

**MAMMALIAN TOXICITY ASSESSMENT  
AND NUTRACEUTICAL PROPERTIES  
OF THE SWALLOW ROOT *DECALEPIS  
HAMILTONII***



A Thesis submitted to the  
*University of Mysore*

For the award of the Degree of  
**DOCTOR OF PHILOSOPHY**

In  
**BIOTECHNOLOGY**

By  
**SHEREEN M.Sc**

Department of Food Protectants and Infestation Control  
**CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE**

**Mysore-570 020**

**INDIA**

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# CHAPTER – I

## LITERATURE REVIEW



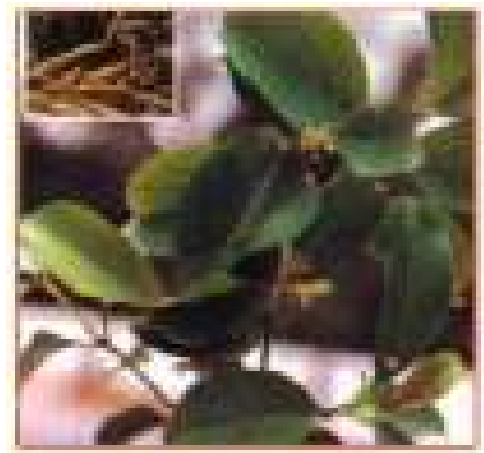
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# ACKNOWLEDGEMENTS

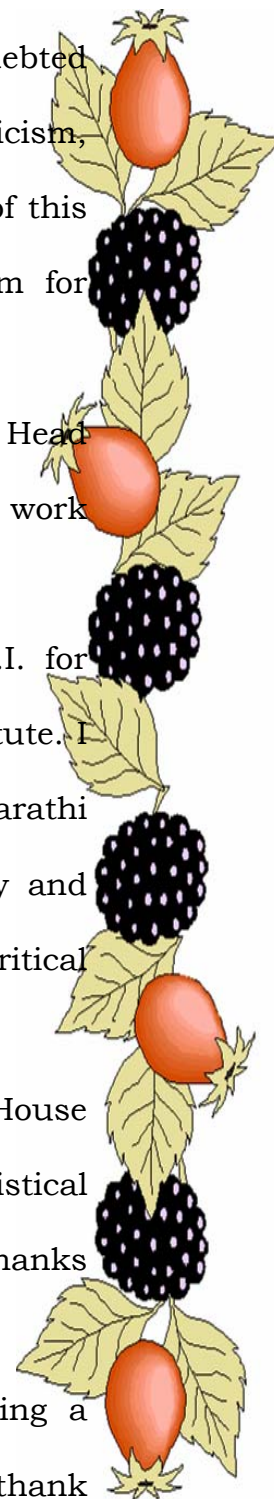
I am extremely happy to express my gratitude to Dr. T. Shivanandappa for his valuable guidance. I am immensely indebted to his guidance in research for his constructive criticism, encouragement patience and perseverance in the fulfillment of this task, at all times during my tenure. I am indebted to him for introducing me to the field of toxicology.

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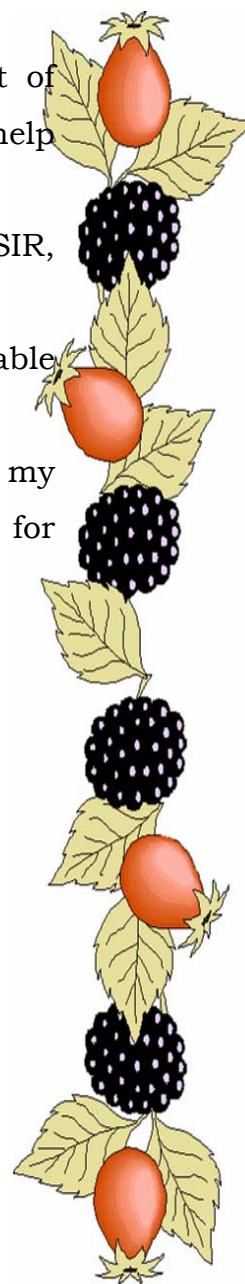


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Finally I wish to place on record the indebtedness to my beloved husband, parents, my dear son, my brothers and for their affectionate support and help throughout my life.





## DECLARATION

I hereby declare that the thesis entitled “MAMMALIAN TOXICITY ASSESSMENT AND NUTRACEUTICAL PROPERTIES OF THE SWALLOW ROOT, “*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 as Senior Research Fellow (CSIR), 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 21<sup>st</sup> OCTOBER 1999 to 31<sup>st</sup> OCTOBER 2002.

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

Date:  
Place:

(SHEREEN)

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## LIST OF ABBREVIATIONS

· CCL <sub>3</sub>	trichloromethyl radical
%	percentage
<	smaller than
>	greater than
µg	microgram
µl	micro litre
·OH	hydroxyl radical
<sup>1</sup> O <sub>2</sub>	singlet oxygen
Abs	absorbance
ALP	alkaline phosphatase
ANOVA	analysis of variance
Aq	aqueous
BHA	butylated hydroxyl anisole
BHT	butylated hydroxyl toluene
BMTD	6-benzamido-4-methoxy-m-toulidenediazonium chloride
BSA	bovine serum albumin
CAT	catalase
CCL <sub>4</sub>	carbon tetrachloride
CDNB	1-chloro 2,4-dinitrobenzene
Cl <sup>-</sup>	chloride
<i>Dh</i>	<i>Decalepis hamiltonii</i>
DTNB	dithiobis-nitrobenzoic acid
e <sup>-</sup>	electron
EDTA	ethylene diamine tetraacetic acid
eg	example
fig	figure
g	grams
GOT	glutamate oxaloacetate transaminase
GPT	glutamate pyruvate transaminase

GPx	glutathioneperoxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-s-transferase
H <sub>2</sub> O	distilled water
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HCl	hydrochloric acid
HMBA	2-hydroxy-4-methoxy benzaldehyde
HOCL	hypochlorous acid
HPLC	high performance liquid chromatography
hr	hours
IC <sub>50</sub>	concentration of inhibitor required to produce 50% enzyme/ activity of inhibition <i>in vitro</i>
LD <sub>50</sub>	concentration required to produce 50% mortality
LDH	lactate dehydrogenase
LDL	lowdensity lipoproteins
LOO <sup>•</sup>	lipidperoxyl radical
LOOH	lipo-peroxide
LPO	lipidperoxidation
M	molar
MDA	malondialdehyde
meth	methanolic
mg	milligrams
mg/kg.b.wt.	milligrams per kilogram body weight.
MgCl <sub>2</sub>	magnesium chloride
min	minutes
ml	milli litre
mM	milli molar



NAD	nicotinamide adenine dinucleotide oxidise form
NADH	nicotinamide adenine dinucleotide reduced form
NADP	nicotinamide adenine dinucleotide phosphate(oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate(reduced form)
NBT	nitroblue tetrazolium salt
O <sub>2</sub>	oxygen
O <sub>2</sub> <sup>•-</sup>	superoxide radical
OCI <sup>-</sup>	hydrochloride ion
OH <sup>-</sup>	hydroxyl ion
OOH <sup>•</sup>	hydroperoxy radical
PMS	Phenazine methoxy sulphate
PUFA	Polyunsaturated fatty acid
ROS	reactive oxygen species
rpm	rotation per minute
secs	seconds
SOD	superoxide dismutase
TBA	thiobarbutaric acid
TBARS	thiobarbuturic acid reactive species
TCA	trichloroacetic acid
Tris	Trichloro (hydroxyl ethyl) aminomethane
UV	ultraviolet
v/v	volume/volume
vit	vitamin
w/v	weight/volume
Xg	gravitational force

## SYNOPSIS OF THE THESIS

### **Mammalian Toxicity Assessment and Nutraceutical Properties of the Swallow Root, *Decalepis hamiltonii***

In the normal course of metabolism, oxidant byproducts due to free radicals formed cause damage to the macromolecules in the cell. Oxidative stress is also caused by various exogenous sources of free radicals that include tobacco smoke, ionizing radiation, pollutants, organic solvents and pesticides. The free radical induced oxidative damage is implicated in several degenerative diseases of aging such as atherosclerosis, rheumatoid arthritis, brain dysfunction, cataracts, immunodeficiency and cancer.

Living organisms have evolved their own antioxidant defenses against free radicals in the form of antioxidant molecules like glutathione and the antioxidant enzymes such as super oxide dismutase, catalase, peroxidase and other enzymes of detoxification.

It is generally recognised that there is an inverse relationship between dietary intake of antioxidant-rich foods and incidence of human diseases. Several studies indicate that antioxidants present in fruits and vegetables offer protective effects against degenerative disease. Therefore, there is great deal of interest in the natural antioxidants or the so-called nutraceuticals as dietary supplements for better health.

Several phytochemicals with antioxidant properties have been isolated from plant sources such as flavanoids and polyphenolic compounds. These phytochemicals possess beneficial properties such as anticarcinogenic, antimutagenic, antidiabetic, anti-inflammatory effects in experimental animals. Many studies have shown specific biochemical action

against DNA damage, inhibition of membrane lipid per oxidation and cellular damage.

Recent surge of interest in the area of herbal health supplements and nutraceuticals has resulted in a huge market potential in excess of 1 billion US \$ in USA alone. In ayurveda, the ancient Indian system of medicine a vast array of plants with medicinal and health promoting potential are known and several medicinal compounds have been isolated. Indian biodiversity offers a rich repertoire of bioactive molecules that are of value in agriculture and medicine. There is a great deal of scientific interest in this area.

*Decalepis hamiltonii* (family: Asclepiaceae) commonly known as the swallow root is a shrubby climber (colloquial names: *kannada-makali беру*; *Tamil-magali kizhangu*; *Telugu-maredu ghaddalu*) with jointed stem and elliptic obviate leaves. It grows wild in the forests of peninsular India. Its roots are elongated and tuberous with fleshy outer layer and woody inner core and possess strong vanilla-like aromatic odour. Traditionally it has been consumed as pickles and the juice made from it is thought to be good for health with “cooling properties”. The roots have also been used as a substitute for *Hemidesmus indicus* used in ayurvedic preparations. Early work on the chemical composition has revealed the presence of amyrins, lupeols, saponins, ketonic substances and 2-hydroxy,4-methoxy benzaldehyde is the flavoring compound present in its volatile fraction. Although the roots of *D.hamiltonii* are in use since ages with the tribal belief that it has positive health benefits, there has been scientific work that has investigated its health promoting potential.

The objective of the work was to investigate the health promoting properties in the roots and its safety to mammals.

Thesis consists of four chapters.

Chapter I gives an overview of the current status of the herbal antioxidants (nutraceuticals) and health.

Chapter II comprises investigation into the antioxidant properties of the root extracts in various *in vitro* systems. Results show that aqueous and methanolic extracts contained strong antioxidant properties based on inhibition of membrane lipid peroxidation in liver and brain, microsomes, and erythrocytes membranes. Inhibition of reactive oxygen species (ROS) such as oxygen superoxide ( $O_2^{\bullet-}$ ) was also observed. Further, 2, hydroxy 4-methoxy benzaldehyde (HMBA), present in the roots extracts was identified from the methanolic extract of the roots and confirmed with authentic standard HMBA was found to possess antioxidant properties and therefore constitutes one of the natural antioxidant molecules from the roots of *D.hamiltonii*.

Antioxidant activity of the roots of *D.hamiltonii* was also demonstrated *in vivo*. Oral intubation of root extracts (100-200 mg/kg/b.wt) to rats showed reduced level of lipid peroxides in the tissue liver and brain and enhanced antioxidant enzymes activities and glutathione content. Therefore, it appears that roots of *D.hamiltonii* possess antioxidant properties both *in vitro* and *in vivo*, an important attribute for its health promoting potential.

Chapter III evaluates the hepatoprotective potential of the root extract of *D.hamiltonii* in the well-known  $CCl_4$  hepatotoxicity model in experimental animals. Both aqueous and methanolic extracts were found to show

protective effect on the CCl<sub>4</sub>-induced liver damage as judged by histopathological observations and the serum marker enzymes (aspartate and alanine-aminotransferases, alkaline phosphatase and LDH). In rats pretreated with the root extracts (100-200 mg/kg b.wt.) the damage was not evident in the liver histopathological sections. Serum enzymes were raised in CCl<sub>4</sub> treated animals indicating liver damage whereas in rats pretreated with the root extract followed by CCl<sub>4</sub> administration, the serum enzyme profile were not raised but comparable to control group. Similar results were obtained with ethyl alcohol as the hepatotoxic agent. Both aqueous and methanolic extracts showed protective action against ethanol-induced hepatotoxicity. These results clearly demonstrate the hepatoprotective property of the roots extracts of *D.hamiltonii*.

Chapter IV comprises mammalian toxicity evaluation study of the root extract and powder of *D.hamiltonii*. Acute toxicity studies were done by administering single oral doses of the root extracts 100-200mg/kg b.wt. No toxic symptoms or mortality was noted. No symptoms of toxicity or mortality were evident. The serum enzyme profile was comparable to the control group. The results indicate that the root extract do not show any significant toxicity at acute exposures

A subchronic toxicity study was done by feeding *D hamiltonii* powder in the diet at 0.5% and 2% for 90 days to weanling rats. Daily food intake and weekly body weight gain were monitored autopsy was done at the end of 90 days. Results showed that food intake and growth in experimental groups were compared to control group. Haematological analysis indicates no change from the control group. Histopathological analysis of the vital

organs showed no changes in rats fed with the experimental diet fed rats. Serum enzyme profiles in the experimental rats were comparable to the control group. Serum cholesterol levels in the experimental diet were significantly lower compared to the control diet fed rats.

An additional study was done to investigate the reproductive potential of male rats fed diets containing *D.hamiltonii* root powder. There was no effect on the fertility or fecundity and survival of the pups born to *D.hamiltonii*-fed rats. Over all, it appears that *D.hamiltonii* shows high margin of mammalian safety. Further, the roots possess health promoting potential.

## Summary and conclusions

- ♣ Roots of *D.hamiltonii* possess antioxidant properties both *in vivo* and *in vitro*.
- ♣ 2-hydroxy 4-methoxy benzaldehyde, a constituent of the root extract, is one of the natural antioxidant molecules.
- ♣ The root extract of *D.hamiltonii* possess marked hepatoprotective potential.
- ♣ The root extracts of *D.hamiltonii* as well as its powder is safe to mammals.
- ♣ In view of the antioxidant properties hepatoprotective potential and high mammalian safety, the roots of *D.hamiltonii* could be considered a scientifically validated source of nutraceuticals and promising therapeutic potential.

## INTRODUCTION

### Free radicals and Diseases

Free radical is an atom or molecule that contains one or more unpaired electrons in the outer orbit which can move in immediate vicinity of its generation and therefore highly unstable leading to cascade of reactions. Free radical reactions are chain reactions, one radical begetting another and converts non radical into a radical. Free radicals are associated with damage to cellular membranes and enzymes as well as to the DNA in the nucleus. Immune cell system uses these free radicals to destroy pathogens and malignant cells and tightly coupled redox reactions. Free radicals are produced during phagocytosis for killing bacteria and in detoxification processes (Elmer Cranton and James Frackelton 1984; Halliwell and Gutteridge 1985).

