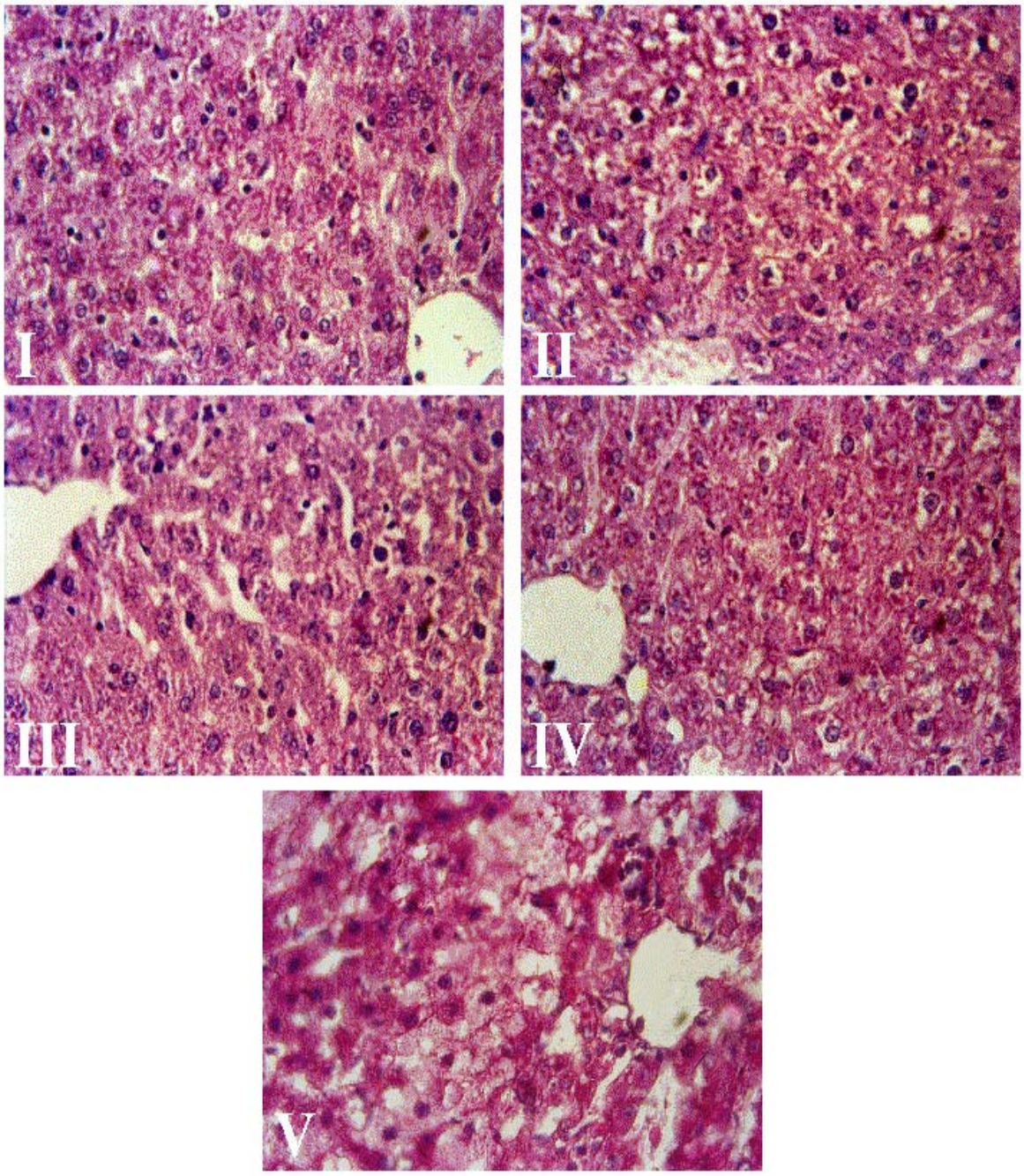


Plate 4.2.: Effects of the aqueous extract of the roots of *D. hamiltonii* pretreatment (multiple dose) on CCl_4-induced liver damage.

H & E staining, magnification, x 400.

Group I- Control; Group II- DHA (100mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group IV- DHA (100mg/kg b.w.) + CCl_4 (1ml/ kg b.w.); Group V- CCl_4 (1ml/ kg b.w.).

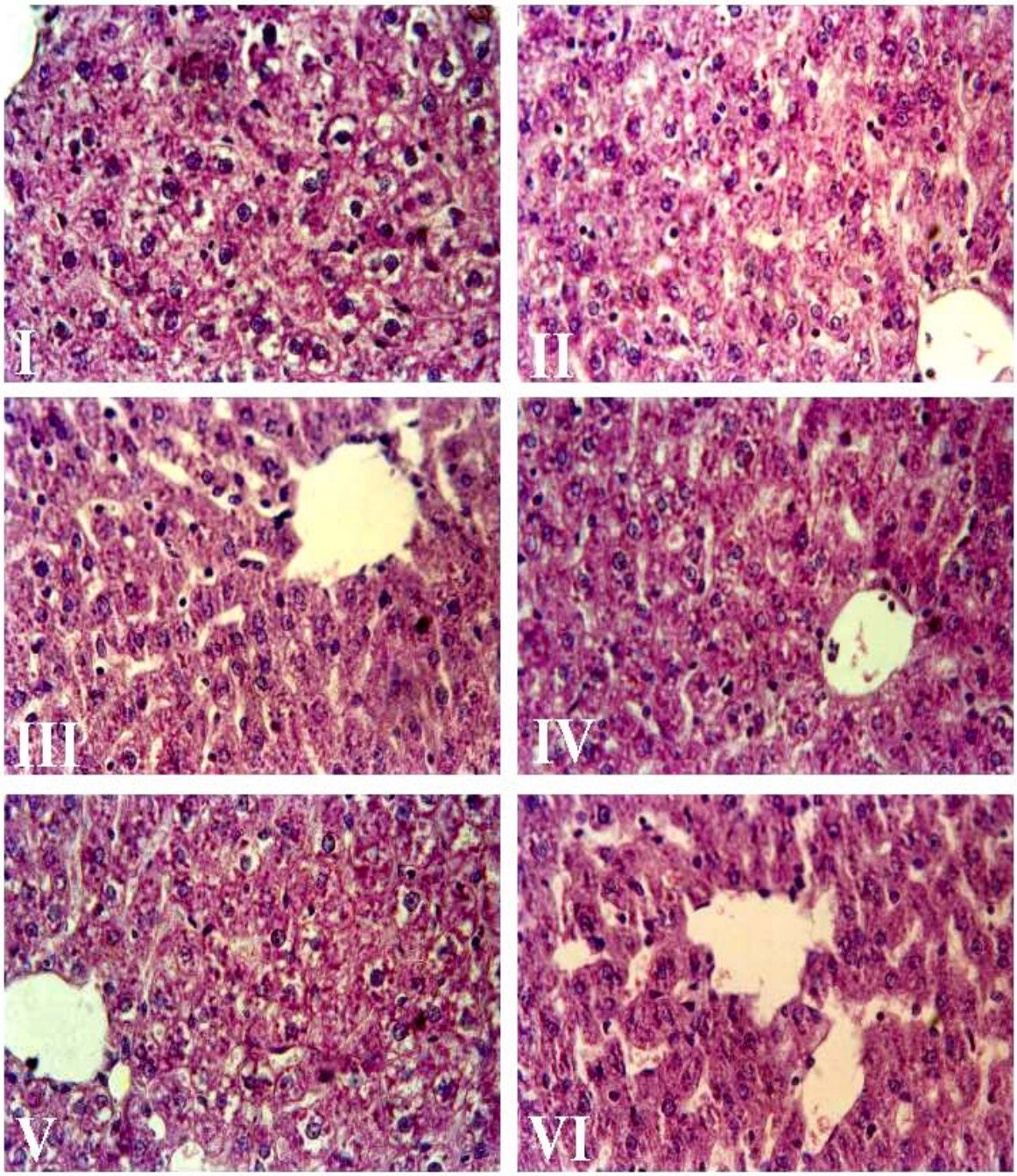


Plate 4.3.: Effects of the aqueous extract of the roots of *D. hamiltonii* pretreatment (single dose) on ethanol-induced liver damage.

H & E staining, magnification, x 400.

Group I- Control; Group II- DHA (200mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + Ethanol (5g/kg b.w.); Group IV- DHA (100mg/kg b.w.) + Ethanol (5g/kg b.w.); Group V- DHA (200mg/kg b.w.) + Ethanol (5g/kg b.w.); Group VI- Ethanol (5g/kg b.w.).