Reduced oxygen supply leads to ischemia in tissues, through lack of blood supply leading to oxidative stress conditions and formation of reactive oxygen species (free radical). Such active oxygen species interact with wide arrays of cellular components including nucleic acids, proteins, carbohydrates, and lipids that jeopardize the tissue structure and function (Halliwell & Gutteridge 1992; Sies 1993). Free radicals may be oxygen centered, sulphur centered or nitrogen centered. For example, carbon centered metabolites are implicated in the toxicity of xenobiotics such as trichloromethyl radical ( $\text{CCl}_3^\bullet$ ),  $\text{CCl}_3^\bullet$  is responsible for the hepatotoxicity of

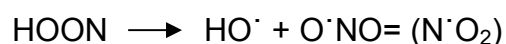
CCl<sub>4</sub>, as a result of reductive cleavage of C-Cl bond in CCl<sub>4</sub> toxicity (Butler, 1961; Aust et. al., 1993).



**Reactive Oxygen Species (ROS):** ROS can be defined as entities of one or more oxygen-containing species and highly reactive. This leads to disturbances in the pro-oxidant and antioxidant balance and increased generation of ROS leads to tissue damage (Sies, 1991, 92, 93; Smith 1992). Imbalance of ROS and antioxidant defense system may be a result of depletion of ROS or enhanced production which can disturb cellular biochemistry and induce oxidative stress (Gutteridge & Halliwell 1994).

Several studies during the last few years have shown that oxidative stress and reactive oxygen species or free radicals are implicated in degenerative diseases, arthritis, asthma, arteriosclerosis, autoimmune diseases, pulmonary diseases, carcinogenesis, cataract, genetic disorders, inflammatory disorders, muscular dystrophy, neuro degenerative diseases, hepatic fibrosis and other hepatic disorders, radiation injury, skin diseases, diabetes mellitus, renal diseases and ageing [Frie, 1994; Peterhans 1997; Domenico et. al., 1998; Beck and Lavender, 1998; Beck, 2000; Christen 2000; Galli et.al., 2000].

Reactive oxygen species produced *in vivo* include superoxide radical (O<sub>2</sub><sup>•-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hypochlorous acid (HOCl), hydroxyl radical, nitric oxide (NO), peroxy nitrous acid, peroxy and alkoxy radicals etc.

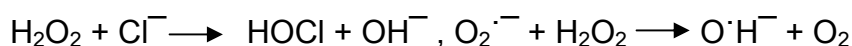




The mechanism of free radical formation may differ. Peroxy nitrous acid is a powerful oxidant, with cytotoxic properties (Aruoma et. al., 1989; Takeuchi 1994).

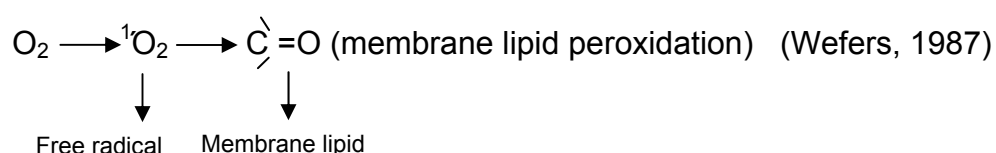
Nitric oxide (NO) has an important role in control of vascular homoeostasis (Kugiyama et. al., 1990). Oxidized LDL inhibits NO and impairs bioactivity of endothelium dependent arterial relaxation and  $\alpha$ -tocopherol restores NO and inhibits LDL oxidation (Wink. et. al., 1991; Nguyen. et. al., 1992; Keaney et. al., 1994; Noguchi and Niki 2000).

Hydroxyl radical is a product of hypochlorous acid and highly reactive.



$\text{Cl}^-$  is a constituent of saline and cytosol. The hydroxyl radical is the most reactive damaging radical with half life of  $10^{-5}$  sec and produced from  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot-}$  by Haber-Weiss reaction (Beauchamp and Fridovich, 1970).

Singlet oxygen is involved in redox reactions in tissues of skin and eye (cataract); it is very unstable and decays fast.



$\text{H}_2\text{O}_2$  easily crosses the cell membrane and attacks at different sites by converting into  $\text{OH}^{\cdot}$ ,  $\text{H}_2\text{O}_2$  generates free  $\text{OH}^{\cdot}$  radicals in presence of transition metal ions (Halliwell. 1978). Metal ions are essential as enzyme cofactors and protein prosthetic groups. A redox-active transition metal mediates oxidation reduction reaction through reversible changes of metal ion. Iron, the most abundant, metal in the living tissue may damage cells

forming potent oxidant radical; for example  $\text{Fe}^{2+}$  reacts with  $\text{H}_2\text{O}_2$  undergoing reduction by Fenton reaction generating hydroxyl radicals ( $\text{HO}^\bullet$ ).

$\text{H}_2\text{O}_2$  produces hydroxyl radicals ( $\text{HO}^\bullet$ ) in the presence of  $\text{Fe}^{2+}$  ions known as superoxide driven Haber Weiss reaction. Super oxide  $^1\text{O}_2^{\cdot-}$  meets many fates and finally gets converted to  $\text{H}_2\text{O}_2$ . These free radicals react with membrane bound polyunsaturated fatty acids and initiate lipid peroxidation (Pyror 1993) leading to cell death, membrane lysis and subsequent degradation to form lipid peroxide radicals. Biochemically, membrane damage due to ROS causes loss of cell structural architecture by disturbing the fluidity and permeability. This affects the influx and efflux of the cellular contents causing cellular swelling, and lysosomal damage by releasing hydrolytic enzymes which lyse the cell and results in serious damage (Wefers and Sies 1983). Autooxidation of cellular components like thiols, hydroquinones, catecholamines,  $\text{H}_2\text{O}_2$  by spontaneous enzyme reaction or dismutation produces  $\text{O}_2^{\cdot-}$  free radicals.

Overall production of free radicals and the mechanism may differ resulting in oxidative stress which can be mild or severe. Mild oxidative stress results in ROS which affects physiological signal for cellular response or signal transduction (Wolin, 1996; Green and Reed, 1998; Bauver and Bauver 1999).

Severe oxidative stress results when the host antioxidant defense is low and damages the biomolecules, tissue damage (Dawson et. al., 1996; Green and Reed 1998; Bauver & Bauver 1999; Evan and Littlewood, 1998; Frank et. al., 2000).

**Free radical injury to biomolecules:** ROS damages proteins, lipids, lipoproteins, nucleic acids, carbohydrates (Beckman & Ames 1997; Steinberg, 1997; Henle & Linn 1997).

*Proteins:*  $\cdot\text{H}$ ,  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\cdot-}$  etc., cause oxidative damage to proteins by attacking side chains, backbone cleavage and protein-protein dimerisation. Oxidation of proteins also results in loss of thiols (Dean et. al., 1997). Proteins with side chain are susceptible to oxidation, for example tryptophan, tyrosine, phenylalanine, histidine, methionine and cysteine. 2-oxo histidine and dityrosine are considered as indicators/ markers of free radical mediated damage to proteins (Acworth and Bailey 1997).

*Lipids:* Lipid peroxidation (LPO) is an important feature of cellular injury due to free radical attack and peroxidation of lipid molecules invariably damage lipids destabilizing the cellular membrane (Yu. 1994). The major fatty acids that are susceptible to lipid peroxidation in cell membranes include poly unsaturated fatty acids (PUFA) like linoleic acid and arachidonic acid (Yu, 1994). Trans-4-hydroxy-2-nonenal and malondialdehyde (MDA) are two byproducts of LPO which cross link and polymerize the membrane components having mutagenic and carcinogenic potential. They attack nucleic acid (Freeman and Crapo, 1982; Valentine 1998).

*Carbohydrates:* oxidation of carbohydrates forms carbonyl compounds and  $\text{H}_2\text{O}_2$  (Valentine 1998).

*Nucleic acids:* ROS causes structural alterations in processes such as nicking, base pair mutations, rearrangements, deletions, insertions and sequence amplifications of nucleic acids (Cuzzocrea et. al., 2001). Oxidation, methylation, depurination and deamination are endogenous reactions (Ames

1989). For example 8-hydroxy guanine replaces guanine under oxidative stress condition and is measured as an index of oxidative damage. Of the five major DNA bases, thiamine and cytosine are most susceptible followed by adenine, guanine and deoxyribose sugar moiety.

**Sources of ROS:** ROS could result from exogenous sources like drugs, toxins, cigarette smoking, electromagnetic radiations, ozone etc. Endogenous ROS are produced during mitochondrial electron transport chain reactions, respiratory burst of phagocytes (Gutteridge 1986), beta oxidation of fats in peroxisomes (Niki, 1997), autooxidation of haemoglobin and ischemia reperfusion injury. Superoxide anion radical regulates metabolism, capable of signalling and communicating between cellular and genetic machinery (Krinsky, 1992; Keher 1993; Ames 1993; Harman, 1996; McCord. 2000). Xanthine oxidase, NADPH oxidase, NO synthase, lipoxygenase are involved in enzymatically generating ROS. These ROS species targets lipids which include polyunsaturated fatty acid leading to lipid peroxidation free radicals which undergo chain initiation and propagation. Lipid peroxidation is one of the free radical chain reactions (Yagi et. al., 1992) and terminates forming more lipid peroxides.

ROS attack proteins, enzyme binding proteins or receptor, DNA damage leading to mutations, genetic damage and premature births. Brain edema and vascular permeability changes in the neural cell are due to changes induced by oxygen derived radicals (Deng et. al., 1993). Reperfusion injury is due to deprivation in oxygen in lungs. ROS from

several cellular responses enhances hypoxia leading to pulmonary diseases (Faber 1990; Reed 1990; Sies, 1991; Smith 1992).

### **Antioxidant defenses**

Any agent that prevents this tissue injury of any free radical catalyzed radical production is an antioxidant. They may be low molecular weight molecules, therapeutic agents; enzymatic agents that dismutate, scavenge or quench partially reduced oxygen, chelate transition metal, oxidant inter conversion and prevent cellular components against oxidative degeneration (Halliwell & Gutteridge 1990; Frei 1994). "Antioxidant is any substance present in low concentration compared with oxidisable substrate and significantly delays or inhibits oxidation/oxidative damage of the target molecules or the substrate" (Halliwell et. al., 1989; 1990; Frei, 1994). Antioxidants may act at different levels in the oxidative process. Scavenging the initiating radicals; by binding to metal ions (by chelation and chelating proteins); scavenging peroxy radicals and removing oxidatively damaged bio molecules.

*Primary antioxidant defense:* Primarily antioxidants interact directly with oxygen radicals and quench their activity and they are enzymatic and non enzymatic components which includes micronutrients like vitamins, water soluble and low molecular weight GSH, ascorbate, urate, bilirubin etc. Lipids soluble are tocopherols (vitamin E)  $\beta$ -carotene (vitamin A)  $\alpha$ -carotene, lycopene, ubiquinol etc and enzymes like superoxide dismutase (SOD) and Catalase CAT (Sies 1992).

*Secondary defenses:* secondary antioxidant defenses include tissue repair enzymes may be either extra cellular or intracellular they are, superoxide

dismutase (SOD), glutathione reductase (GRT), glutathione peroxidase (GPx), endogenous chelators, protease, lipases and DNA repair enzymes (Keher 1993).

Antioxidant defenses may vary in mechanisms as it comprises of different types of cellular component and can be classified as

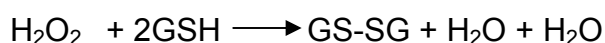
- a) Preventive antioxidants or the first line of defense.
- b) Radical scavenging antioxidants or the second line of defense.
- c) Repair enzymes the third line of defense and finally
- d) The defenses of therapeutic agents and phytochemicals, dietary antioxidants which also includes vitamins and other macro and micronutrients.

*Preventive antioxidants or first line of defense:* Preventive antioxidants act by quenching of superoxide radical  $O_2^{\cdot-}$ . Decomposition of  $H_2O_2$  and sequestration of metal ions results in more damaging  $HO^{\cdot}$  radicals (hydroxyl) (Fenton driven reaction). The antioxidant enzymes, SOD, CAT, GPx GRT and GST, non enzymatic molecules like GSH, trace metals, some proteins and SOD mainly acts on superoxide  $O_2^{\cdot-}$  and  $H_2O_2$  and protect against viral infections, cardiac reperfusion injury (Sharonov 1991; Beckman and Crow, 1993). SOD increase in the central nervous system can have protective effects. SOD is Mn, or  $Cu^+$  or Zn dependent; Mn-SOD is found in plasma extracellularly, whereas SOD- $Cu^+$  and SOD-Zn are intracellular and therefore consumes metal ions for its activity and inhibits metal ion radicals (Chan 1984; Sharonov 1991; Beckman and Crow, 1993). SOD also inhibits  $Cu^+$  induced LDL oxidation, and blocks free radical reaction to protect the cells, reduces  $O_2^{\cdot-}$  to  $H_2O_2$ . SOD is the first trap for superoxide anion  $O_2^{\cdot-}$

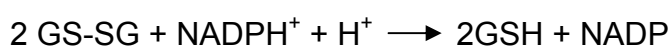
and converts to  $\text{H}_2\text{O}_2$ . Catalase a tetrameric enzyme present in cytosol acts by catalyzing the decomposition of  $\text{H}_2\text{O}_2$  to water and oxygen.



Catalase (CAT) removes  $\text{H}_2\text{O}_2$  in human erythrocytes and scavenges  $\text{H}_2\text{O}_2$ . CAT activity can ameliorate myocardial ischemia injury (Galinanes, 1992).  $\text{H}_2\text{O}_2$  is toxic to cytosol as it can attack different sites and can be removed by glutathione peroxidase. Glutathione peroxidase is a selenium dependent enzyme acts on, lipid hydro peroxides ( $\text{CO}_2\text{H}$ ) using GSH as substrate.



The companion enzyme Glutathione reductase reduces GS-SG to two molecules of GSH.



GSH protects the cells from oxidants by getting oxidized to form GSSG. GSSG further reduces to form GSH by NADPH dependent glutathione reductase. GSH is also a substrate for hydro peroxide reducing glutathione peroxidase reactions (Costagliola & Menzione 1990; Strong et. al., 1993; Mark and Ames 1994). It was also observed in research studies that GSSG in diabetics is higher due to lower GSH reductase activity and increased oxidative stress. This reduction of GSH-reductase activity is believed to be due to inadequate NADPH (cofactor) (Costagliola 1990; Strong 1993). Glutathione peroxidase and glutathione reductase are indirectly regarded as inhibitors of lipid peroxidation (Wefers & Sies 1988; Frei 1991; Buettner, 1993; Ernst Peterhans, 1994; Frei, 1994; Akerboom, & Sies 1994).

Trace elements are metals like selenium (Se), Zinc (Zn) Manganese (Mn) and Copper ( $\text{Cu}^+$ ) etc are primarily required by metalloenzymes.

Glutathione peroxidase is selenium dependent; Mn-SOD in mitochondria dismutates superoxide anion. Zn is a component of several cytosolic enzymes including SOD, alkaline phosphatase etc (Salonen et al. 1985; Evans, 1994; Meier et. al., 1998; Mac, 1998; Yamakura et. al, 1998; Powell, 2000).

*Radical Scavenging antioxidants or second line of defenses:* The radical scavenging antioxidants include GSH, vitamin C, uric acid, albumin, bilirubin, vitamin E (tocopherols), carotenoids, flavonoid and ubiquinol. Glutathione and vitamin E help in balancing of pro-oxidant and antioxidants under oxidative stress condition by reducing and oxidising capacity of nicotinamide adenine dinucleotide phosphate NADPH (Sies 1991; Smith 1992).

$\beta$ -Carotene is an excellent scavenger of singlet oxygen, produced during photo sensitivity. Carotenoids are lipid soluble and precursor of vitamin A (Esterbauer et. al., 1989; Krinsky, 1989; Romiu et. al., 1990). Vitamin E, vitamin C and GSH at water lipid interface of membrane prevent membrane damage by redox reactions; vitamin E and vitamin C are dietary antioxidants. Vitamin E scavenges peroxyl radical of LPO and protects poly unsaturated fatty acid (PUFA) present in cell membrane and low density lipoproteins (LDL) against LPO. Frei et. al., (1991) suggested that uric acid protects LDL against macrophages mediated oxidation. Therefore vitamin E stabilizes the cellular membranes and prevents proliferation of free radicals (Negre 1993).

Ascorbic acid inhibits nitrosamine formation as it is a precursor for activity of enzymes during carcinogenesis. Ascorbic acid, tocopherols and flavonoids scavenge by conjugation reactions. Isoflavones, phytoestrogens,



epigallocatechin gallate (EGCG) etc suppress oxidative radicals and acts as suppressing agents (Starvic 1994).

*Repair enzymes, third line of defense:* These are complex group of enzymes inhibiting peroxide radicals, which causes cell membrane damage and form chain reactions. For example, DNA repairs enzymes, transferase, methionine sulfoxide reductase, lipase, proteases phospholipases (Henle and Linn, 1997; Macio et. al., 1991; Beyer, 1994; Berger et. al., 1997).

### **Diet and health- “Nutraceuticals”**

It has been reported that there is an inverse relationship between dietary intake of nutrient rich foods and the incidence of number of human diseases (Negri et. al., 1991; Kelio et. al.,1996; Rice et. al., 1997; Lu and Foo 2000). However, a large number of foods with such functional properties are being investigated and reviewed in Europe, USA, Japan, Canada and India. (Gopalan, 1994; Thomas, 1994; Bellisle & Diplock et. al., 1998; Sochi et. al., 2002). Such foods are scientifically investigated for their disease prevention and therapeutic effects and consequently new terminologies such as “functional foods”, “medicinal foods” and “nutraceuticals” etc have been introduced. In fact, food could also be medicine. Bio-active components of food, drugs, food additives, carotenoids, flavonoids from fruits and vegetables contribute to health benefits and constitute “nutraceuticals”.

“A nutraceutical is any food or food ingredient considered to provide health benefits including prevention and treatment of diseases” (De Felice 1993; 1994). This definition applies to all categories of foods, fruits and vegetables, pharma foods etc which are also rich source of antioxidants. Some examples are the Ginseng, Ginko biloba, carotenoids phytochemicals, prim rose oil and folic acid in orange juice,  $\beta$ -carotene and dietary fibre in apple, garlic and its medicinal components, omega- 3-fatty acids, cruciferous vegetables, soyabean, flax seeds etc (Miesel, 1997; Fitz Gerald and Meisel, 2000; Klein et. al., 2000; Greeson et. al 2001; Kruger and Mann 2003). Nutraceuticals may be in the form of functional foods, (eg;Tarralin) designer foods,(eg;silymarin), medicinal foods (eg;salacinol) (Kowsalya et. al.,1995; Krishna et. al., 1999; and Wolf and Weisbrode, 2003;

David et. al., 2004). Nutraceuticals could also be antibiotic enhancers, antiviral, anti-tumor, anti-arthritis, anti-atherosclerotic, hepatoprotective and cardioprotective. There are claims of resistance development, increased antibiotic and antimicrobial activity by nutraceuticals of garlic derived products, Echinacea and zinc products. Silymarin (taxifolin and silichristin) derived nutraceuticals include silychristin, silybinin A, silybinin B, silidianin (Ward, Fasitsas, Katz 2002, Wallace et. al., 2003; Barret et. al., 2003). Nutraceutical production may also be possible with food grade micro-organisms, such as *Lactococcus lactis*, lactic acid producing bacteria.

An overview of metabolic engineering activities for nutraceutical productions can be found on website [www.nutraceuticals.com](http://www.nutraceuticals.com) (Hungeholtz et. al., 2002). Nutraceuticals are biological therapeutics promoting health benefits (Whitman. 2001) (see table 1.1). The bioactive molecules in purified active form or the so called nutraceuticals are becoming popular for their molecular, cellular and medical aspects or molecular therapeutics (Colic and Pavelic 2002).

**Table 1.1: Nutraceuticals and their importance in health**

<b>Food/ nutraceuticals</b>	<b>Importance in health</b>
1. Consumption of oat bran	Reduces serum cholesterol
2. Calcium from oranges	Prevents osteoporosis
3. Green tea	Enhancer of humoral and cell mediated immunity
4. Ginseng	Enhance the production of immune cells.
5. Glucosamine ribose and derivatives	Repairs cartilagenous tissue and connective tissue and maintains cellular energy.
6. <i>Artemisia asiatica</i>	Attenuates cerulin induced pancreatitis etc
7. Soya	Extracts prevent polycystic kidney
8. Epidermal growth factor opiod peptides lactoferrin-found in milk	Food supplement and nutrients for growth

So such findings on plant based food have made nutraceuticals acceptable for enhancing the well being. In the “era of nutraceuticals” therefore, there is a great demand to isolate, identify and market the active ingredients from fruits and vegetables that promote healthy lives.

Plant based foods, fruits, vegetables, whole grains, legumes, nuts, higher intake of dietary fibre vitamins, ascorbic acids, tocopherols, carotenoids plant derived phytochemicals present in significant amount in plants provide health benefits and reduce risk of chronic diseases. Moderate consumption of wine provides protection against cardiac diseases due to the antioxidant activity of wine; grapes are rich sources of polyphenols like quercetin and anthocyanins and are antioxidants (Block, 1992; Willet, 1994; 1995; Meisel. 1997; Jaya Prakash et. al., 2001; Sujatha, 2003).

The concept that certain leaves, roots, plant extracts are useful as health aids, because they diminish the risk of diseases since these plants contain phytochemicals such as ferulic acid, caffeic acid, p-coumaric acid, flavonoids, anthocyanins, quercetin, carotenoids etc. These plant polyphenols are ubiquitous in fruits, vegetables and other culinary herbs and have been reported to have multiple biological effects on human health like anti-inflammatory, inhibition of platelet aggregation, anti-microbial, anti-tumor activities (Formica et. al., 1985; Wang, Cao, Prior, 1997; Cao et. al., 2001; Mazza et. al., 2002; Lampe 2003).

There are many other polyphenols and phytochemicals, such as lignans, isoflavones found in wide range of plant species belonging to the families dipterocarpaceae, cyperaceal, gnetaceae, pinaceae, leguminoceae, myrtaceae, moraceae, fabaceae, liliaceae, vitaceae. Polyphenols are

commonly found in the roots, barks, rhizomes and leaves. Major dietary sources like spices, grape, wine, peanuts do contain bioactive nutraceuticals (Cassidy et. al., 2000; Jayaprakash et. al., 2001).

**Spices:** Spices are the dried aromatic parts of plants, such as seeds, berries, roots, pods and sometimes leaves. They form variety and complexity to human diet. Several studies have shown that spices elicit protective actions and good health. Preliminary screenings of 35 different Indian spices and herbs for antibacterial and antiviral effects have been screened, out of which only a handful of spices and their constituents have been tested for antioxidant activity (Krishna and Banerjee, 1999). Besides spices, herbs contain phenolic antioxidative and antimicrobial constituents. Many leafy spices belonging to labiatae family such as sage, rosemary, oregano and thyme show strong antioxidant activity (Nakatami, 1997; Krishna and Banerjee, 1999; Shobana and Naidu, 2000; Wargovich et. al., 2001). Other phytochemicals glucosinates from brassicaceae family, apiaceae family (cumin, funnel, caraway) contain monoterpenes and carotenoids that have beneficial health effects (Lampe; 2003).

**Antioxidants:** Dietary antioxidants can attenuate free radical mediated disease conditions and ameliorate the risk of cardio vascular disease (Rimm, et. al., 1993). Research has shown that dietary antioxidants can limit cell death induced by oxidative stress and therefore may regulate cell death. (Miller et. al., 2001) *Wizened Oinotria* olives and their products are biophenols possess antioxidant properties; traditional olive foods have improved health within mediterranean diet. Antioxidant (Nicola 2000)

properties were also found in herbs of mediterranean diet, *Agaricus campestris*, *Cynara cardunculus*, *Thymus pulegioides* and *Vicia faba* were responsible for health beneficial effects. (Schaffer et. al 2002) glucurinated flavonoid isolated from *spinach* was effective in combating oxidative damage (Bergman et. al., 2003). *Oats* extracts were also known for antioxidant activity (Bratt et. al., 2003). There was high antioxidant activity by asian dark soya sauce. (Long et. al., 2000); *Oats* (*Avena sativa*) exhibited higher antioxidant activity (Handelman et. al., 1999); lemon oil, a novel antioxidant, has biotechnological applications. Many polyphenols are potent antioxidants in foods and through their redox activities they may confer health benefits (Calabrese et. al., 1999). Turmeric and several spices, artichoke, devils claw, garlic and salmon oil have been reported to show antioxidant activity with therapeutic uses (Fernandez et. al., 2003).

**Table 1.2: Dietary antioxidants**

<b>Antioxidants</b>	<b>Dietary Sources</b>	<b>Health effects</b>
Ascorbate	Citrus fruits, fortified cereals/dry cereals, spinach, cabbage, onion, melons, cruciferous, vegetables etc	Strong protection cancer prevention and various other diseases (Block, 1991).
Flavonoids	Fruits and vegetables, apples, lemons, oranges, cauliflower, tea, bilberries, grapes etc	Free radical scavengers; metal ion chelators; prevents LPO (Pincemail et. al., 1986; Harborne 1988).
Retinoids and carotenoids	Carrots, tomatoes, grapefruit, bean, broccoli, orange, mangoes	Scavenges singlet oxygen effectively scavenges peroxy radical and inhibits LPO and prevents oxidative damage (Romiu et. al., 1990).
Vitamin A	Milk, egg, fish, liver	Effective against triplet sensitizers (oxygen free radicals) photo and electrons and singlet oxygen (Romiu et. al., 1990; Frei, 1994).
Vitamin E	Dietary intake of PUFA containing edible-oils	Prevents membrane damage protects LDL and scavenges lipid peroxy radicals (Stocker et. al., 1991, Frei and Gaziano 1993).
Ubiquinone	Soyabean, meat, fish (mackerel and sardine) wheat germ, vegetables, spinach, garlic, Cow's milk, broccoli, carrots wheat grain, fruits and vegetables.	Prevents membrane lipid peroxidation;Involved in mitochondrial electron transport reactions; regenerates vitamin E, prevents LDL oxidation (Stocker et. al., 1991, Frei and Gaziano 1993).



Block and her colleagues (1992) have recently reviewed 172 studies that relate to consumption of fruits and vegetables and the incidence of diseases, lower risk of degenerative diseases (Block et. al., 1992). Dietary antioxidants ascorbate, tocopherol, carotenoids, urate, bilirubin, carnosine, ubiquinone are implicated in many of the degenerative diseases as these antioxidants prevent or reduce mutagenesis and carcinogenesis since DNA damage can be prevented by antioxidant rich foods. (Stocker et. al., 1991; Frei and Ames 1992; Seis et. al., 1992 and Wolf 1992) (see table 1.2). Induction of phase II detoxification enzymes protects against carcinogenesis. Edible plants belonging to Cruciferaceae are inducers of phase II detoxification enzymes (Block, 1992).

Synthetic lipid lowering drugs also possess antioxidant activity which includes probucol, lovastatin, amino guanidine, leumedin etc (Cristol et. al., 1992; Aviram 1992; Picard et. al., 1992; Naveb et. al., 1993). Some commercially used antioxidants in food industry include  $\alpha$ -tocopherol, propylgallate (PG), butylated hydroxy anisole (BHA) butylated hydroxy toluene (BHT), Tertiary butyl hydroquinone (TBHQ), santolquin, nordi hydro guaiaretic acid (NDGA) (Richie, 1986; Kikugawa et. al., 1990; Kocchar et. al., 1990). Natural antioxidants from plant extracts are potential sources of natural antioxidant drugs and therapeutic agents which can replace synthetic antioxidants (Namiki, 1990).

## Plant derived antioxidants

Cereals, pulses, nuts, spices, beverages, tea, coffee, cocoa, wine and several other culinary and medicinal herbs possess diverse pharmacological properties and have shown to be sources of chemopreventive agents. Many of these bioactive constituents other than vitamins and minerals are known as phytochemicals or phyto-protectants. (Ames 1983; Hausteen 1983; Pratt 1992; Caragay, 1992; Ames 1993; Dakora 1995).

Phytochemicals are found to be anti-inflammatory, anti-atherogenic antioxidants, therefore their clinical importance in health and diseases. This is evident from traditional practices of folk medicine which involves numerous plant extracts to treat various health disorders for example *Lycoris radiata* have shown pharmacological and therapeutic use in Alzheimers disease (Mark 2004).

Therefore much interest is currently on phytochemicals as bioactive antioxidants which include polyphenols like flavonoids, coumarins such as *Scoparone* from *Artemisia scoparia*, *Osthole* from *Angelica pubescens*, Morin hydrate a plant derived antioxidant, gossypol, quercetin, myricetin are strong antioxidants. Coumarins are identified from natural sources (plants) with synergistic antioxidant activity (Wu et. al., 1993; Beukelman 1995; Fylaktakidon 2004). Plant derived monoterpene-bakuchiol was found to possess antioxidant activity (Adhikari et. al., 2003).

There are more than a dozen classes of phytochemicals (Lampe, 2003) most of which are plant secondary metabolites and pigments (see table1.3).

**Table 1.3: Phytochemicals derived from various plant species**

<b>Class of phytochemicals</b>	<b>Sources</b>
<u>TERPENES</u> Monoterpenes Tetraterpenes (carotenoids)	cumin, fennel ,caraway, saffron, cinnamon, juniper, ginger, turmeric (Lampe, 2003).
<u>Terpene derivatives</u> Phenyl propanoids cinnamic acid, Eugenol, Vanillin	cinnamon, cloves, vanilla bean (Lampe, 2003).
<u>Diarylheptanoids</u> Curcumin	turmeric (Lampe, 2003).
<u>Isothiocyanates</u> Allyl isothiocyanate 6-methylsulfinyl isothiocyanate allyl compounds	mustard seeds, wasabi wasabi garlic- c).
<u>Sulphur compounds</u> Thiols, sulphides, di-&poly sulphides	garlic, asafoetida (Lampe 2003).
<u>Phenolic acids</u> Ferulic acid, caffeic acid P-coumaric acid	coffee, tea, and cocoa (Nardini et. al., 1995; 1998; Daglia et. al., 2000; Hammerstone. et. al., 2000) .
Phenolics Flavonoids Catechins Gallic acid Iso flavonoids Anthocyanidins	onions, (Boyle et. al., 2000) bean, sprouts, (Tomohirosawa et. al., 1999) spices, (Madsen et. al., 1995) Vaccinium myrtillus (bilberries) (Morazzoni et. al., 1996).
Alkaloids Glucosinolates Indoles	Brassicaceae (Lampe, 2003).
Hypericin and Pseudohypericin	St. John's Wort( Ernst 2000).
<u>Terpenoids</u> Phytosterols Limonoids Triterpenols	leguminosae, myrtaceae, liliaceae, (Rice et. al., 1996; Pool et. al., 1999 ; Cassidy et. al., 2000).