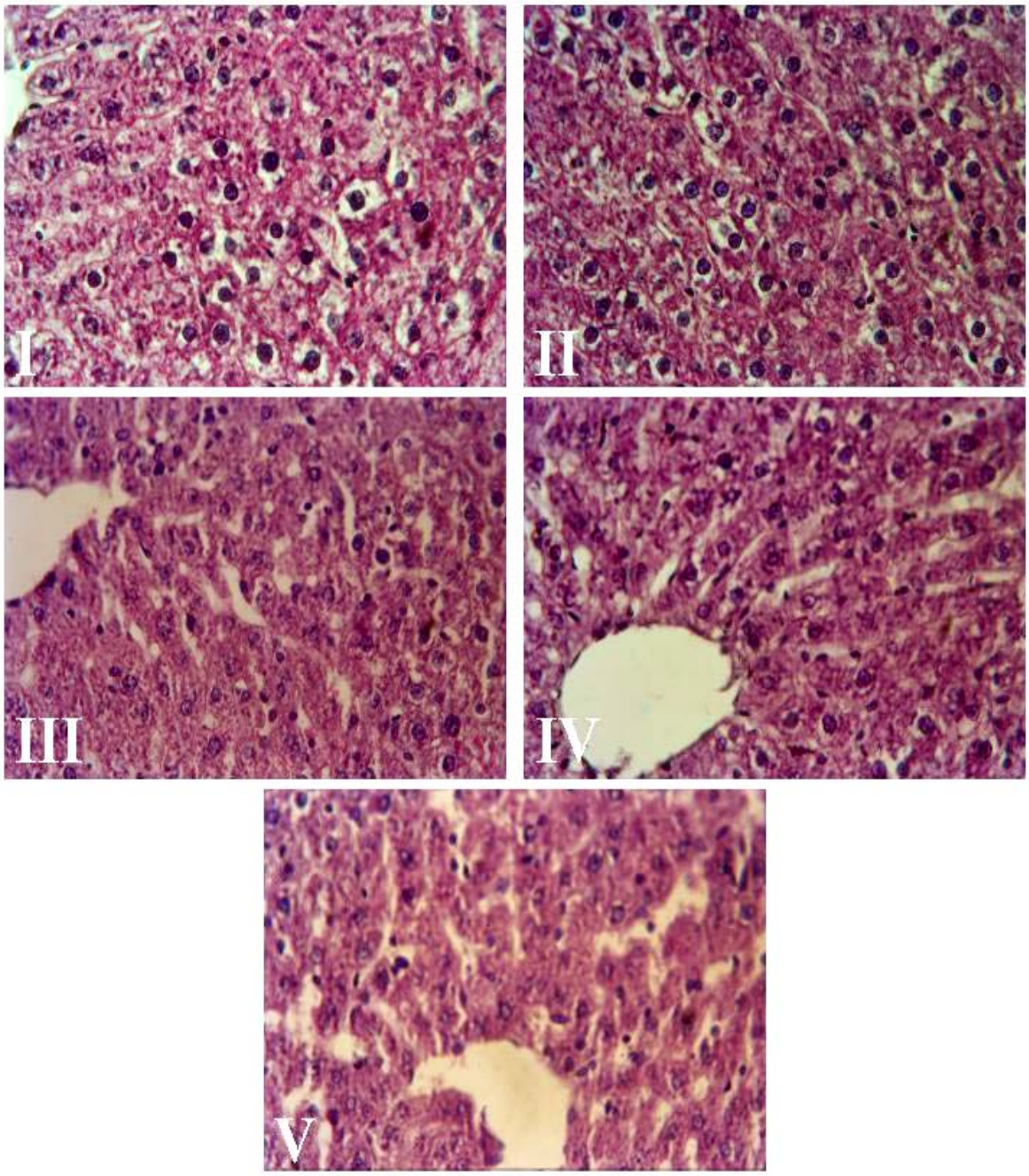


Plate 4.4.: Effects of the aqueous extract of the roots of *D. hamiltonii* pretreatment (multiple dose) on ethanol-induced liver damage.

H & E staining, magnification, x 400.

Group I- Control; Group II- DHA (100mg/kg b.w.); Group III- DHA (50mg/kg b.w.) + Ethanol (5g/kg b.w.); Group IV- DHA (100mg/kg b.w.) + Ethanol (5g/kg b.w.); Group V- Ethanol (5g/kg b.w.).

DISCUSSION

Roots of *D. hamiltonii* are traditionally consumed for their alleged health benefits. Although the roots are not specifically recommended or used for any ailment, it is believed to possess "cooling" properties (Nayar et al., 1978). However, there are no studies on the health promoting potential of *D. hamiltonii*. We have shown for the first time shown that the roots of *D. hamiltonii* possess antioxidant properties (Srivastava et al., 2005). Recently, we have identified some of the active principles responsible for the antioxidant activity in the aqueous extract of the roots of *D. hamiltonii*. It has been shown that antioxidants or plant extracts with antioxidant activity, exhibit hepatoprotective activity (Sheweita et al., 2001; Rajagopal et al., 2003; Wang et al., 2004). The present investigation was undertaken to evaluate of the efficacy of aqueous extract of roots of *D. hamiltonii* for its hepatoprotective effect against xenobiotic-induced oxidative stress and liver damage.

In our study, administration of a single acute dose of CCl_4 or ethanol to rats, led to raised levels of the serum enzymes, AST, ALT, ALP and LDH, indicating liver injury (Lin et al., 1996). The leakage of these enzymes into the blood stream was associated with massive centrilobular necrosis, fatty changes and cellular infiltration of the liver as judged by the histopathological observation. Pretreatment of rats with DHA prevented the CCl_4/ethanol-induced increase in the serum enzyme levels implying that the liver damage was prevented and therefore the leakage of enzymes was reduced. This is consistent with the histopathological observations. Our results on the roots extracts of *D. hamiltonii* add to a number of plants that show hepatoprotective potential including the Indian medicinal

plants (Lee, 2004; Shahjahan et al., 2004). However, the biochemical basis of the hepatoprotective action has been studied only for a few plants.

It is believed that the hepatoprotective effects of plant extracts against xenobiotic-induced liver injury possibly involve mechanisms related to free radical scavenging effects by the antioxidants (Lin and Huang, 2000). Membrane lipid peroxidation induced by CCl_4 and ethanol has been implicated in the pathogenesis of hepatic injury (Bandoyopadhyay et al., 1999). In the present study, marked increase in MDA, an index of lipid peroxidation, observed in the liver of CCl_4 and ethanol administered rats is indicative of membrane damage of the liver cells. Pretreatment of DHA prevented lipid peroxidation which could be attributed to its free radical scavenging activity present in the extract (Srivastava et al., 2005).

ROS (including superoxide anion and H_2O_2) are generated constantly by a number of cellular sources. Since ROS is injurious to biomolecules, cells have evolved a variety of antioxidant enzyme defences to counteract the ROS generated during normal cell metabolism and/or various pathophysiological processes. Among the cellular antioxidants, GSH, SOD, catalase, GPx, and GR have been extensively studied. SOD catalyses the dismutation of superoxide anion to H_2O_2 and O_2. Since H_2O_2 is harmful to cells, catalase and GPx further catalyze the decomposition of H_2O_2 to water. In reactions catalyzed by GPx, GSH is oxidized to GSSG, which is reduced back to GSH by GR. GR, therefore plays central role in maintaining the GSH level. GSH is also a cofactor/substrate for GST (phase II enzymes), primarily involved in the detoxification of electrophilic xenobiotics via the formation of GSH-electrophile conjugate (Hayes et al., 2005). Recent studies have also demonstrated that GST plays an important role in

protecting cells against ROS-mediated injury by the detoxification of lipid hydroperoxides derived from oxidative damage (Yang et al., 2001). Thus, the coordinate actions of various cellular antioxidants in mammalian cells are critical for effectively detoxifying ROS and maintaining the redox state of the cells.

In our study, CCl_4 and ethanol administration to rats, led to reduced antioxidant capacity of the liver as evident in the decreased activity of the antioxidant enzymes. These results are consistent with earlier reports (Valenzuela et al., 1980; Shahjahan et al., 2004). DHA pretreatment restored the antioxidant enzyme profile and prevented the oxidative hepatic damage. The multiple dose pretreatment of DHA to rats was much more effective and substantially enhanced the antioxidant enzyme activities. Positive effects of plant–derived polyphenols on antioxidant enzyme activities *in vivo* have been reported (Molina et al., 2003; Lieber, 2003). Therefore, the enhanced antioxidant enzymes by DHA is attributed to the antioxidant constituents.

Glutathione (GSH), the tripeptide γ-glutamylcysteineglycine, is the major non-enzymatic regulator of intracellular redox homeostasis, ubiquitously present in all cell types at millimolar concentration (Meister and Anderson, 1983). GSH directly scavenges free radicals or acts as a substrate for GPx and GST during the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds. In our study, CCl_4 and ethanol administration led to marked depletion of the glutathione level which predisposes cells to oxidative stress (Speisky et al., 1985). Our results show that hepatoprotection by *D. hamiltonii* root extracts against CCl_4 and ethanol toxicity could involve restoration of GSH level as seen in the multiple dose pretreatment. The exact mechanism by which the extract enhanced GSH levels is not clear but could be attributed to increased synthesis of

GSH through the enzymes such as γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase, the key enzymes in the biosynthesis of glutathione. There is some evidence for induction of γ–GCS activity by the plant extracts and the enhancement of GSH levels (Scharf et al., 2003).

One of the major consequences of oxidative stress is irreversible protein modification such as generation of carbonyls or loss of thiol residues (Berlett and Stadman, 2001; Levine, 2002). These oxidative modifications alter the biological properties of proteins leading to their fragmentation, increased aggregation and enzyme dysfunction. Increasing evidence suggests that irreversible oxidative modifications of proteins are important factors in the pathophysiology of several degenerative diseases (Beal, 2002). Free radical mediated modification of protein thiol groups, specifically cystein residues, is repaired by cellular antioxidant systems, such as the GSH or thioredoxin systems. In our study significant increase in the protein carbonyl content in the liver of CCl_4 and ethanol treated rats was observed suggesting oxidative damage to proteins. DHA pretreatment prevented the oxidative changes which is attributed to the antioxidant potential of DHA.

The mechanism by which the plant extracts enhance the antioxidant enzyme levels is not clearly understood. Some studies suggest that the enhancement of phase II enzymes by antioxidants, such as polyphenols, is achieved by upregulating the corresponding genes by interaction with antioxidant response elements (AREs) that transcriptionally regulate these genes (Ferguson, 2001; Shay and Banz, 2005). It has also been shown that the γ-glutamylcystein synthetase (γ-GCS), a key enzyme in the *de novo* glutathione synthesis, is also transcriptionally regulated by AREs (Myhrstad et al., 2002).

It is known that several treatments that induce expression of phase II detoxifying enzymes also result in elevated γ-GCS activity as well as increased intracellular GSH levels (Mulcahy et al., 1997). Therefore, it is reasonable to assure that the antioxidant compounds present in the DHA could induce the phase II enzymes by acting on the genes. However, this hypothesis needs to be confirmed by future studies.

In conclusion, our study has demonstrated for the first time, that DHA protects liver against hepatotoxic injury. The hepatoprotective activity of DHA could, atleast partly, be a result of free radical scavenging or inhibition of inflammatory mediators in CCl_4 and ethanol mediated lipid peroxidation. The bioactive antioxidant principles of the aqueous extract could be responsible for the observed hepatoprotective effect *in vivo*. Further, DHA exhibits antioxidant activity by inhibition of lipid peroxidation and enhancement of the antioxidant status of cells by induction of antioxidant enzymes and GSH. These results provide a scientific basis for the hepatoprotective effect and perhaps may underlie many other health promoting attributes of *D. hamiltonii*.

Chapter v

NEUROPROTECTIVE ACTIVITY

INTRODUCTION

All aerobic organisms are susceptible to oxidative stress simply because the toxic molecular species of oxygen such as superoxide and hydrogen peroxide are produced in mitochondria during respiration (Balaban et al., 2005). It is estimated that about 2% of the total oxygen consumed during respiration is converted to ROS. Brain is considered highly sensitive to oxidative damage as it is rich in easily peroxidizable fatty acids, consumes an inordinate fraction (20%) of the total oxygen for its relatively small weight (2%) and is relatively deficient in its antioxidant defenses (Chong et al., 2005). High level of iron in the brain makes it susceptible to oxidative stress via the iron-catalyzed formation of ROS. In addition, those brain regions that are rich in catecholamines are exceptionally vulnerable to free radical generation. The catecholamine adrenaline, noradrenaline and dopamine can spontaneously break down (auto-oxidise) to free radicals or can be metabolized to radicals by the endogenous enzymes such as monoamine oxidases (Schmidt and Ferger, 2004).

There is a substantial evidence that oxidative stress is a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases including parkinson's disease, alzheimer's disease, amyotrophic lateral sclerosis as well as in cases of stroke, trauma and seizures (Cui et al., 2004). Decreased level of antioxidant activity and increased lipid peroxidation and oxidative modifications of DNA and proteins especially in substantia nigra of the brain have been reported in patients with parkinson's disease. A number of *in vitro* studies have shown that antioxidants- both endogenous and dietary- can protect nervous tissue from damage by oxidative stress (Lau et al., 2005). Uric acid, an endogenous antioxidant, is reported to prevent neuronal damage in rats,

from the metabolic stresses of ischemia, oxidative stress as well as exposure to the excitatory amino acid, glutamate and the toxic compound, cyanide. Vitamin E is shown to prevent cell death in rat neurons subjected to hypoxia followed by oxygen reperfusion (Zhang et al., 2004). Both vitamin E and beta-carotene were found to protect rat neurons against oxidative stress from exposure to ethanol (Lamarche et al., 2004).

Excessive ethanol consumption has been shown to result in damages to a number of organs including the brain due to the induced oxidative stress, leading to the increased production of ROS and induction of lipid peroxidation (Sergent et al., 2001). Lipid peroxidation is associated with a progressive loss in membrane potential, increase in membrane permeability to ions and, finally, cell death. Ethanol may cause oxidative stress to tissues and cells through a number of mechanisms. One possible mechanism is through the induction of cytochrome P450 2E1 (Cohen-Koren and Koren, 2003). A second mechanism could possibly be due to the ability of ethanol to cause overexcitation of neurons, which triggers a number of events including release of excitatory neurotransmitters, loss of Ca^{2+} homeostasis, altered intracellular signaling cascades and cell death (Yamamoto et al., 2004).

Hexachlorocyclohexane (HCH), an organochlorine insecticide, is widely used in agriculture and public health. HCH enters animal tissues via food chain, respiration or dermal contact and gets accumulated in cells (Lopez-Aparicio et al., 1994). Technical HCH is a mixture of atleast five isomers of which γ-HCH (lindane) is the main insecticidal component. At acute doses, HCH induces neurotoxic effects such as convulsive seizures and increased neuronal activity (Woolley et al., 1985), enhanced transmitter release (Baker et al., 1985), alterations in the activities of acetylcholinesterase

(Raizada et al., 1994), Na^+, K^+-ATPase (Parries and Hokin-Neaverson, 1985) and Mg^{2+}-ATPase (Sahoo et al., 1999). At subchronic exposure, HCH is reported to induce changes in neurotransmitter levels (Martinez and Martinez-Conde, 1995; Anand et al., 1998). Organochlorine pesticides including HCH induce oxidative stress in neural tissues of rat (Sahoo et al., 2000). Involvement of reactive oxygen species (ROS) has been postulated as a possible mechanism for HCH toxicity (Junqueira et al., 1994; Samanta and Chainy 1997; Srivastava and Shivanandappa, 2005). The brain shows distinct variation in the regional distribution of the antioxidant defenses and metabolic rates that could be responsible for differential oxidative damage in the brain regions (Goss-Sampson et al., 1988; Ansari et al., 1989; Srivastava and Shivanandappa, 2005).

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate - DDVP), an organophosphorus (OP) compound, is a contact and stomach - acting insecticide with fumigant action. Neurotoxicity from OP exposure is generally related to inhibition of acetylcholinesterase (AChE) (Aldridge, 1985). All OPs elicit their primary effects by phosphorylating or phosphonylating the active site of the enzyme AChE, causing accumulation of acetylcholine (ACh) in synapses, which results in excessive stimulation of cholinergic receptors on postsynaptic cells, leading to cholinergic toxicity (Agarwal, 1993). Inhibition of brain AChE is generally regarded as a sensitive biochemical marker and most sensitive measure of OP toxicity (Worek et al., 2005).

Tubers of *Decalepis hamiltonii* (Wight and Arn.)(family: Asclepiadaceae) are consumed as pickles and juice for its alleged health promoting properties. The roots are used in folk medicine and as a substitute for *Hemidesmus indicus* in ayurvedic preparations (Nayar et al., 1978). We have earlier shown that the roots of *D. hamiltonii*

possess potent antioxidant properties which could be associated with their alleged health benefits (Srivastava et al., 2005). We have also shown the hepatoprotective potential of the roots of *D. hamiltonii* (see Chapter IV). In this study, we have examined the neuroprotective potential of the aqueous extract of the roots of *D. hamiltonii* in rats against (a) ethanol-induced oxidative alterations in brain, (b) HCH-induced oxidative stress in different parts of brain, and (c) DDVP-induced AChE inhibition in the different regions of brain.