**Polyphenols:** Polyphenols are heterogeneous diverse class of compounds constituting major classes of antioxidants (see table 1.4). These occur in all parts of plant including fruits, vegetables, seeds, leaves, roots, bark and species of leguminoseceae plants such as soyabeans, sesame seeds, flowers etc (Pratt et. al., 1990).

Antioxidant activity of phenols towards peroxy radicals is by the dissociation of energy of the phenolic heterocyclic ring and O-H bond. The redox potential and the steric hindrance are due to abstraction of phenolic hydrogen by peroxy radicals. The peroxy radicals are considered to be electrophobic substituents (Burton et. al., 1985; 1986; Mukai et. al., 1989). Plant phenols like flavonoids are polyphenols with cancer blocking property which have a common skeleton of di-phenyl pyrons, two benzene rings (A-B) linked through hetero cyclic pyran or pyrone ring (C). The basic ring structure allows a multitude of substitution patterns giving rise to flavonoids, flavones, catechins anthocyanidines and isoflavonoids. They are low molecular weight compounds having 3 phenolic rings (Pyrone) based on flavone nucleus. The conjugation ring structure and the heterogeneous group allow the phenol to actively scavenge and stabilize free radicals. The carboxylic groups inhibit lipid peroxidation by metal chelation (Rice-Evans et. al., 1996).

Flavonoids are ubiquitous in various fruits and vegetables reported to have multiple beneficial biological effects as antioxidants, anti-inflammatory, anti-microbial, anti-tumor etc. Polyphenolic flavonoids have been reported from fruits and vegetables (Formica and Regelson, 1995).

**Table 1.4: Polyphenols- flavonoids and other phenols from different plant sources**

<b>Classes of Phytochemicals</b>	<b>Sources</b>
<u>Polyphenols</u>	fruits, vegetables, spices, herbs, culinary herbs, beverages etc.
<u>Flavonoids:</u> flavonols, catechin, epicatechin gallate, quercetin, epicatechin, anthocyanidins, apigenidin, flavones, pyro cyanidins anthocyanidins	green and black tea, coffee, cocoa, red wine, grape seed (Nardini et. al., 1995; 1998; Coa et. al., 1996 ; Evans et. al., 1997; Daglia 2000 ; Hammerstone et. al., 2000; Jayaprakash et. al., 2001).
<u>Flavonols</u> Narigenin, Taxifolin	eucalyptus, citrus fruits (Rice-Evans et. al., 1994).
<u>Other polyphenols include</u> Tannins Sesamin, Sesamamolin, Sesaminol,	labiatae (Bravo 1998). sesame seed oil (Lee-Wenchang et. al., 2002).
Caffetannins	cereals, legumes and nuts
Stilbenes, Anthraquinones  Lignans	vegetables, fruits are rich sources of polyphenols (Bravo 1998)  common in leguminose and fabaceae
Napthoquinones $\gamma$ -linolenic acid Stilbenes Resveratrol Carnosol, Carnosic acid Isoflavones	primrose oil (Wu. et. al.,1982 Aruoma et. al.,1992 Wang et. al.,1994) grapes and peanuts. rosemary,(Pratt, et. al., 1990) soyabeans, (Cassidy. et. al., 2000)

Soya, isoflavanones, ferulic and p-coumaric acid have been reported to show estrogenic and anti-estrogenic activity (Gehm et. al., 1997; Setchella and Cassidy 1999). Phytosterols possess serum cholesterol lowering properties and they are incorporated into functional foods that are being developed and marketed. Some of the phytosterol products contain common unsaturated sterols and a subset called phytostanols (saturated sterols called stanols) (Robert. et. al., 2003). Several dietary studies of phytoestrogen rich diets (Soya) on menopausal symptoms, cardio vascular functions, endocrine regulation of the menstrual cycle etc have shown positive effects that may be interpreted as beneficial. Red wine, a major source of resveratrol, shows protective effects by various mechanisms (Renaud et. al., 1992; Cassidy et. al., 2000).

## **Indian medicinal plants as source of health promoting**

### **Phytochemicals**

The ancient Indian system of medicine “*Ayurveda*” is based on plants. Several herbal plants have been described to possess curative as well as restorative properties. (CSIR-Wealth of India). The results of collaborative work of National Institute of Health, USA and Central Drug Research Institute, Lucknow, India, where the botanical, chemical and pharmacological studies on 2000 Indian, medicinal plant materials were screened in two phases, first phase was based on the actual use in the traditional Indian system of medicine. The second phase was broad-screening of a large number of natural products for biological activity irrespective of the fact whether they were used as medicine or not, of which 1973 plant species were found to be effective against various diseases and health disorders (see table 1.5). The plant derived phytochemicals include alkaloids, terpenoids, quinones etc. (Dhar et. al., 1968; 1973; 1974, Bhakuni, et. al 1969; 1975; 1974; Dhawan, 1977; 1980).

Scartezzone and Speronio (2000) have reviewed Indian medicinal plants used in ayurveda system of medicine like *Emblica officinalis*, *Curcuma longa*, *Mangifera indica*, *Momordica charantia*, *Santalum album*, *Swertia chirata*, *Withania somnifera*. These plants exhibit antioxidant properties including other pharmacological properties.

**Table 1.5: Phytochemicals from Indian medicinal plants and their effects**

<b>Polyphenols</b>	<b>Medicinal plant</b>	<b>Effects</b>
Bisbenzy lisoquinoline (alkaloid, hayatin)	<i>Cissampelos pareira</i>	Neuromuscular blocking agent (Bhattacharji et. al., 1952).
Rauwolscine alkaloid	<i>Rauwolfia canescens</i>	$\alpha$ -adrenergic blocking activity (Dhar et. al., 1955; Kohli et. al., 1961).
Furano-coumarin	<i>Psoralea corylifolia</i>	Anticancer (Mukerji et. al., 1961).
7-alkanin naptha quinines areneben-1	<i>Arebia nobilis</i>	Anticancer inhibits reverse transcriptase (Katti et. al., 1979).
Cocsulinine di-benzo-1, 4-dioxy bisbenzylisoquinoline	<i>Cocculus pendulus</i>	Anticancer (Gupta et. al., 1970).
Glycoside-ruvoside cardenolide, asclepin	<i>Asclepias curassavica</i>	Cardiotonic activity (Patnaik et. al 1978).
Kutkin	<i>Picrorrhiza kurrooa</i>	Protection against lanthanum and galactosamine Induced liver damage (Kar et. al., 1976).



Several approaches have been followed to develop therapeutic aids from natural products. Further, active constituents vary under different ecological conditions based on the plant's geographical region. One of the examples is the polyphenol lupeols content in plant varies with the ecological conditions. Lupeols loses its property on storage; for example plants of labiatae family (Rake et. al., 2002).

**Phytochemicals as antioxidants:** Plants are abundant sources of important phytochemicals. Uses of natural and herbal medicines have gained importance in India and all over the world (see table 1.6). Few such examples where plant are exploited for antioxidant activity are *Passiflora alata* (*passifloraceae*) a plant of folk medicine in South America used as sedative and tranquilizer possesses antioxidant properties (Rodrigues et. al., 2005). *Equisetum arvense* (*Equisetaceae*) is an oriental medicinal herb with both antioxidant and hepatoprotective property (Hyuncheol et. al., 2004). *Rhus verniciflua* Stokes herbal plant with antioxidant and therapeutic potential (Lim et. al., 2001, Le et. al., 2002). Prenyl flavones isolated from *Artocarpus heterophyllus* possessed antioxidant potential (Ko.FN et. al., 1998). Butein isolated from *Dalbergia odorifera* was found to be versatile scavenger and powerful antioxidant (Cheng et. al., 1998). *Withania somnifera* (*Ashwa gandha*) used in rasayana of Indian system of medicine has antioxidant properties (Gupt et. al., 2003), Knot wood extracts possess high antioxidative potency (Willfor, 2003). *Artemisia campestris* is a medicinal herb with hepatoprotective and antioxidant properties (Aniya et. al., 2000). These studies have proved the importance of plant derived products in medicine and therapeutics.

**Table 1.6: Medicinal plants with phytochemical antioxidant compounds**

<b>Medicinal plants with antioxidant properties</b>	<b>Phytochemical antioxidant components</b>
<i>Emilia sochifofoha</i> (Asteraceae): leaves extract	Pyrrolizidine, alkaloids and (flavanoids) (Shylesh and Padikkala 1999)
<i>Centella asiatica</i> (umbelliferae): rejuvenator, whole plant extract.	Polyphenols (Jayashree et. al.,2003)
<i>Similax china</i> (liliaceae) :root extracts	Iso-serine, S-methyl cysteine sulfoxide in rhizome, leaves-starch glycoside, gums, tannins, amide steroidal saponins N-acylarginine in plant. steroidal saponins in roots (Tripathi et. al.,2001)
<i>Embllica officinalis</i> amla (euphorbiaceae):fruit	Fruits rich in vitamin C, tannins, gallic acid ellagic acid, emblicanin, punigluconin penduculagin (cocktail of chyavanprash) (Bhattacharya et. al.,1999)
<i>Ginko biloba</i> :leaves extract	Flavanoidic heteroside components, terpenoids heteroside quercetin and kaemferol. (Pincemail et. al.,1989)
<i>Rumex crispus</i> :leaves and seeds used as vegetable in Turkey	Phytochemicals rich in antioxidants (Ali-Yildrin et. al., 2001)
Piper beetle : leaf	Triterpenes, beta sistosterol (Saravanan et.al.,2002)
<i>Sideritis javalanbrensis</i> labiateae :ariel parts of plant used	Flavonoid, aglycones, cirsilol, cirsimaritin, methoxy-cirsilineol (Jose- luis Rois et. al.,1992)
<i>Phellinus baumi</i> mushroom:folk-medicine	Rich in phenolics (Mi-yaeshon et. al., 2003)
<i>Morinda citrifolia</i> (Indian mulberry): Roots, fruits and leaves	Anthroquinones, aglycones, glycosides (Thomas 1971, Zenk, 1975;Zmohdain et.al.,2002)

**Table 1.7: Some of the investigations of medicinal plants with hepatoprotective activity**

Sl. No	Botanical name	Part of the plant for medicinal use	Family	Authors
1	<i>Pongamia pinnata</i> (Mess)	Seeds (oil)	Fabaceae	Sasmal et. al., (1997)
2	<i>Cichorium intybus</i>	Stem and bark	Asteraceae	Gadgoli & Mishra, (1997)
3	<i>Bauhinia vahlii</i>	Bark	Caesalpinaceae	Gupta et. al.,(1997)
4	<i>Embllica offinalis</i>	Fruit	Euphorbiaceae	Jose & Kuttan (2000)
5	<i>Boerhaavia diffusa</i> L	Roots	Nyctaginaceae	Chandan et. al.,(1991) Rawat et. al.,(1997)
6	<i>Cassia occidentalis</i>	Leaves	Leguminosceae	Jafri. et. al., (1999)
7	<i>Glycyrrhiza glabra</i>	roots/ whole plant	Fabaceae	Vijaya Padma et. al., (1998)
8	<i>Mamordica subangulata</i> and <i>Naragamia alata</i>	Fruit	Cucurbitaceae	Asha. (2001)
9	<i>Terminalia bellerica</i> (roxb)	Fruit	Combretaceae	Anand. et. al.,(1994)
10	<i>Acillea millefolius</i>	Seeds	Combretaceae	Gadgoli & Mishra (1995)
11	<i>Eclipta alba</i>	Leaves	Combretaceae	Singh et. al., (1993;2001)
12	<i>Ricinus communis</i>	Leaves	Euphorbiaceae	Visen, et. al.,(1992)
13	<i>Acanthus ilicifolius</i>	Leaves, stem	Acanthceae	Babu ,et.al., (2001)
14	<i>Ficus hispida</i> (Linn)	Leaves	Moraceae	Mandal, et. al.,(2000)
15	<i>Phyllanthus amarus</i>	Leaves	Euphorbiaceae	Tripathi. et al., (1993)
16	<i>Capparis spinosa</i>	Bark	Capparaceae	Vijaya Padma et. al., (1998)
17	<i>Terminalia arjuna</i>	Bark	Combretaceae	
18	<i>Achillea millefolium</i>	Seeds	Astraceae	
19	<i>Tamarix gallica</i>	Whole plant		
20	<i>Terminalia chebula</i>	Fruits	Combretaceae	
21	<i>Azadirachta indica</i>	Leaves	Meliaceae	
22	<i>Sidacordifolia</i>	Roots		

Brahma rasayana, a polyherbal formulation of a group of Indian medicinal plants possess antioxidant potential (Ramadasan Kuttan. et. al., 2001). Geriforte, a herbo-mineral preparation, is a stimulant for antioxidant system and prevents radical damage (Vandana et. al., 1998). Lot of information on prevention of oxidative stress related diseases and other chronic diseases using nutraceuticals, therapeutic agents drugs etc. from plant derived bioactive principles is available (Vinitha Singh et. al.,1992; Bindiya saraswat et. al.,1993; Govindarajan et. al., 2003; Visen et. al., 1996; Ansari et. al., 1998).

**Phytochemicals as hepatoprotectants:** Many of the plants belonging to Indian system of medicine show both antioxidant and hepatoprotective activity against liver damage caused by xenobiotics and other infections (see table 1.7and 1.8). The involvement of free radicals in liver diseases has been explained by Poli, 1993. ROS and lipid peroxidation play a role in hepatic fibrosis with loss of normal liver cell architecture. Antioxidants inhibit this process of fibrosis which induces cell damage of liver. Therapeutic claims of traditional medicinal plants could be due to their scavenging properties and are considered as antioxidants (Thresiamma et. al., 1996).

Hepatoprotective effects of some Indian medical plants against liver damage by CCL<sub>4</sub>, CCl<sub>4</sub> produces toxic effect by free radical generation (CCl<sub>3</sub>), damages bile duct or ductile, canalicular membrane of hepatocytes and blocks transcanalicular transport of bile acids (Bindiya et. al.,1993), ethanol toxicity and *Plasmodium berghei* were productive. The protective effects were due to the presence of various phytochemicals including polyphenols present in the medicinal plants.