MATERIALS AND METHODS

Chemicals: Technical HCH was obtained from Tata chemicals (Mithapur, India) which had the following composition of isomers: alpha-72%, beta-5%, gamma-13.6 and delta-8%. Technical grade dichlorvos (DDVP; 2.2-dichlorovinyl dimethyl phosphate) was obtained from All India Medical Corporation (Mumbai, India). Nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), cumene hydroperoxide (CHP), pryogallol, bovine serum albumin (BSA), tetraethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), acetylthiocholine iodide (ATCI), 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India. All the chemicals used were of highest purity grade available.

Animals and treatment: Sixty day old adult male Wistar rats (180-200g) were divided into different groups, of eight each. The Institute Animal Ethics Committee guidelines were followed for the animal experiments. Experiments on neuroprotective activity of the aqueous extract of the roots of *D. hamiltonii* were done with multiple dose (7 days) oral pretreatment at the doses of 50 and 100 mg/kg b.w.. The toxicant was given orally on the 7^{th} day 1h after the *D. hamiltonii* extract administration. The dose of the toxicant was selected as a factor of the LD_{50} in rats: in case of ethanol it was ½ of LD_{50}; for HCH the dose selected was 1/3 of LD_{50}; and for DDVP a dose of 1/3 of LD_{50}. Animals were sacrificed by ether anesthesia after 16h of toxicant administration; the brain perfused with

saline was dissected on ice to get different parts/regions, which were processed immediately for biochemical assays.

Experimental design and groupings

Four separate experiments were done and following were the treatment groups:

A. Ethanol neurotoxicity:

Groups: Group I- Control; Group II- DHA (100mg/kg b.w.) (7 days); Group III- DHA (50mg/kg b.w.) (7 days) + ethanol (5g/kg b.w.); Group IV- DHA (100mg/kg b.w.) (7 days) + ethanol (5g/kg b.w.); Group V- ethanol (5g/kg b.w.).

B. HCH neurotoxicity:

Groups: Group I- Control; Group II- DHA (100mg/kg b.w.) (7 days); Group III- DHA (50mg/kg b.w.) (7 days) + HCH (500mg/kg b.w.); Group IV- DHA (100mg/kg b.w.) (7 days) + HCH (500mg/kg b.w.); Group V- HCH (500mg/kg b.w.).

C. DDVP neurotoxicity:

Groups: Group I- Control; Group II- DHA (100mg/kg b.w.) (7 days); Group III- DHA (50mg/kg b.w.) (7 days) + DDVP (47mg/kg b.w.); Group IV- DHA (100mg/kg b.w.) (7 days) + DDVP (47mg/kg b.w.); Group V- DDVP (47mg/kg b.w.).

D. Antioxidant potential of DHA:

Multiple doses for 5, 15 and 30 days.

Groups: Group I- Control; Group II- DHA (50mg/kg b.w.); Group III- DHA (100mg/kg b.w.).

Biochemical assays

Lipid peroxidation: Lipid peroxidation (LPO) in the tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS)(Ohkawa et al., 1979). Tissue homogenate (10% w/v in 50 mM phosphate buffer, pH 7.4) was mixed with TCA (10 %) and TBA (0.34 %) and boiled in a water bath for 15 minutes, cooled and centrifuged. Absorbance of the supernatant was read at 535 nm. TBARS was calculated using tetraethoxypropane as the standard.

Glutathione: A 10% (w/v) tissue homogenate prepared in 5% (w/v) trichloroacetic acid, centrifuged at 2,000g for 5 min and glutathione (GSH) in the deproteinized supernatant was estimated by Ellman's reagent with a standard curve (Ellman, 1959).

Protein carbonyls: Tissue homogenates (10% w/v) were prepared in 20mM tris-HCl buffer, pH 7.4 with 0.14 NaCl, centrifuged at 10,000g for 10 min at 4^{o}C and 1.0ml of the supernatant was precipitated with an equal volume of 20% TCA and centrifuged. The pellet was resuspended in 1.0ml of DNPH (10mM in 2M HCl) and allowed to stand at room temperature for 60 min with occasional vortexing. 0.5ml of 20% TCA was added to the reaction mixture and centrifuged, the pellet obtained was washed 3 times with acetone and solubilised in 1.0ml of 2% of SDS (in 20mM tris-HCl, 0.1M NaCl, pH 7.4). The absorbance was read at 360nm in a UV-Visible Spectrophotometer and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} (Levine et al., 1990).

Protein content was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Enzyme assays

Brain tissue was homogenized (10% w/v) in ice-cold 50 mM phosphate buffer (pH 7.4), centrifuged at 10,000g for 20 min. at 4^{o}C and the supernatant was used to assay the enzyme activities.

Antioxidant enzymes:

Superoxide dismutase (SOD) activity was measured using pyrogallol (2mM) autoxidation in tris buffer (Marklund and Marklund, 1974).

Catalase (CAT) activity was measured using H_2O_2 (3 %) as the substrate in phosphate buffer (Aebi, 1974).

Glutathione peroxidase (GPx) activity was measured by the indirect assay method using glutathione reductase. Cumene hydroperoxide (1mM) and glutathione (0.25mM) were used as substrates and coupled oxidation of NADPH by glutathione reductase (0.25 U) in tris buffer (50mM, pH 7.6) was monitored at 340nm (Mannervik, 1985).

Glutathione reductase (GR) activity was assayed in a reaction mixture containing oxidized glutathione (20mM) and NADPH (2mM) in potassium phosphate buffer (Calberg and Mannervik, 1985).

Glutathione transferase (GST) activity was assayed by the method of Warholm et al. (1985) using glutathione (20mM) and CDNB (30mM) as the substrates in phosphate buffer, change in absorbance at 344nm was monitored in a UV-Visible Spectrophotometer. (For details see Chapter IV)

Acetylcholinesterase: AChE activity was assayed spectrophotometrically by measuring the rate of hydrolysis of the substrate ATCI, according to the method of Ellman et al. (1961). The reaction mixture (3.0 ml) in 0.1M phosphate buffer (pH 7.4) contained

0.33mM DTNB and 0.5mM ATCI at 37^{o}C; the reaction was started by the addition of substrate and change of absorbance every 30 sec was followed at 412 nm in a UV-Vis spectrophotometer. The enzyme activity was calculated by the extinction coefficient (E_{412}= 1.36 x 10^4 M^{-1} cm^{-1}). The AChE activity was expressed as nmoles of ATCI hydrolyzed min^{-1} mg^{-1} of protein.

Statistical analysis: The data were expressed as means ± S.E. of eight observations (n = 8) and significant difference between each of the groups was statistically analyzed by Duncan's multiple range test (Statistica Software, 1999), represented by alphabets for each level of significance. A difference was considered significant at $p<0.05$.

RESULTS

The food consumption and body weights of the DHA treated rats were comparable to the control group.

Lipid peroxidation

Ethanol induced lipid peroxidation (LPO) (95% increase) in the brain (Table 5.1.). Increased LPO was observed in all the brain regions of HCH treated rats, being high in the cortex and cerebellum than that of midbrain and brain stem (Table 5.2.). DHA pretreatment ameliorated the HCH and ethanol induced LPO (Table 5.1. and 5.2.). DHA, the extract *per se* decreased the basal level of lipid peroxide content (Figure 5.3.).

Antioxidant enzymes

Ethanol administration led to decreased activity of the antioxidant enzymes, SOD, CAT, GPx, GR and GST; DHA prevented the ethanol-induced decrease in the activity of all the antioxidant enzymes, (Table 5.1.).

The distribution of the antioxidant enzymes in the brain regions of normal (control) rats shows distinct variation. SOD activity was highest in the stem and lowest in the midbrain whereas CAT activity was high in the midbrain but low in the cortex. Activities of GPx, GR and GST, on the other hand, were highest in midbrain but, low in cerebellum and cortex, respectively (Table 5.3.).

Effect of HCH on the antioxidant enzymes also showed distinct regional variation. A significant decrease in the activity of SOD in the brain regions of HCH treated rats was seen. Brain stem and cortex showed marked decrease followed by midbrain and cerebellum. CAT activity was markedly increased in the brain of HCH treated rats. The increase in activity was highest in the cortex followed by cerebellum, brain stem and

lowest in midbrain. An induction of GPx activity by HCH treatment in all the brain regions was observed which was in the following order: cortex > cerebellum > brain stem > midbrain. Marked induction of GR in the brain regions of the HCH treated rats was seen which was highest in the cortex followed by cerebellum, midbrain and, least, in the brainstem. Similarly, GST activity was elevated in the brain regions of HCH treated rats. Different brain regions responded to a different degree, with cortex showing highest induction followed by brain stem and least in midbrain and cerebellum (Table 5.3.).

DHA pretreatment induced the antioxidant enzyme activities in all the regions of the rat brain (Table 5.3.). The multiple dose pretreatment of DHA enhanced the antioxidant status of the rat brain (Fig. 5.2. and 5.3.)

Glutathione

A significant reduction in GSH content was observed in the brain regions of ethanol and HCH treated rats. In HCH treated rats, cortex showed maximum reduction, and brain stem, the least. DHA pretreatment not only prevented the decrease in GSH due to the xenobiotic treatment but also enhanced GSH levels in the rat brain (Table 5.1., 5.2. and Fig. 5.3.)

Protein carbonyls

Ethanol induced carbonylation of proteins which was prevented by DHA pretreatment. (Table 5.1.).

Acetylcholinesterase

Results show differential AChE activity in the regions of the brain. Among the brain regions, the highest AChE activity was found in striatum and the lowest in cerebellum.

The AChE activity in the brain regions was in the order: striatum > pons > thalamus > medulla > cortex > hippocampus > cerebellum (Table 5.4.).

A single dose of DDVP (1/3 LD_{50}) elicited differential AChE inhibition in the brain regions which ranged from 58% (striatum) to 22% (cerebellum) and the inhibition was in the order: striatum > hippocampus > medulla > pons > cortex > whole brain > thalamus > cerebellum (Table 5.4.). Administration of DHA alone did not produce any significant effect on AChE activity. Pretreatment of rats with DHA provided significant protection against AChE inhibition due to DDVP in all the brain regions (Table 5.1.).

Table 5.1.: Neuroprotective effect of *D. hamiltonii* aqueous extract pretreatment (multiple dose) against ethanol toxicity in rats: Oxidative biochemical changes in the brain.

Group	LPO[1]	SOD[2]	CAT[3]	GPx.[4]	GR[4]	GST[5]	GSH[6]	PC[7]
I	1.73[a] ±0.14	0.57[c] ±0.04	1.17[b] ±0.09	17.64[b] ±1.44	91.23[b] ±8.23	172.48[c] ±14.38	14.56[c] ±1.23	30.23[a] ±2.94
II	1.69[a] ±0.13	0.71[d] ±0.03	1.4[c] ±0.13	20.04[c] ±1.83	120.36[c] ±10.28	193.25[d] ±16.74	16.95[d] ±1.49	30.09[a] ±2.71
III	2.13[b] ±0.19	0.48[b] ±0.04	1.15[b] ±0.11	16.32[b] ±1.47	87.48[b] ±7.15	148.22[b] ±12.27	11.23[b] ±0.09	35.36[b] ±3.21
IV	1.87[a] ±0.16	0.59[c] ±0.03	1.21[b] ±0.11	17.54[b] ±1.51	93.61[b] ±8.27	170.83[c] ±14.35	13.87[c] ±0.11	33.29[b] ±2.93
V	3.38[c] ±0.28	0.22[a] ±0.02	0.91[a] ±0.08	14.03[a] ±1.23	64.24[a] ±5.83	121.54[a] ±11.06	9.56[a] ±0.08	39.18[c] ±3.76

Group - I: Control, II: DHA (100mg/kg bw), III: DHA (50mg/kg bw) + Ethanol (5g/kg bw), IV: DHA (100mg/kg bw) + Ethanol (5g/kg bw), V: Ethanol (5g/kg bw).

[1]nmoles MDA/mg protein, [2]Units/mg proteins, [3]µmole H_2O_2/min/mg protein, [4]nmoles NADPH/min/mg protein, [5]µmole CDNB conjugate/min/mg protein, [6]µg/ mg protein, [7]µmole/mg protein.

Means with different superscript letters differ significantly ($p<0.05$).

Table 5.2.: Neuroprotective effect of the aqueous extract of the roots of *D. hamiltonii* against HCH-induced oxidative stress in the brain regions of rat.

Parameters	Group	Brain Regions			
		Cortex	**Cerebellum**	**Stem**	**Midbrain**
TBARS[1]	I	$1.19^{b}\pm0.09$	$1.01^{b}\pm0.08$	$1.33^{a}\pm0.11$	$1.23^{b}\pm0.11$
	II	$1.08^{a}\pm0.10$	$0.91^{a}\pm0.07$	$1.26^{a}\pm0.10$	$1.06^{a}\pm0.09$
	III	$1.34^{d}\pm0.12$	$1.19^{d}\pm0.10$	$1.42^{a}\pm0.12$	$1.29^{b}\pm0.10$
	IV	$1.21^{c}\pm0.11$	$1.08^{c}\pm0.11$	$1.37^{a}\pm0.12$	$1.25^{b}\pm0.11$
	V	$1.46^{d}\pm0.13$	$1.28^{d}\pm0.12$	$1.47^{a}\pm0.13$	$1.39^{b}\pm0.12$
Glutathione[2]	I	$14.35^{c}\pm1.21$	$15.88^{c}\pm1.34$	$16.34^{b}\pm1.35$	$14.33^{b}\pm1.28$
	II	$15.84^{d}\pm1.34$	$17.6^{d}\pm1.49$	$17.61^{b}\pm1.58$	$15.61^{b}\pm1.37$
	III	$12.56^{b}\pm1.18$	$13.57^{b}\pm1.18$	$15.02^{b}\pm1.32$	$13.12^{b}\pm1.26$
	IV	$12.88^{b}\pm1.14$	$13.69^{b}\pm1.25$	$15.21^{b}\pm1.26$	$13.36^{b}\pm1.17$
	V	$10.48^{a}\pm0.97$	$11.99^{a}\pm1.08$	$13.29^{a}\pm1.19$	$11.27^{a}\pm1.03$

Group- I: Control; II: DHA (100mg/kg bw); III: DHA (50mg/kg bw) + HCH (500mg/kg bw); IV: DHA (100mg/kg bw) + HCH (500mg/kg bw); V: HCH (500mg/kg bw).

[1]nmoles MDA/mg protein, [2]mg/g protein.

Means with different superscript letters differ significantly ($p<0.05$).

Table 5.3.: Neuroprotective effect of the aqueous extract of the roots of *D. hamiltonii* against HCH-induced oxidative stress in rats: Antioxidant enzymes