**Table 1.8: Medicinal plants with hepatoprotective properties**

<b>Medicinal plants. Or culinary herbs.</b>	<b>Phytochemical content polyphenol constituents</b>	<b>Effects</b>	<b>Means of liver damage</b>
<i>Picrorhiza kurroa</i> (leaves)	Picroliv- irridoid glycoside fraction picroside Kutkin- picroside1 Kutkoside catapol cinnamoyl, vanillyl- Kutkoside Kutkiolacetate kutikisterol	Hepatoprotective Hepatoprotective	CCl <sub>4</sub> <i>Plasmodium berghei</i> ethanol toxicity galactamine
(Singh and Rastogi 1972; Das et. al.,1976; Raj et.al.,1990; Chander et. al.,1992; Vinitha Singh et. al.,1992; Bindiya saraswat et. al.,1993; Govindarajan et. al.,2003; Visen et. al., 1996; Ansari et. al., 1998 )			
<i>Silymarin marianum</i> (leaves)	Flavolignan –sily marin	Hepatoprotective	CCl <sub>4</sub> galatosamine <i>Plasmodium berghei</i>
(Rameshchander et. al.,1989)			
<i>H.rosasinensis</i> (fruits flower petals)	Anthocyanins glycosides of anthocyanidines flavonoids, Alkylgallates	Hepatoprotective	CCl <sub>4</sub>
Frederick et. al. ,1998			
<i>Garcinia kola</i> (seeds)	Kolaviron- Garcinia biflavanoid kolaflavanone	Hepatoprotective	2-Acetyl amino glucosamine
Farombi, et. al., 2000			
<i>Wedelia calendulaceae</i> (leaves)	Coumestans (wedelolactone dimethyl benzofurannon wedelic acid	Hepatoprotective effective for liver skin and neural system	Paracetamol
(Emmanuel et. al.,2001)			
<i>Combretum dolichopetalum</i> leaf <i>Morinda lucida</i> (root bark)	Alkaloids flavonoids tannins saponins	Hepatoprotective	CCl <sub>4</sub>
(Asuzu et. al.,1989; Udem et. al.,1997)			
<i>Lawsonia alba</i> (lan) (bark)	Polyphenols etc. may be present	has antioxidant and hepatoprotective properties	CCl <sub>4</sub>
(Manoj Bhandarkar et.al., 2003)			

<i>Andrographis paniculate</i>	Andrographolide (kalmegh)	Liver disorders general debility dyspepsia hepatoprotective	CCl <sub>4</sub>
(Choudhary et.al., 1984; Neha Trivedi and Rawal, 2001)			
<i>Inula racemose</i> hook f. (roots)	Alkaloids Amino acids sugars, fixed oils phenols, Tannins steroids	Used in all poly herbal formulation of Indian medicine (pushkarmoola)	CCl <sub>4</sub>
(Rao, and Mishra, 1997)			
<i>Paedenia foetida</i> leaf	Alkaloids flavonoids steroids polyphenols etc.	Mainly used in liver disorders	CCl <sub>4</sub>
(Dave, Bhavsar 1992; 1993;1996)			
<i>Ocimum sanctum</i> (green tulsi)	Ocimum flavanoids essential oils	Chemo protective effects hepatoprotective effects	CCl <sub>4</sub>
(Devi, Ganasoundari Rao et. al. ,1999; Uma devi, et. al., 2000)			
<i>Solanum nigrum</i> (fruits)	Solamin alkaloids saponins, carotene glycosides , tanins steroids and Terpenoids	Liver diseases	CCl <sub>4</sub>
(Nadeem, Dendiya et. al.,1997)			
<i>Fumaria indica</i>	Mono methyl fumarate	Antihepatotoxic activity liver disorders	CCl <sub>4</sub>
(Rao and Mishra, 1998)			
<i>Propolis or beeglue</i>	Organic aromatic acids ketones, caffeic acid ferulic acid isoferulic acid cinnamic and 3,4 dimethoxy cinnamic acid various flavonols)	Hepatoprotective activity anti-inflammatory, antihyperglycemic antimutagenic	CCl <sub>4</sub>
(Aiman, et. al., 2002)			
<i>Sarcostemma brevistigma</i>	Bregenin, brevine brevinine, sarcogenin sarcobiose flavonoids	Hepatoprotective effects liver disorders anti rheumatic anti allergy broncho dilator	CCl <sub>4</sub>
(Sethuraman 2003)			

Some of the poly herbal formulations of Indian system of medicine for Haridradighirta (Based on panchagarya – ancient Ayurvedic text) are a combination of *Terminalia chebula* (fruit), *Emblica officinalis* (fruit), *Azadirachta indica* (leaves), *Sida cardifolia* (roots), *Glycyrrhiza glabra* roots, is effective against liver disorders (Satturwar et. al 2003). Another example of such poly herbal formulation effective against liver disorders is liv-52 and hepatomed (Sudhir and Gill 1991). Hepatomed is combination of *P. kurrooa*, *A. paniculate*, *S. nigrum* and *C.intybus* was found to be safe and hepato stimulant.

**Safety consideration of phytochemicals:** The plant derived phytochemicals, pharma foods, and functional foods and, medicinal foods are rich in antioxidant properties; so such foods with health benefits posses' physiological and beneficial functions. They are health foods with disease preventing effects and not pharmaceutical drugs (Soichi et. al., 2002). Pharmacological doses are used clinically to treat specific diseases. Physiological doses are used to improve or maintain good health such as dietary supplements, antioxidants, micronutrients, trace metals etc. They follow recommended dietary allowance (RDA). Natural phytochemicals present in low concentrations are beneficial. For purified mega/large dose usage, safety study is necessary to reduce risks and ensure safety and efficacy factor (Soichi et. al., 2002).

The classification of the bioactive substances as dietary supplement or functional food and their food safety determination by means of scientific

standards (“generally recognized safe substances”) should be comparable. Dietary supplement include vitamins, minerals, herbs and amino acids which is to increase the dietary intake. Plant extracts and combination of other ingredients are not applicable under dietary supplements. Dietary supplements have to be scientifically approved by Food and Nutrition board (USA).

Basically the product or bio active molecule as therapeutic agent or a plant extract is a simple or complex mixture derived from respective novel sources. Its exposure and toxicological studies provides the margin of safety and determines toxic levels and also the health benefits as well adverse health effects (Ernst, 1999; Bryan et. al., 2001; Kruger and Mann 2003).

Plant extracts are complex mixtures with myriad components that are different classes of chemicals. Key information about the plant is necessary which includes documentation of herbal sources, chemical class of active principal, if known, processing formulations, administrations contradictions, and its traditional geographical areas as this would be easy to predict the toxicity profile. This could also include animal studies such as repeat- dose general toxicity studies, toxico-kinetic studies, reproductive toxicology, genotoxicity and cariogenicity studies, further modes and site of action. From these primary clinical evidences magnitude of risk factors consistency, exposure effect, latency, dose-response mechanism of action, experimental design involving laboratory animals are evaluated.



Several investigators emphasize on safety and toxicological evaluation of plant extracts because of the side effects. Screening of herbal bio active components or phytochemicals from various plant sources are potential sources of bio-dynamic, therapeutically; valued phytochemicals (Hays, 1989; Thomas, 1994; Kruger and Mann, 2003; Shoji et. al., 2004; Wolf and Weisbrode, 2004).

## OBJECTIVE

*Decalepis hamiltonii* commonly known as the swallow root, (family - asclepiadaceae ) grows in the forests of Peninsular India. *Decalepis hamiltonii* is a stout climber with jointed stem, opposite, simple ovate leaves, flowers small white, roots enter deep in the soil often transversing the crevices of large rocking boulds (Nayar et. al., 1978). *Decalepis hamiltonii* colloquially known as *Makali беру* (Kannada), *Makalikizhangu* (Tamil). The roots of this plant have sweet sarsaparilla like taste and strong aromatic odour. Roots are fleshy with woody core. The tubers of *Decalepis hamiltonii* are consumed as pickles and beverage (health drink). The roots are also used in ayurvedic preparations as a substitute for *Hemidesmus indicus* (Nayar. et. al., 1978).

Recently, a process for the debittered beverage and pickles has been developed from Central Food Technological Research Institute (C.F.T.R.I) (Chauhan et. al., 2000). Research work at C.F.T.R.I has shown that roots of *D. hamiltonii* protect the stored grain from insect infestation and a biopesticide has been formulated (Jacob et. al., 1998). The roots exude white latex and a strong odour. Earlier work on chemical composition of the swallow root has shown that it contains aldehydes, inositols, saponins a crystalline resin acid, saponins, ketonic substances, with sterols, resinols (Murti and Sheshadri, 1941; a, b, c). The scientific basis of its health promoting properties needs to be investigated. Further, the mammalian toxicity and safety needs to be addressed since the roots are consumed by man.

Our major objective was to study the nutraceutical value of the tubers of *D.hamiltonii* by investigating the antioxidant property, a feature associated health promoting potential and the mammalian safety.

## INTRODUCTION

A free radical is defined as any chemical substance or molecules that contain one or more unpaired electrons in the outer most orbital. Oxygen produced in the cell is referred to reactive oxygen species (ROS), (Nakazawa et. al., 1996). There is a cellular defense against ROS to avoid oxidative stress (Gutteridge and Halliwell 1994). However imbalance in the cell defense may affect cellular function and oxidative degradation of macromolecules like DNA proteins and lipids. It is believed that ROS is involved in a number of health disorders or diseases (Cochrane et. al., 1992; Ames et. al., 1993).

Antioxidants counter the effects of free radicals and significantly delay or inhibit oxidative damage (Gutteridge and Halliwell 1994). Endogenous antioxidants include low molecular weight scavengers of oxidising species and enzymes which inactivate superoxide and hydroperoxides and prevent the formation of free radical species and their reactions (Chaudiere and Ferrari 1999).

Hydrophilic scavengers in the cytosol, mitochondria and nuclear compartments such as ascorbate and glutathione scavenge oxidising free radicals by means of hydrogen of one electron.

Hydrophobic scavengers found in the cellular membranes scavenge lipid peroxidation chain reactions. Alpha-tocopherols (vitamin E) are quenchers of singlet oxygen. The antioxidant enzymes include SOD, catalase, GPx, GRT, GST which work at the expense of the cofactors such as NADPH. Some of the physiological antioxidants like urate, bilirubin carnosine, and ubiquinol are important in preventing many of the diseases.

The exogenous antioxidants are the synthetic antioxidants in food industry such as propylgallate, santoloin, butylated hydroxy anisole, butylated hydroxy toluene and pharmaceutical drugs like, lovastatin, probucol etc. Natural antioxidants derived from various plant sources are exploited for their therapeutic use and replace the use of synthetic antioxidants (Namiki, 1990).

**Dietary antioxidants:**

Ascorbate, tocopherol, carotenoids, important vitamins and other important chemical ingredients of fruits and vegetables are major contributors for health benefits. Low incidence of various diseases like cancer, cardiovascular disease, stroke etc. in humans due to intake of dietary antioxidants present in fruits and vegetables has been reported and several reports strongly support the protection against diseases by vitamin C containing foods (Block et. al., 1991). Dietary intake of natural antioxidants reduces the risk of cardiovascular diseases (Rimm et. al., 1993). Another example is the association of lens protein oxidation and cataract formation and the role of lens antioxidants in particular vitamin C, vitamin E, carotenoids and retinoids, in the protection of lens from oxidative stress (Krinsky 1989; Romieu et. al., 1990; Taylor et. al., 1991; 92). Fruits like apples, lemons, oranges, cauliflower, grape, mangoes, bilberries, citrus, and other plant foods such as bean, tea, cruciferous vegetables etc contain polyphenols like flavonoids, carotenoids, catechins, quercetins and anthocyanins which exhibit antioxidant activity (Negri et. al., 1991; Rice et. al., 1994; Formica et. al., 1995; Middleton et. al., 2000; Chakroborty et. al., 2000). Commonly consumed beverages like cocoa, coffee and tea possess

antioxidant potential (Nardini et. al., 1995, 1997; Cao et. al., 1996; Daglia et. al., 2000; Hammerstone et. al., 2000).

In view of the role of antioxidants in ameliorating oxidative stress, there is a surge for antioxidant molecules from natural sources.

### **Health promoting potential of plant derived antioxidants:**

Phytochemicals as antioxidants and protective therapeutic agents are found in numerous Indian medicinal plants and their preparations are used in preventive medicines. “Brahma rasayana” is a herbal tonic derived from Indian plants which contains potent antioxidants (Praveen et. al., 1999). Maulik and coworkers (1997; 1999) have evaluated herbal medicinal plants for antioxidant activity. Most of all the medicinal plants with inflammatory activity may also possess antioxidant activity (Dhawan 1980; Rastogi and Dhawan 1982; Jang et. al., 1997). *Hypericum androsaenum* a medicinal plant of family guttiferaceae contains polyphenols with antioxidants activity (Patricia 2002). Plants of labiatae family such as *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Salvia officinalis*, *Sidertis javalambrensis*, *Satureja hortensis*, *Majorama hortensis* (Jose et. al., 1992; Rice et. al., 1995; Rake et. al., 2002) also possess antioxidant activity *in vitro*. Plants of laminaceae family have also been reported to possess antioxidant properties (Damien et.al., 2004). Although antioxidant activity of several plants has been reported their *in vivo* efficacy is not known.

### ***Decalepis hamiltonii*:**

*Decalepis hamiltonii* (family: Asclepiadaceae) commonly known as the swallow root (colloquial names: *Kannada-Makaliberu*; *Tamil-Magali*

*kizhangu*; Telugu-Maredu ghaddalu) is a climber with jointed stem and elliptic obviate leaves. It grows wild in the forests of peninsular India. Its roots are elongated and tuberous with fleshy outer layer and woody inner core and possess strong vanilla-like aromatic odour. Traditionally the roots have been used as pickle and the juice is also consumed as it is believed to give health benefits with “cooling properties”. The roots are used in ayurvedic preparations as a substitute for *Hemidesmus indicus* (Nayar et. al., 1978). Early work on the chemical composition has revealed the presence of amyriins, lupeols, saponins, ketonic substances (Murti and Seshadri 1941, a,b,c) 2 hydroxy 4-methoxy benzaldehyde is the flavoring compound present in its volatile fraction (Nagarajan et. al., 2001).

Earlier work at C.F.T.R.I. has shown that the roots possess insecticidal properties (George et. al., 1998). A process for the debittered juice from the roots of *D. hamiltonii* has also been reported from C.F.T.R.I, Mysore (Chauhan, et. al., 2000) although the roots of *D. hamiltonii* are consumed in the southern India for their alleged health benefits there has been no scientific study to prove its health promoting potential. Therefore this work was undertaken to investigate whether the roots possess antioxidant properties which is generally associated with nutraceuticals present in herbal products. This chapter describes antioxidant activity of the extracts of the roots in *in vitro* assay and the *in vivo* effects on the antioxidant enzymes in the laboratory rat.

## MATERIALS AND METHODS

**CHEMICALS:** Butylated hydroxy anisole (BHA). Butylated hydroxyl toluene (BHT), 2- hydroxy, 4-methoxy benzaldehyde (HMBA), ethylene-diaminetetra acetic acid (EDTA) disodium salt, glutathione reductase (GRT) 5,5-dithiobis 2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), hydrogen peroxide ( $H_2O_2$ ), 1-chloro2,4-dinitrobenzene (CDNB), were purchased from Sigma chemical company, St. Louis, MO(USA), nitroblue tetrazolium (NBT), phenazine metho sulphate (PMS), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (oxidized) (GSSG) were purchased from Sisco research laboratories Mumbai, India, Solvents were purchased from E-Merck (Merck) limited, Mumbai, India. Rest of the chemicals was purchased from Qualigens fine chemicals, Mumbai, India.