Antioxidant enzymes	Group	Brain regions			
		Cortex	Cerebellum	Stem	Midbrain
Superoxide dismutase[1]	I	$0.3^{c}\pm0.02$	$0.52^{b}\pm0.04$	$1.21^{d}\pm0.09$	$0.21^{c}\pm0.01$
	II	$0.41^{d}\pm0.03$	$0.61^{c}\pm0.05$	$1.73^{e}\pm0.12$	$0.256^{d}\pm0.01$
	III	$0.22^{b}\pm0.02$	$0.41^{a}\pm0.03$	$0.84^{b}\pm0.06$	$0.19^{b}\pm0.01$
	IV	$0.29^{c}\pm0.02$	$0.49^{b}\pm0.04$	$1.09^{c}\pm0.09$	$0.21^{c}\pm0.01$
	V	$0.19^{a}\pm0.01$	$0.39^{a}\pm0.03$	$0.73^{a}\pm0.05$	$0.16^{a}\pm0.01$
Catalase[2]	I	$0.88^{a}\pm0.07$	$1.15^{a}\pm0.09$	$1.84^{a}\pm0.16$	$3.02^{a}\pm0.28$
	II	$1.26^{b}\pm0.09$	$1.41^{b}\pm0.11$	$2.22^{b}\pm0.2$	$3.48^{b}\pm0.31$
	III	$3.05^{c}\pm0.29$	$1.65^{c}\pm0.13$	$2.66^{c}\pm0.22$	$3.66^{b}\pm0.33$
	IV	$2.86^{c}\pm0.24$	$1.58^{c}\pm0.13$	$2.43^{c}\pm0.20$	$3.52^{b}\pm0.34$
	V	$4.41^{d}\pm0.38$	$1.99^{d}\pm0.17$	$2.82^{c}\pm0.25$	$4.02^{c}\pm0.38$
Glutathione peroxidase[3]	I	$11.56^{a}\pm1.32$	$3.17^{a}\pm0.27$	$13.87^{a}\pm1.16$	$29.74^{a}\pm2.46$
	II	$14.98^{b}\pm1.43$	$4.12^{b}\pm0.32$	$17.61^{b}\pm1.52$	$33.46^{b}\pm3.07$
	III	$18.68^{c}\pm1.78$	$5.89^{c}\pm0.48$	$18.74^{b}\pm1.64$	$36.48^{b}\pm3.21$
	IV	$17.88^{c}\pm1.69$	$5.64^{c}\pm0.41$	$18.56^{b}\pm1.59$	$36.03^{b}\pm3.38$
	V	$24.33^{d}\pm1.99$	$6.97^{d}\pm0.58$	$20.61^{c}\pm1.78$	$39.91^{c}\pm3.51$
Glutathione reductase[3]	I	$50.84^{a}\pm4.67$	$102.72^{a}\pm9.21$	$91.38^{a}\pm7.76$	$192.44^{a}\pm17.45$
	II	$76.6^{b}\pm6.54$	$139.56^{b}\pm11.54$	$116.7^{b}\pm10.27$	$252.09^{b}\pm23.67$
	III	$165.92^{d}\pm14.87$	$158.34^{b}\pm13.84$	$118.94^{b}\pm10.72$	$263.76^{b}\pm25.24$
	IV	$132.38^{c}\pm11.89$	$149.22^{b}\pm12.94$	$117.18^{b}\pm10.66$	$257.33^{b}\pm23.18$
	V	$238.34^{e}\pm21.69$	$172.46^{c}\pm13.06$	$119.28^{b}\pm11.08$	$268.38^{b}\pm24.83$
Glutathione-S-transferase[4]	I	$55.22^{a}\pm4.68$	$82.82^{a}\pm7.54$	$128.38^{a}\pm10.98$	$315.27^{a}\pm30.22$
	II	$74.84^{b}\pm6.59$	$90.75^{a}\pm8.46$	$143.78^{b}\pm11.25$	$359.1^{b}\pm31.28$
	III	$99.12^{c}\pm8.74$	$95.36^{a}\pm8.79$	$155.31^{b}\pm14.67$	$368.37^{b}\pm32.57$
	IV	$97.26^{c}\pm9.23$	$94.85^{a}\pm8.99$	$152.46^{b}\pm13.89$	$364.57^{b}\pm34.12$
	V	$159.26^{d}\pm13.88$	$99.69^{a}\pm9.23$	$167.36^{b}\pm15.61$	$378.77^{b}\pm35.65$

Group- I: Control; II: DHA (100mg/kg bw); III: DHA (50mg/kg bw) + HCH (500mg/kg bw); IV: DHA (100mg/kg bw) + HCH (500mg/kg bw); V: HCH (500mg/kg bw).

[1]Units/mg protein, [2]nmoles H_2O_2/mg protein, [3]nmoles NADPH/mg protein, [4]nmoles GS-CDNB/mg protein.

Means with different superscript letters differ significantly ($p<0.05$).

Table 5.4.: Neuroprotective effect of *D. hamiltonii* root extract (aqueous) pretreatment against DDVP-induced AChE inhibition in the brain regions of rat.

Group	AChE* activity in brain region							
	Hippocampus	Thalamus	Pons	Cortex	Medulla	Striatum	Cerebellum	Whole Brain
I	21.35^{d} ±1.98	41.23^{c} ±3.25	52.87^{d} ±4.28	21.42^{d} ±1.88	39.61^{c} ±2.93	92.33^{d} ±7.18	15.38^{b} ±1.33	34.12^{d} ±2.39
II	21.22^{d} ±1.29	42.34^{c} ±4.12	51.67^{d} ±4.36	21.84^{d} ±1.74	39.24^{c} ±2.62	93.15^{d} ±8.24	15.22^{b} ±1.26	34.29^{d} ±2.31
III	11.28^{c} ±1.02	33.17^{a} ±1.47	32.78^{b} ±2.91	14.18^{b} ±1.24	20.45^{a} ±1.76	51.24^{b} ±4.83	12.35^{a} ±1.09	22.27^{b} ±1.95
IV	14.62^{b} ±1.33	35.34^{b} ±1.22	36.94^{c} ±3.41	16.09^{c} ±1.32	26.39^{b} ±2.18	64.33^{c} ±4.11	12.72^{a} ±1.07	24.57^{c} ±1.89
V	9.39^{a} ±0.74	31.75^{a} ±2.54	28.54^{a} ±2.16	12.42^{a} ±1.03	18.65^{a} ±1.43	38.77^{a} ±3.29	11.99^{a} ±1.07	20.14^{a} ±1.81

* nmoles ATCI hydrolysed/min/mg protein.

Group: I- Control; II- DHA (100mg/kg bw); III- DHA (50mg/kg bw) + DDVP (47mg/kg bw); IV- DHA (100mg/kg bw) + DDVP (47mg/kg bw); V- DDVP (47mg/kg bw).

Means with different superscript letters differ significantly ($p<0.05$).

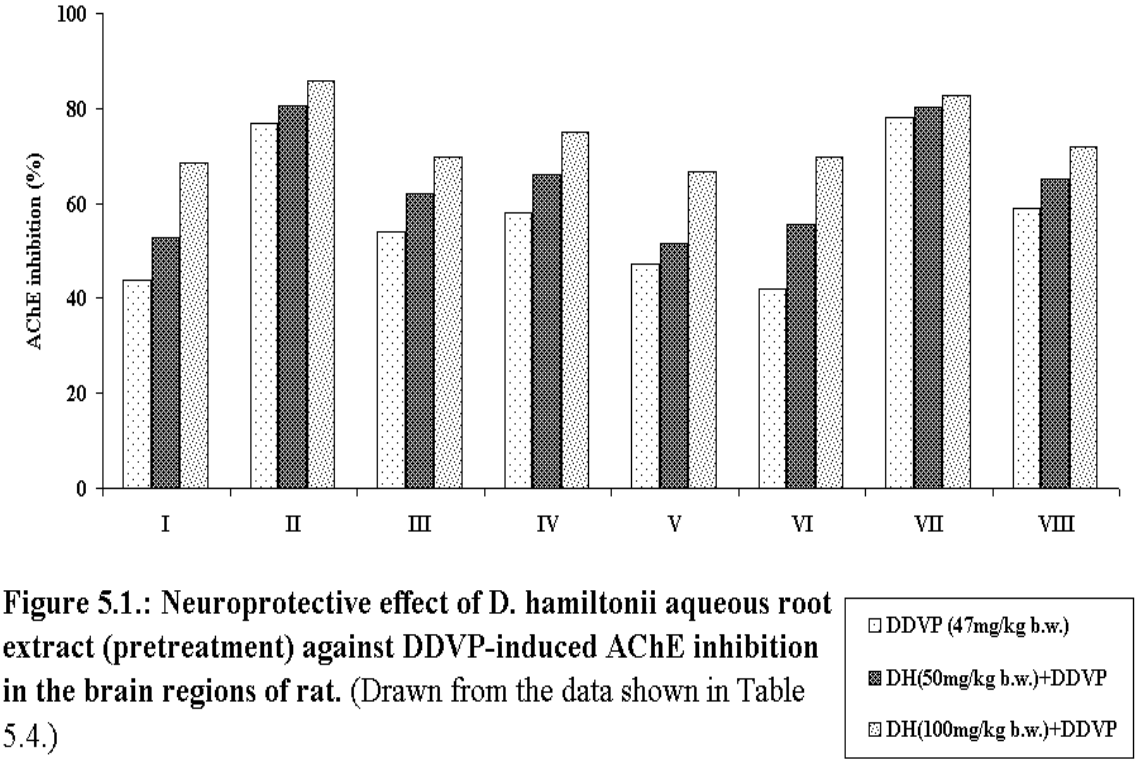


Figure 5.1.: Neuroprotective effect of D. hamiltonii aqueous root extract (pretreatment) against DDVP-induced AChE inhibition in the brain regions of rat. (Drawn from the data shown in Table 5.4.)

I: hippocampus; II: thalamus; III: pons; IV: cortex; V: medulla; VI: striata; VII: cerebellum; VIII: whole brain.