**Preparation of extract:** Fresh roots of *D. hamiltonii* (5 kg) were cleaned the fleshy portion was separated from the woody central core, dried at room temperature and powdered in a food and fruit mill grinder. The root powder was lyophilized and the yield was 1.71 kg.

To prepare the aqueous extract, 100 g of the root powder was homogenised in warm distilled water (300 ml) and blended for 15 minutes and then placed on a shaker at 37°C for 12-14 hrs. The extract was then filtered through Whatman # 1 filter paper. The extract obtained was further lyophilized and the yield was 10.62 g.

To prepare the methanolic extract, 100 g of root powder was Soxhlet extracted with methanol and concentrated using a rotary flash evaporator



(40<sup>o</sup> C); yield of the extract was 53.10 g. The extract was suspended in oil (500 mg/ml) for administration to animals.

**Phenolic content:** Total phenolic content in the *D.hamiltonii* extracts was determined by using Folin-Ciocalteu reagent as described by Yildrin, Mavi et. al., (2001) with slight modification. 100 microliters of the extracts was added to 10ml of distilled water and 2 ml of Folin-Ciocalteu phenol reagent. The mixture was then allowed to stand for 5 mins at room temperature followed by the addition of 2.0 ml of sodium carbonate to the reaction mixture. The absorbance was recorded at 765 nm in spectrophotometer. Results were expressed in milligrams of guaicol (O-methoxy phenol) equivalent / g extract.

**HPLC analysis:** In view of the earlier report of the presence of 2-hydroxy 4-methoxy benzaldehyde (HMBA) in the roots of *D. hamiltonii* its presence in the methanolic extract was detected by reverse phase HPLC using C18 column in a Shimadzu system.

Methanolic extract (100µl) was injected and eluted isocratically with methanolic: water (60:40) and UV detector at 277nm. The peak was compared with the Standard HMBA

### **I. Antioxidant activity (*in vitro*)**

2-Hydroxyl, 4-methoxy benzaldehyde (HMBA), butylated hydroxy anisol (BHA), butylated hydroxy toluene (BHT) (commercially available) 20 µl were used as standards from a stock of 2mg/ml in *invitro* experiments.

**Microsomal lipid peroxidation:** Microsomes were isolated by the calcium aggregation method (Kamath, et. al., 1972) from liver, Brain and Erythrocytes. 100µl of microsomal (or erythrocyte membrane)suspension was added to 0.7ml of phosphate (0.1 mM,pH7.4) buffer to which was added 100µl each of ferrous sulphate(0.1mM)and ascorbate (0.1mM)with or without the root extract incubated for 1 hour at 37°C. This was followed by the addition of 0.5ml of 2.8% TCA and 0.5ml of 1% TBA. The mixture was heated in a boiling water bath for 10 mins, cooled centrifuged and the supernatant was read at 535 nm in spectrophotometer. Lipid peroxidation was calculated as malonaldehyde formed by the extinction coefficient  $1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$  (Buege and Aust 1978)

**Superoxide scavenging activity:** Superoxide anion was generated by the reaction of NADH and phenazine methoxy sulphate (PMS) coupled to the reduction of nitroblue tetrazolium chloride (NBT) (Nishikimi and Yagi 1972) with slight modification. The reaction mixture contained NBT (0.1mM), NADH (0.1mM) with or without the extracts in a total volume of 1 ml tris buffer (0.02 M (pH 8.3). The reaction was started by adding PMS (0.01mM) to the mixture and the absorbance change was recorded at 560 nm every 30 sec for 1 min. Percent inhibition was calculated against control without the extract.

## **II Antioxidant activity *in vivo***

The *in vivo* antioxidant activity of *D. hamiltonii* root extracts was investigated by administering single, multiple doses of extracts to albino rats.

**Animals:** Albino rats (CFT-Wistar strain) weight range 150-180 grams were housed in polypropylene cages and provided with feed and water ad libitum. The animals were maintained in the animal house of C.F.T.R.I and acclimatized (Brooke bond, Lipton India Ltd, Calcutta, India).

**Single dose study:** Animals were divided into three groups of four animals each. Group I served as the control, group II and III were treated with *D.hamiltonii* extract. The animals were administered with single dose each of 200 mg/Kg b.w. of *D.hamiltonii* extracts to group II and III. The control group was treated with carrier, groundnut oil.

**Multiple dose study:** Animals were divided into three groups of four animals each. Group I served as the control and the other two were treated with *D.hamiltonii* extract groups (II and III). Daily dose of 100 mg/Kg b.w. of *D.hamiltonii* extracts were orally administered to group II and III for 6 days. Autopsy was done 16 hrs after the last administration by ether anaesthesia.

### **Biochemical Assays**

Liver, brain and testes were dissected out, washed with cold saline and a 10% homogenate of the tissues was prepared in 0.1 M tris-HCl buffer (pH 7.4) and used for enzyme assays.

**GSH assay:** Reduced glutathione in brain, liver and testes was determined based on Ellmans (1959) method with slight modification as described by Benke et. al., (1974). 10 % homogenate was prepared with 5% TCA containing 0.1 mM EDTA, centrifuged at 2000 X g for 5 mins. To 0.1 ml of the supernatant aliquot 2.8 ml of 0.2 M Tris-HCl buffer (pH 8) and 50 µl of

10mM DTNB were added. The yellow colour developed was read at 412 nm and GSH concentration was calculated from a standard curve.

### **Enzymes:**

**Superoxide dismutase (SOD EC. 1.15.1.1):** SOD was assayed by the method of Marklund and Marklund (1974). The degree of inhibition of pyrogallol autooxidation at an alkaline pH, by the supernatant was measured as the enzyme activity.

**Assay:** Pyrogallol autooxidation reaction mixture consisted of 2 ml of Tris HCl (pH 8.2) 0.5 ml of 2 mM pyrogallol and 1.5 ml distilled water. Initial rate of pyrogallol auto-oxidation per minute was recorded for 3 minutes at 420 nm in spectrophotometer which was considered as 100% auto oxidation.

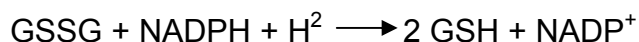
The assay mixture for the enzyme assay contained 2 ml of tris-HCl (pH 8.2) 0.5 ml of 2ml pyrogallol and of the enzyme sample (0-0.5 ml) and volume made upto 4 ml with distilled water. The reaction rate of auto-oxidation after the addition of the enzyme was noted. Amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation is considered as 1 unit of enzyme activity. The enzyme activity was then calculated using the formula:

$$\text{Units of inhibition} = \frac{\text{Assay volume}}{\text{Vol of tissue extract}} \times \text{dilution factor} \times \frac{1}{\text{mg of protein}}$$

### **Catalase: (CAT EC.I. 11.1.6)**

**Assay:** The assay mixture consisted of 25µl of 3 % H<sub>2</sub>O<sub>2</sub> as the substrate in 3 ml of 50 mM phosphate buffer of pH 6.8. The aliquots of enzyme sample (0-25 µl) were added to initiate the reaction and absorbance change per minute was recorded at 240 nm for 3 minutes. Activity was calculated by extinction co-efficient  $\Sigma=0.40 \text{ cm}^2/\text{mole}$  (Bergmeyer and Bernt 1974).

**Glutathione peroxidase: (GP<sub>x</sub> EC.1.11.1.9)** Glutathione peroxidase catalyzes the reduction of hydrogen peroxides with GSH as the reductant



The reduction of GSSG is coupled to the oxidation of NADPH through glutathione reductase (Tappel 1978; as modified by Paglia and valentine 1967).

**Assay:** 100 µl of the enzyme sample was incubated for 5 minutes at 37° C in a reaction mixture containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit of glutathione reductase in tris buffer (0.1 mM pH 7.6) and final volume was made upto 1.65ml; to this 50 µl of cumene hydroperoxide 1 mg/ml were added to initiate the reaction and absorbance at 340 nm was recorded. The amount of enzyme that transforms 1 µ mol of NADPH to NADP per minute at 37° C is expressed as units /mg protein.

**Glutathione reductase: (GR EC. 1.6.4.2)**

Glutathione reductase is a flavoprotein catalyzing the NADPH-dependent reduction of glutathione disulphide (GSSG) to glutathione (GSH) (Inger Carlberg and Manervik 1985)

**Assay:** To 0.5 ml of phosphate buffer at 30° C 50 ml of 2 mM NADPH, 50 ml of GSSG (20 mM in Distilled water) and a final volume was made upto 1 ml with distilled water. The reaction was initiated by the addition of the enzyme sample (0-100 µl) 1 unit of glutathione reductase activity is the amount of enzyme that catalyses the reduction of 1 µ mol of NADPH per minute extinction Co-efficient of NADPH  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

**Glutathione-S-transferase: (GST EC. 2.5.1.18)**

GST activity was assayed as according to Booth et. al., (1961) as modified by Moron, et. al., (1979).

**Assay:** The enzyme sample 50  $\mu$ l was added to 3 ml of buffer (0.1 M, pH 7.4) 0.5 mM GSH and preincubated in a water bath at 30<sup>o</sup> C. The reaction was initiated by adding 50  $\mu$ l, 0.5 mM of CDNB and the absorbance was recorded at 344 nm in a spectrophotometer. The enzyme activity was expressed as n moles of CDNB conjugated per minute per mg protein and calculated by the extinction co-efficient for the S-2, 4-dinitrophenyl Glutathione  $\sum_{340}=9.6 \text{ mM}^{-1} \text{ cm}^{-1}$

**Statistical analysis:**

The data were statistically analysed by ANOVA and Duncan's multiple range test to evaluate differences among the groups at 0.05 significance level using the statistical package (CRD- analysis for equal replicates).

## RESULTS

*In vitro* antioxidant activity: *in vitro* microsomal lipid peroxidation assay results, when compared to that of control standard like HMBA and commercially used antioxidants in food industry BHA and BHT, extract (table 2.1). 80% inhibition, where as *D.hamiltonii* extracts showed 90 % inhibition of liver, brain, erythrocyte membranes (fig 2.1) showed 70-Separation and identification of the compound HMBA in *D.hamiltonii* roots as one of the constituents was shown by HPLC. The retention time of the authentic standard was similar to the peak in the extract; the phenolic content was higher in the methanolic extract of than that aqueous *D.hamiltonii*.

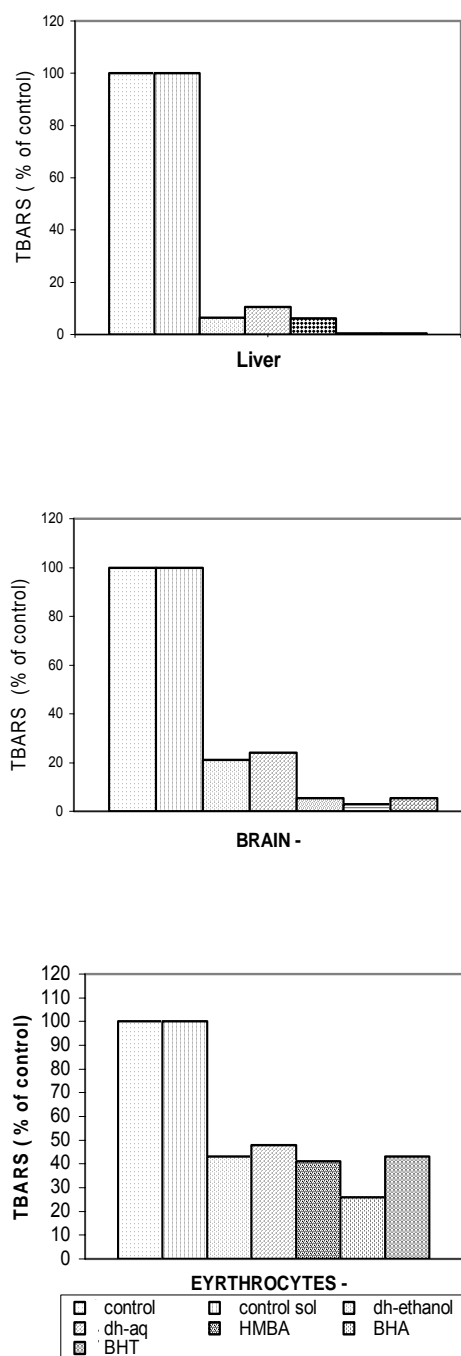
Erythrocyte membranes lipid peroxidation inhibition by HMBA, BHA, BHT and *D.hamiltonii* extracts showed 50 % inhibition. Further IC 50 of the *D.hamiltonii* extracts HMBA and BHA was determined by microsomal lipid peroxidation of the tissues liver, brain and Erythrocyte membranes (table 2.2). Lipid peroxidation was also noticed *in vivo* fig 2.9, 2.16 by *D.hamiltonii* extract. *In vitro* scavenging of superoxide by *D.hamiltonii* extracts, HMBA and BHA compared to control showed quenching of oxygen free radical by a non-enzymatic system (fig 2.2).

### ***In vivo* antioxidant activity of *D.hamiltonii* extracts:**

Animals treated with *D.hamiltonii* extracts did not show any significant changes. Administration of *D.hamiltonii* extracts resulted in increase of GSH levels in brain, liver and testes (fig 2.9, 2.15). *D.hamiltonii* extract treated rat showed 20% of SOD level (fig 2.4, 2.10) increase compared to control. *D.hamiltonii* extracts treated showed 20-30% of GPX level (fig 2.5, 2.11)

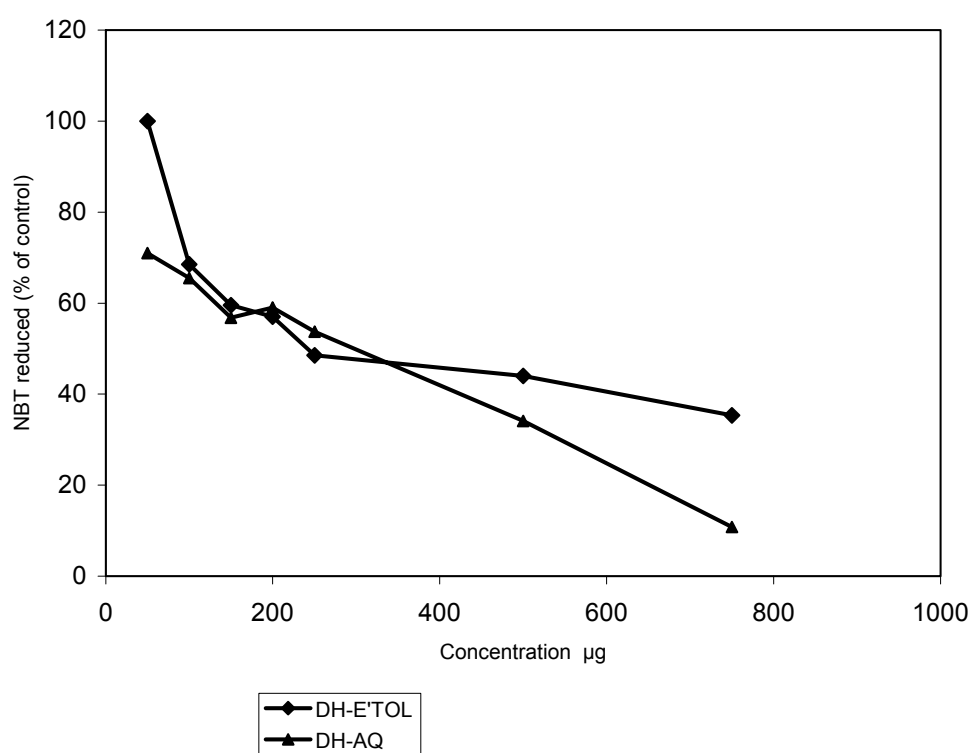
increase than control in liver, brain, and testes. Glutathione reductase (fig 2.6, 2.12) of the liver, however, remained unaltered compared to control. An increase of 20 % glutathione reductase level in brain and testes was observed. GST (fig 2.7, 2.13) and CAT (fig 2.8, 2.14) showed no alteration in enzyme levels due to administration of *D.hamiltonii* extracts. *D.hamiltonii* methanolic extract was more significantly effective than *D.hamiltonii* aqueous.





**Fig 2.1: Inhibition of membrane lipid peroxidation of brain, liver and erythrocytes ( *in vitro*) by *Dh* extracts.**

Dh (aq) = *Decalepis hamiltonii* aqueous extract (10mg/ml)  
Dh ethanol = *Decalepis hamiltonii* ethanol extract (10mg/ml)  
HMBA = 2 Hydroxy-4-methoxy-benzaldehyde (2 mg/ml)  
BHA = Butylated Hydroxy Anisole (2 mg/ml)  
BHT = Butylated Hydroxy toluene (2 mg/ml)



**Fig.2.2: Quenching of oxygen free radical by *Decalepis hamiltonii***

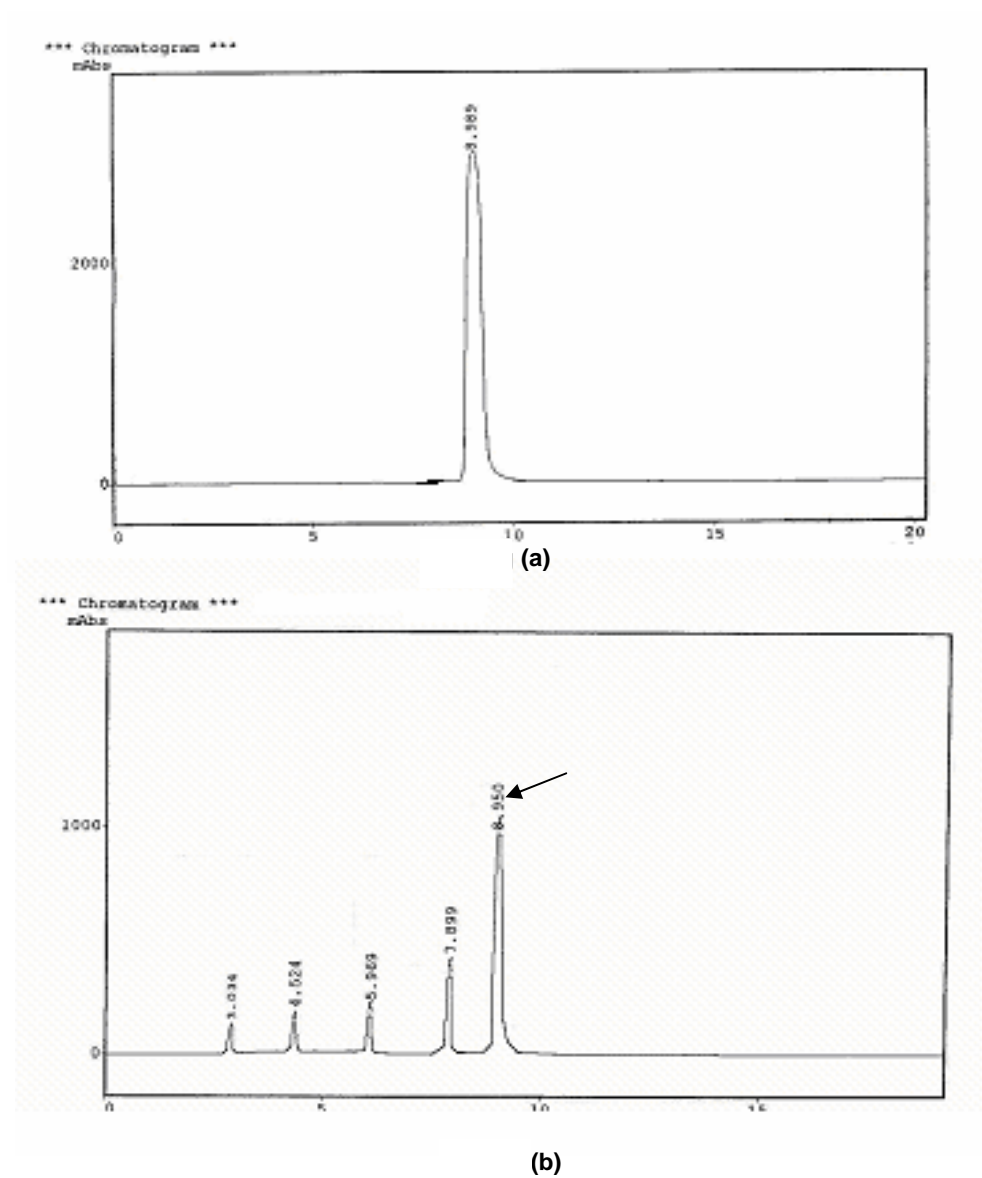
Table 2.1: Phenolic content of *D. hamiltonii* root extracts

Extracts	Phenolic content (mg/g)
Aqueous extract	12.67
Methanolic extract	34.67

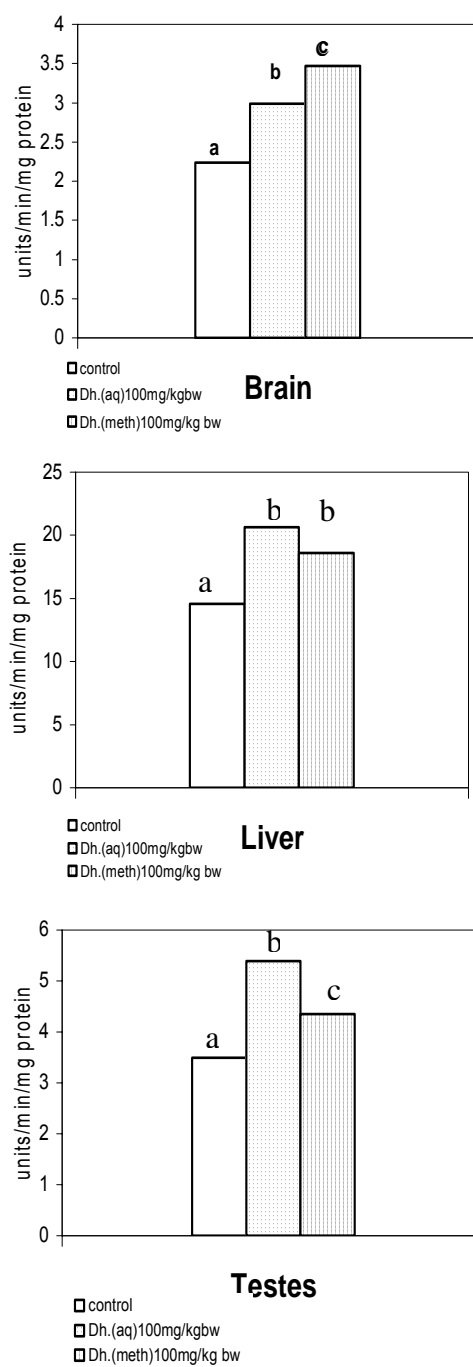
\* Phenolic content estimation as guaicol equivalents

Table 2.2: Antioxidant activity of the extracts of *D. hamiltonii*

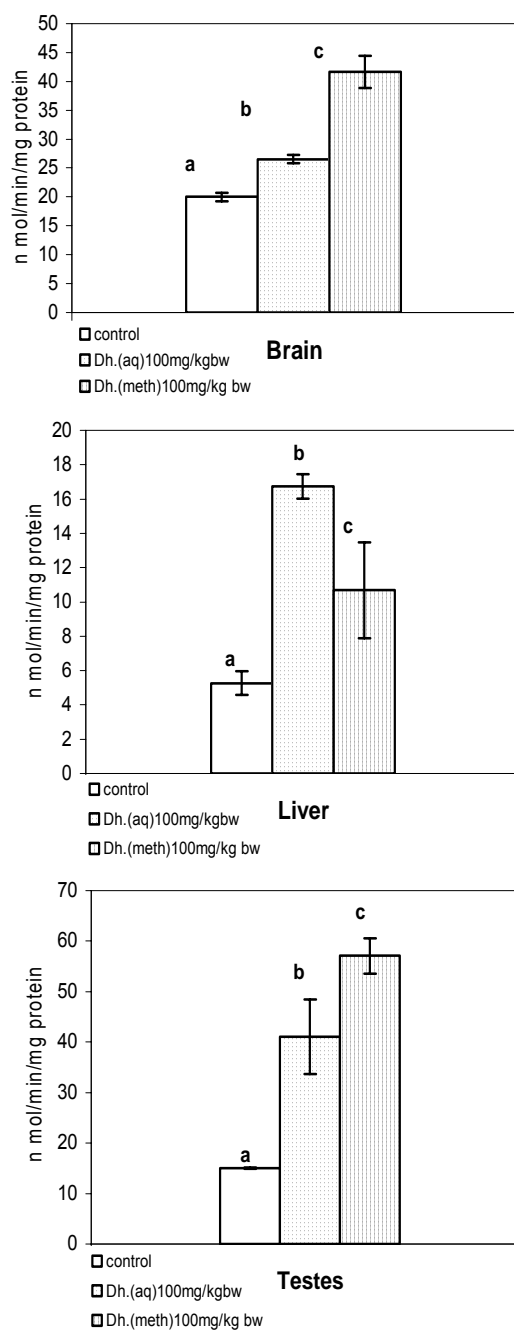
Sample	LPO IC <sub>50</sub> (µg /ml)	ROS IC <sub>50</sub> (µg /ml)
Methanolic extract	108.89	302
Aqueous extract	217.27	743
HMBA	0.115	-
BHA	0.036	-



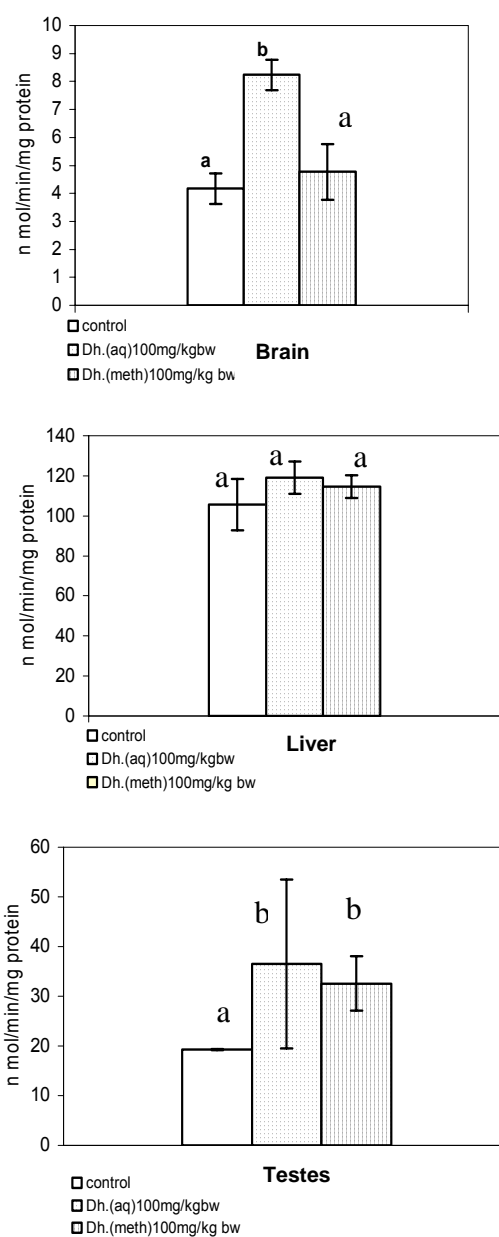
**Fig 2.3:** HPLC analysis of 2-hydroxy 4-methoxy benzaldehyde in the methanolic extract of *D.hamiltonii* roots (a) standard HMBA profile (b) profile of the root extract, arrow marks indicates HMBA with the same retention time.



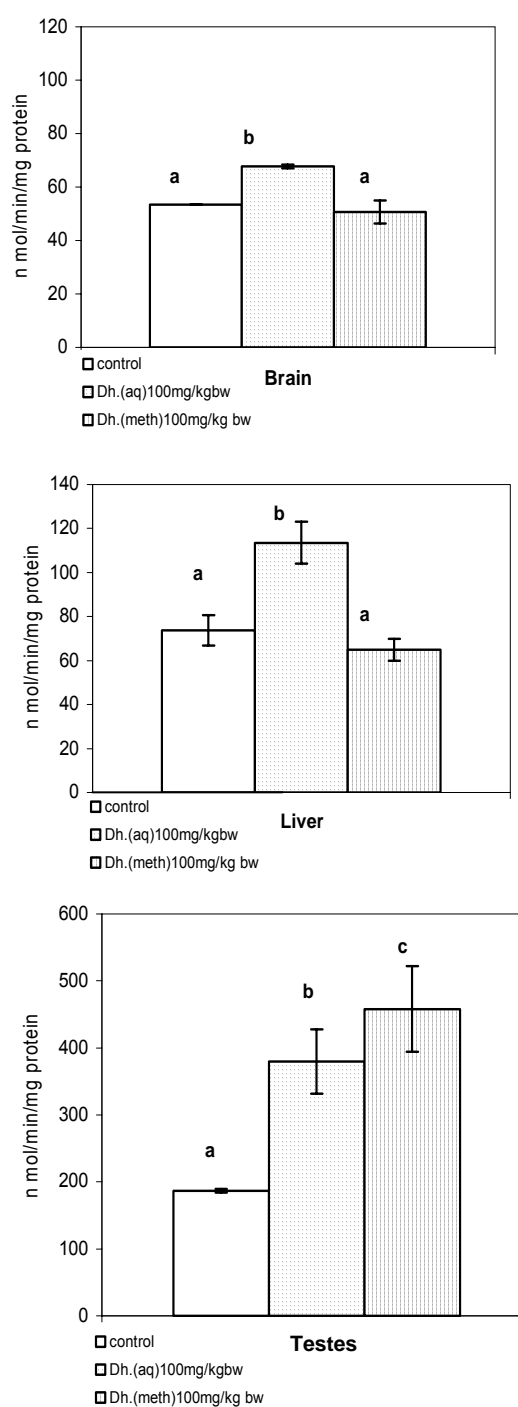
**Fig 2.4: Effect of *Dh.* root extract (single dose) on antioxidant enzyme superoxide dismutase,** each bar represents the mean  $\pm$  SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  level. (DMRT)



**Fig 2.5: Effect of Dh. root extract (single dose) on antioxidant enzymes: Glutathione peroxidase**, each bar represents mean  $\pm$ SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)