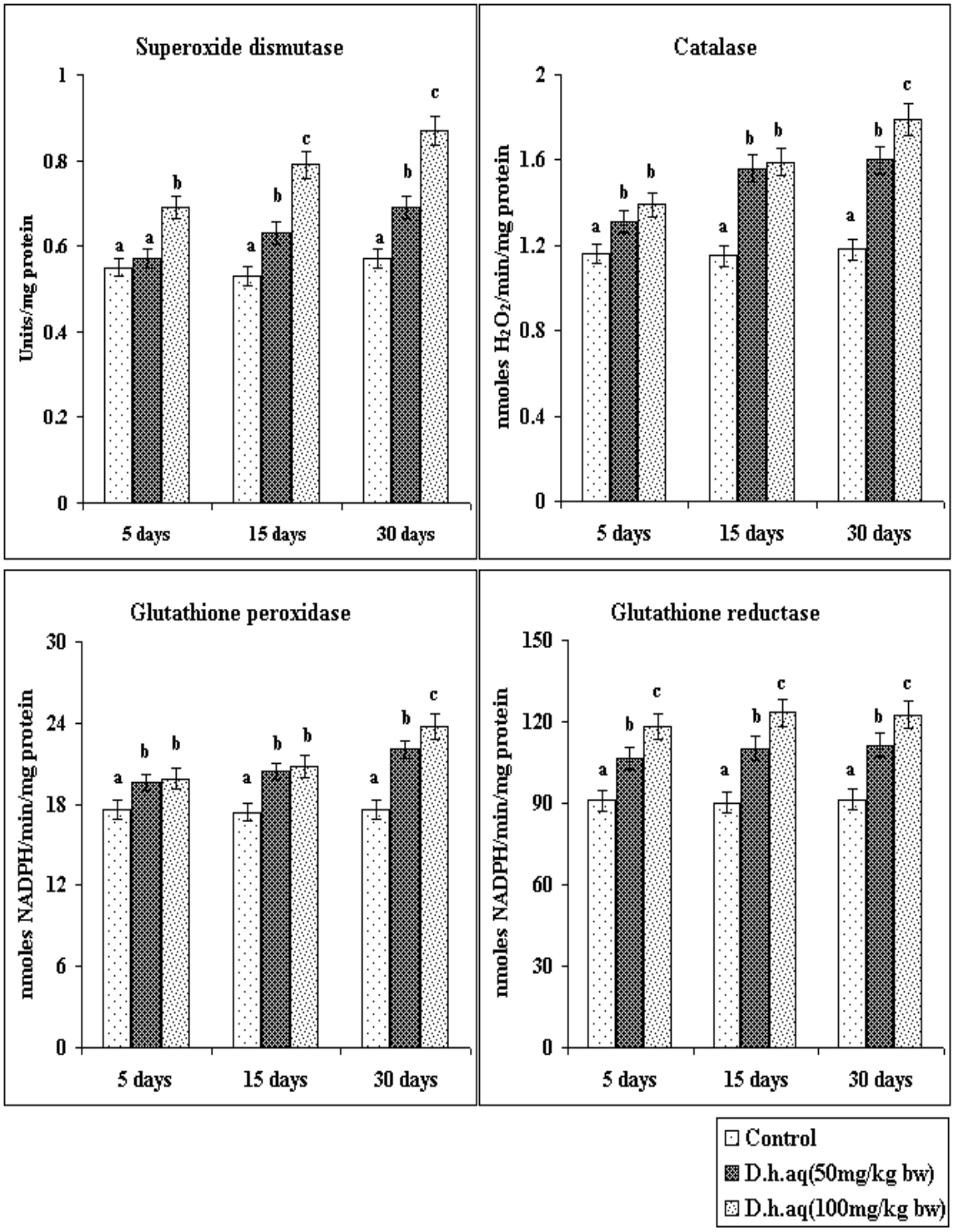


Figure 5.2.: Influence of DHA (**multiple dose**) on the antioxidant enzyme profile in the rat brain.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at $p < 0.05$ level (DMRT).

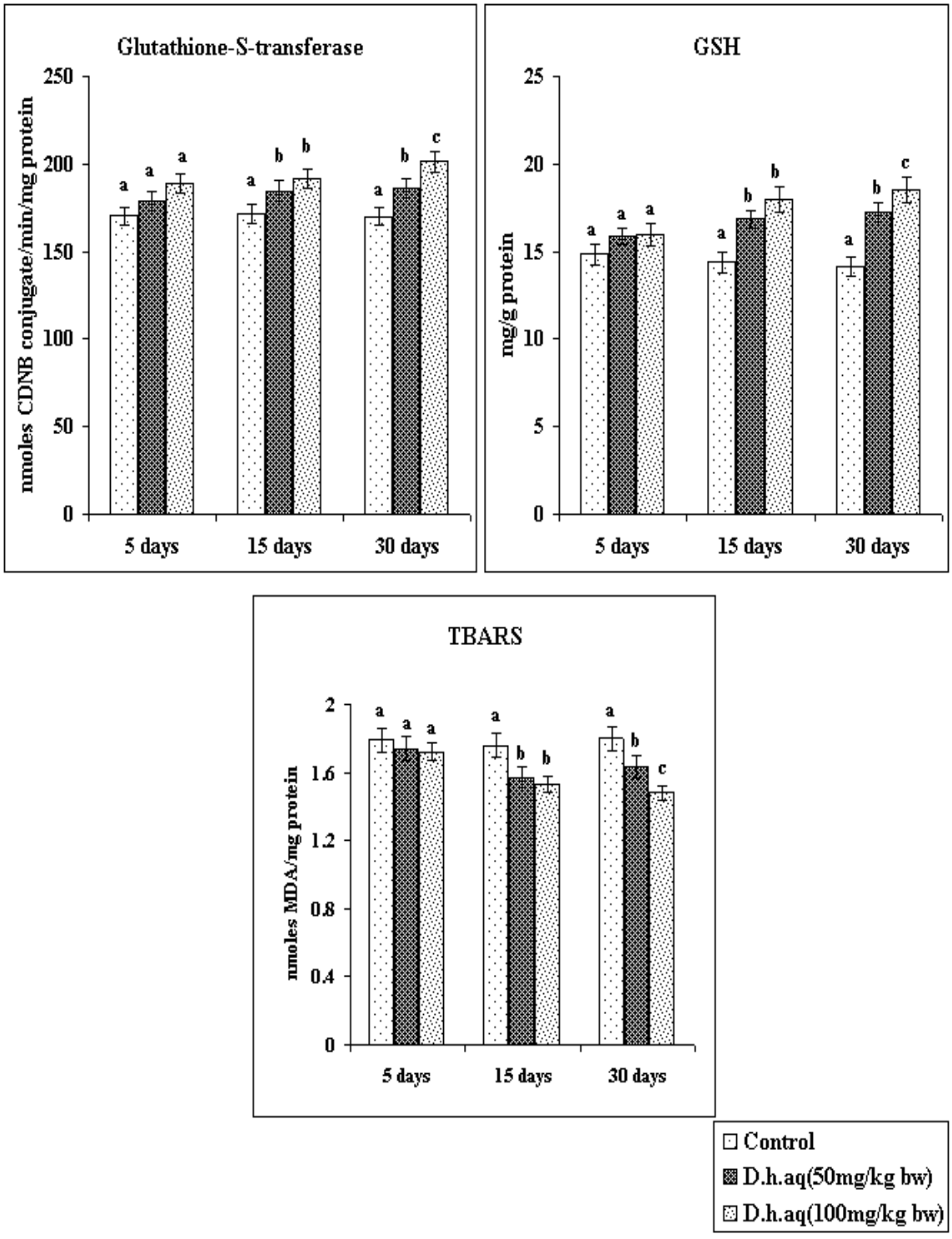


Figure 5.3.: Influence of DHA (**multiple dose**) on the antioxidant profile in the rat brain.

Each bar represents the mean ± SE, n = 8; bars with different alphabets differ significantly at p< 0.05 level (DMRT).

DISCUSSION

Potential deleterious effects of xenobiotics are manifested in tissues due to peroxidation of membrane lipids, particularly the PUFA (Yang and DiSilvestro, 1992; Bagchi and Stohs, 1993). Brain is considered highly vulnerable to oxidative stress than other organs of the body as it consumes high amounts of oxygen; contains high amounts of PUFA and low levels of antioxidant enzymes (Somani et al., 1996).

Our study shows that pretreatment of DHA boosted the antioxidant status of the brain. 30 day pretreatment with DHA alone was most effective in reducing the basal (endogenous) LPO and enhancing the GSH content, and in addition, increased the antioxidant enzyme activities in the brain. Some studies suggest that the enhancement of phase II enzymes by antioxidants such as polyphenols present in aqueous plant extracts, is achieved by upregulating the corresponding genes by interaction with antioxidant response elements (AREs) that transcriptionally regulate these genes (Ferguson, 2001). It has also been shown that the γ-glutamylcystein synthetase (γ-GCS), a key enzyme in the glutathione synthesis, is also transcriptionally regulated by AREs (Myhrstad et al., 2002). It is also known that treatments that induce expression of phase II detoxifying enzymes also result in elevated γ-GCS activity as well as increased intracellular GSH levels (Mulcahy et al., 1997). The possibility that, also in this case, the interaction of some compounds present in the DHA with AREs *in vivo*, would result in a higher antioxidant status apart from its free radical scavenging property.

Acute ethanol intake induces an increment in TBARS levels in brain homogenates (Uysal et al., 1989), an effect that is abolished by antioxidant treatments (Kumral et al., 2005). Ethanol-induced brain lipid peroxidation takes place concomitantly with a partial

depletion in biocomponents related to antioxidant activity, alpha-tocopherol, ascobate, selenium, zinc, copper and GSH (Houze et al., 1991). Ethanol is reported to lower SOD activity in the brain of rats (Ribiere et al., 1987). The mechanism of ethanol induced damage involves several processes related to alcohol metabolism: a) changes in the NAD/NADH ratio resulting from alcohol breakdown by alcohol dehydrogenase, b) production of ROS due to metabolism by the microsomal ethanol-oxidizing system, c) lowering of GSH, and d) decreased activity of antioxidant enzymes (Nordmann, 1994; Lieber , 2005). Both, increased ROS production and decreased antioxidant potential, results in the formation of toxic compounds that leads to cellular damage and scarring, thereby contributing to disease. Our study is in agreement with earlier observations on ethanol-induced oxidative stress in the rat brain (Saravanan et al., 2003). In this study, the aqueous extract of the roots of *D. hamiltonii* inhibited LPO and protein carbonylation induced by ethanol in the rat brain. Further, *D. hamiltonii* aqueous extract pretreatment restored the GSH level and antioxidant enzyme profile of the brain.

HCH has been reported to cause lipid peroxidation in the tissues of rat (Junqueira et al., 1994; Samanta and Chainy, 1995; Samanta and Chainy, 1997). Oxidative stress in the brain regions of rats was evident from induction of LPO by HCH treatment. Among the brain regions, cortex and cerebellum showed high induction as compared to the midbrain and brain stem. Differences in the fatty acid composition of the brain regions could also account for differential lipid peroxidation since white matter is rich in myelin with lower PUFA than the grey matter (Svennerholm, 1968). The midbrain and brain stem are relatively heavily myelinated regions and therefore could be expected to be more resilient to peroxidative stress (Macevilly and Muller, 1996). Our results are in

agreement with earlier reports of decreased SOD activity in the cerebral cortex of rats due to HCH treatment (Sahoo and Chainy, 1998; Sahoo et al., 2000). SOD activity was reduced in all the brain regions, the cortex and stem being the most affected. Our results showing high induction of CAT and GPx in the brain regions are suggestive of enhanced biochemical defenses to scavenge the over production of H_2O_2. This correlates with the results showing increased production of TBARS (LPO) which is indicative of corresponding overproduction of free radicals due to HCH action. Induction of GR could be viewed as a mechanism for replenishment of GSH, the antioxidant molecule vital for cells to detoxify the toxic xenobiotics or their metabolites. Depletion of GSH content in the brain regions by HCH is consistent with the earlier reports on the cerebral cortex (Barros et al., 1988; Sahoo et al., 2000). GSH depletion affects the metabolic detoxication of lipid hydroperoxides formed due to HCH action in the brain (Kosower and Kosower, 1979). GSH-conjugation of HCH metabolites is known (Portig et al., 1979). Induction of glutathione-S-transferase (GST) activity in the brain regions suggests GSH utilization.

Oxidative stress induced by HCH in the brain could be independent of its neurotoxic action. Regional differences in the action of HCH on the brain imply differential distribution in the brain regions and/or its metabolism (Sanfeliu et al., 1988; Martinez and Martinez-Conde, 1998). Another possible reason for the regional differences in the oxidative stress could also involve differences in the neurotransmitter profile (Sunol et al., 1988). The cortex, among the brain regions, shows lower basal level of antioxidant enzymes and, therefore, is likely to be relatively prone to oxidative stress (Hassoun et al., 2003). Our study adds to the evidence that the vulnerability to oxidative stress of the brain is region-specific. Altered activity of antioxidant enzymes in the brain

regions may indicate an adaptive biochemical response to HCH-induced oxidative stress (Carvalho et al., 2001). DHA pretreatment boosted the antioxidant enzyme activity in different regions of the rat brain but HCH induced increase in the activities of CAT, GPx, GR and GST was due to the oxidative stress induced which was evident as increased LPO and decreased GSH level. Pretreatment with the aqueous extract of the roots of *D. hamiltonii* to rats prevented the HCH-induced oxidative stress in rat brain as evident from the decreased LPO and increased GSH level.

OP compounds are neurotoxic primarily through inhibition of AChE, leading to accumulation of acetylcholine and subsequent activation of cholinergic, muscarinic and nicotinic receptors (Bagchi et al., 1995). OPs may also induce oxidative stress on acute exposure (Banerjee et al., 1999). Several pesticides exert their biological effects through electrophilic attack on the cellular constituents of hepatic and brain tissues (Samanta and Chainy, 1995) and generation of reactive oxygen species (Lemaire et al., 1994). The common remedies for OP poisoning are combinations of atropine and oxime reactivator, such as 2-pyridine aldoxime methiodide (2-PAM) aimed at relieving AChE inhibition. The OP-induced oxidative damage could be attenuated through the use of antioxidants which offer promise in the treatment of OP poisoning (Cankayali et al., 2005). *D. hamiltonii* root aqueous extracts are a cocktail of antioxidants (Srivastava et al., 2006). This study has provided evidence that antioxidant rich plant extract offer protection against OP-induced neurotoxicity as evident from lesser AChE inhibition due to pretreatment with the extract. The exact mechanism of prevention is not clear but could involve the free radical scavenging activity as well as the boosting of the brain's antioxidant defenses.

Antioxidant intervention in therapeutic strategy for treatment of neurological disorders is gaining significance (Boyd-kimball et al., 2005; Joshi et al., 2005). Natural plant products are being used in antioxidant therapy for neurodegenerative disorders as they have minimal pathological and toxic side effects in contrast to side effects of a number of synthetic drugs (Butterfield et al., 2002). It is known that dietary antioxidants and herbal extracts can significantly contribute to the modulation of complex mechanisms of neurodegenerative diseases. Given their potential contribution to immune modulation, use of traditional medicine and food plant extracts in the management of the neurological disorders is of great interest. Understanding the molecular mechanisms of neuroprotection, oxidative stress and immune function will facilitate future therapeutic use of antioxidants (Aruoma, 2002; Wang et al., 2003). Research is now proceeding in parallel with efforts to demonstrate clinical efficacy of the secondary metabolites on traditional medicine and food plants.

Our study shows that *D. hamiltonii* enhances the antioxidant status of the brain much more than in the liver (see Chapter IV). There are very few studies showing neuroprotective action of plant extracts. This study is one such, which demonstrates the neuroprotective potential of *D. hamiltonii*. Because failure to cope with oxidative stress is a common factor in the aetiology of many diseases DHA's effects on the improvement of the antioxidant response could provide an explanation for the health promoting properties attributed to it. The role of free radical mediated oxidative injury in acute insults to the nervous system including stroke or trauma, as well as in chronic neurodegenerative disorders, is being just recognized. Tackling free radical offers a novel therapeutic target in such diseases. However, only when the mechanisms and involvement of free radicals

in the pathogenesis of neurodegeneration as well as neurological and CNS diseases are understood, will approaches to antioxidant therapy be designed effectively and targeted.

CONCLUSIONS

CONCLUSIONS

- Natural antioxidants are thought to prevent or slow down free radical induced oxidative stress and, therefore, possess health promoting potential.

- Six antioxidant compounds were isolated from the edible roots of *D. hamiltonii* of which five are novel antioxidant molecules, reported for the first time. The purified compounds showed free radical scavenging activity, reducing power, metal chelation and inhibited protein carbonylation and human LDL oxidation *in vitro*.

- The antioxidant compounds isolated from *D. hamiltonii* ameliorated xenobiotic-induced cytotoxicity in EAT cells and rat primary hepatocytes. The mechanism of cytoprotective action appears to involve inhibition of lipid peroxidation, suppression of ROS and maintaining GSH level.

- The aqueous extract of the roots of *D. hamiltonii* showed hepatoprotective potential in rats. The root extract protected against hepatotoxicity induced by CCl_4 and ethanol. Further, the extract boosted the antioxidant status of the rat liver.

- The root extract also shows neuroprotective potential; it protected the rat brain against xenobiotic (ethanol, HCH, DDVP)-induced neurotoxicity and enhanced the antioxidant status of the brain.

- The bioactive principles of the aqueous extract of *D. hamiltonii* could be responsible for the protective effect by enhancing the antioxidant status *in vivo*.

- The edible roots of *D. hamiltonii* are a source of novel nutraceuticals.

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