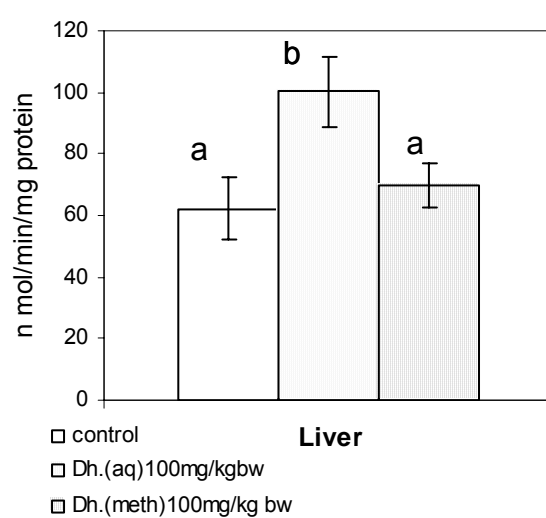


**Fig 2.6: Effect of *Dh.* root extract 00(single dose) on antioxidant enzymes: Glutathione reductase**, each bar represents mean  $\pm$  SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)

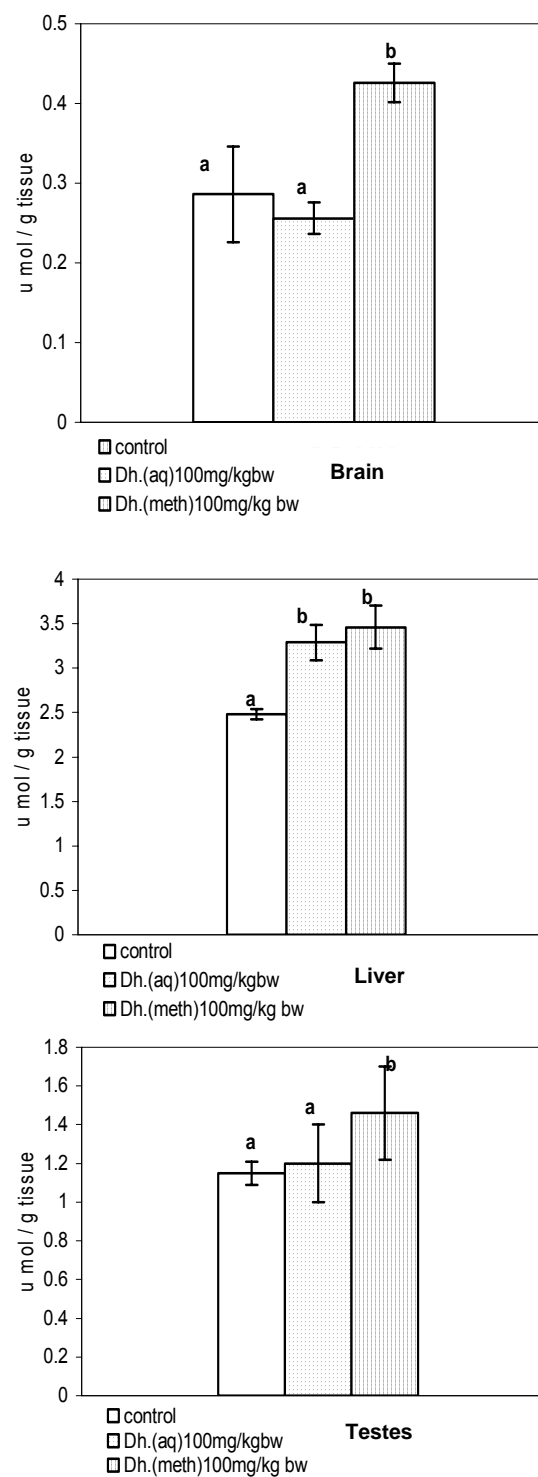


**Fig 2.7: Effect of *Dh.* root extract (Single dose) antioxidant enzymes: Glutathione-S-transferase**, each bar represents mean  $\pm$  SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)

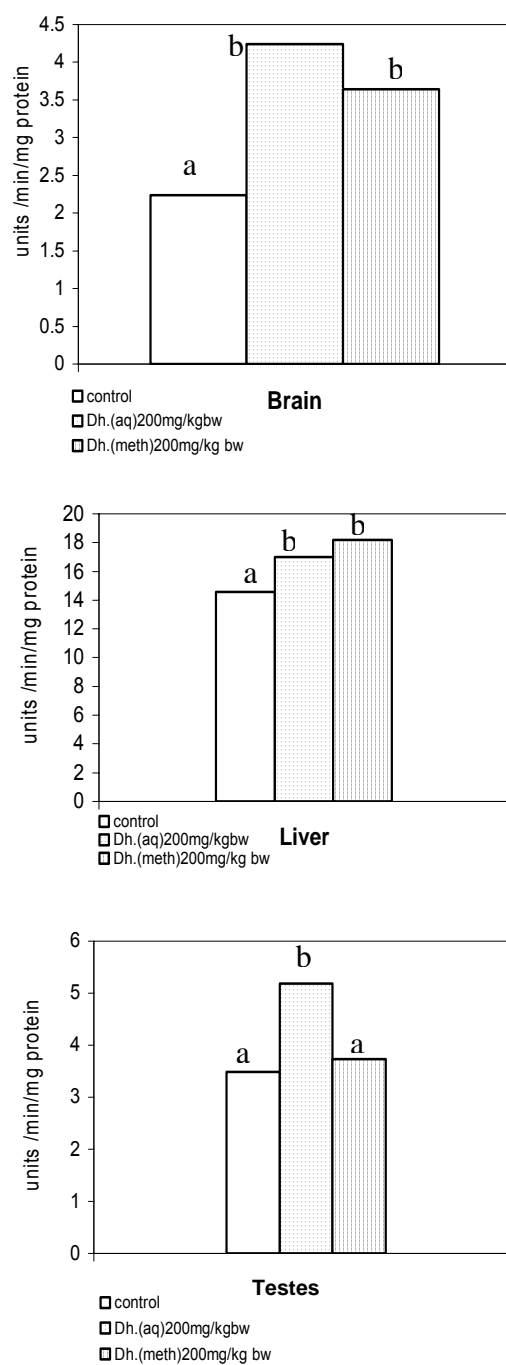




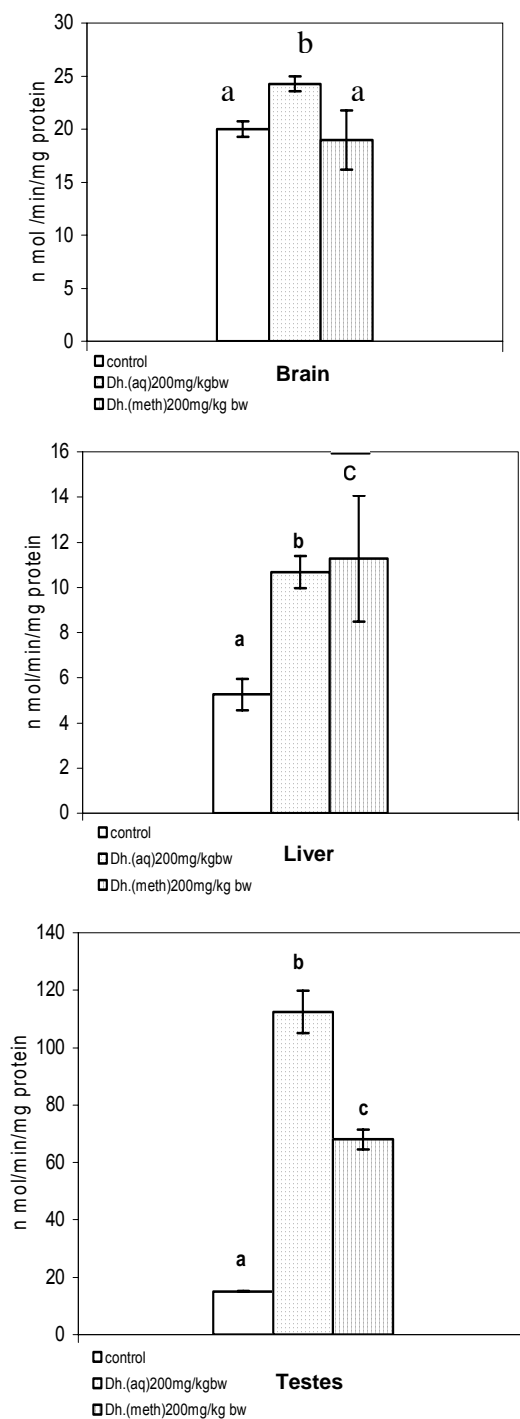
**Fig 2.8 : Effect of *Dh.* root extract (single dose) on antioxidant enzymes: Catalase**, Each bar represents mean  $\pm$ SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)



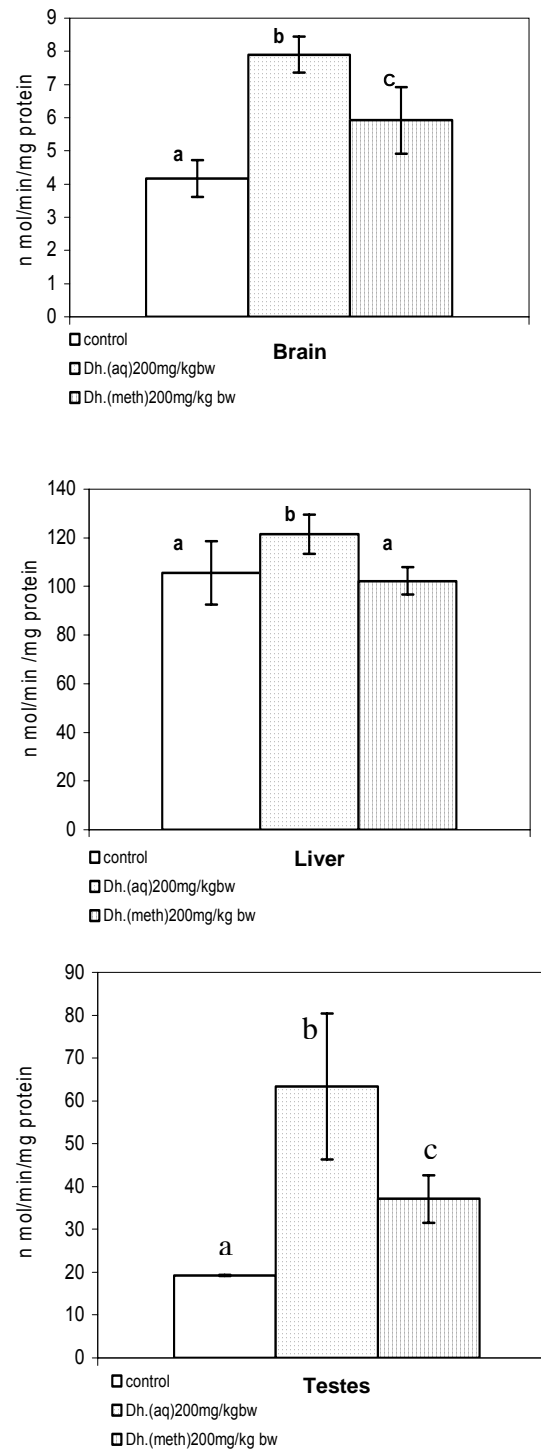
**Fig 2.9: Effect of Dh. extract (single dose) on GSH levels** each bar represents mean ± SE, n=4; bars with different alphabets indicate statistically significant difference at p<0.05 (DMRT)



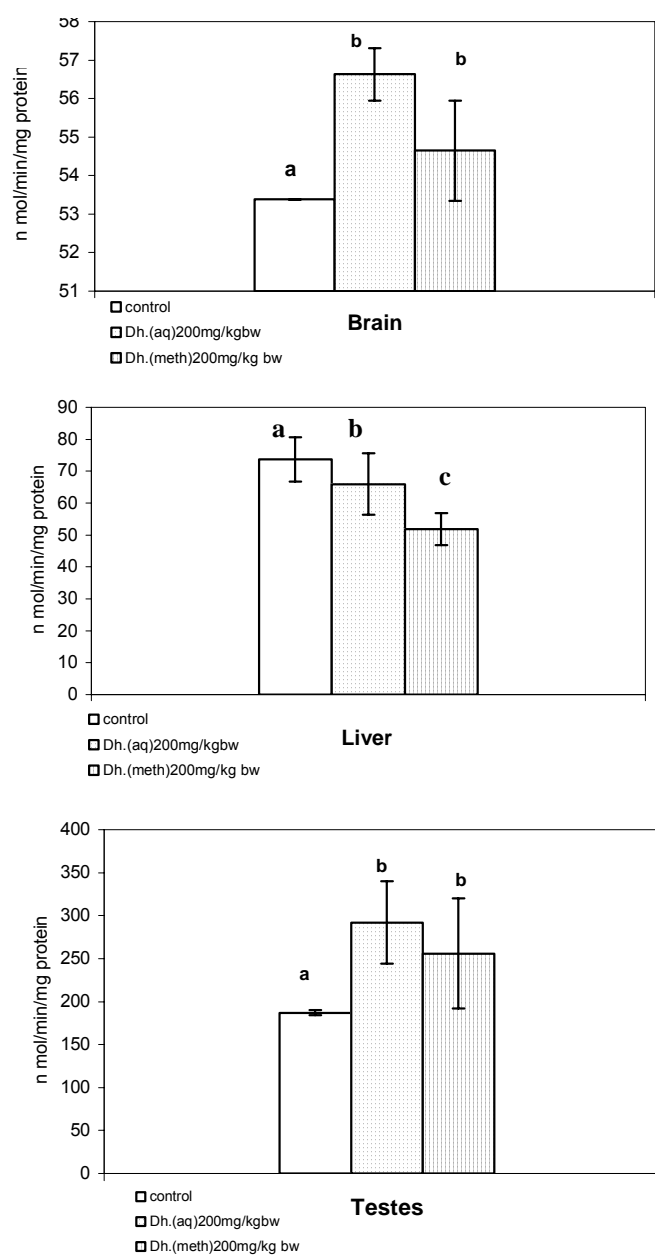
**Fig 2.10: Effect of Dh. root extract (multiple dose) on antioxidant enzymes: Superoxide dismutase**, each bar represents mean  $\pm$ SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)



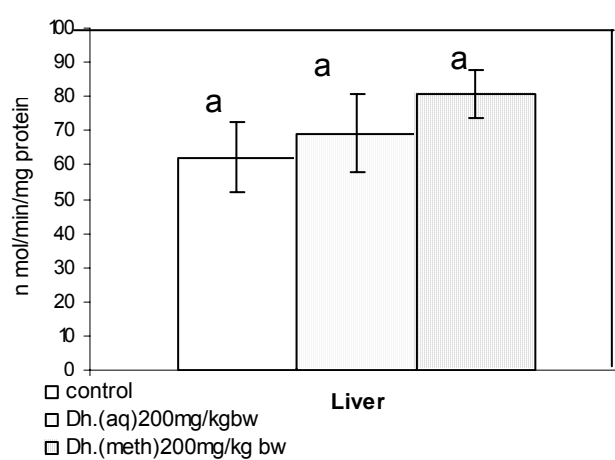
**Fig 2.11: Effect of Dh. root extract (multiple dose) on antioxidant enzymes: Glutathione peroxidase**, Each bar represents mean SE, n=4. bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)



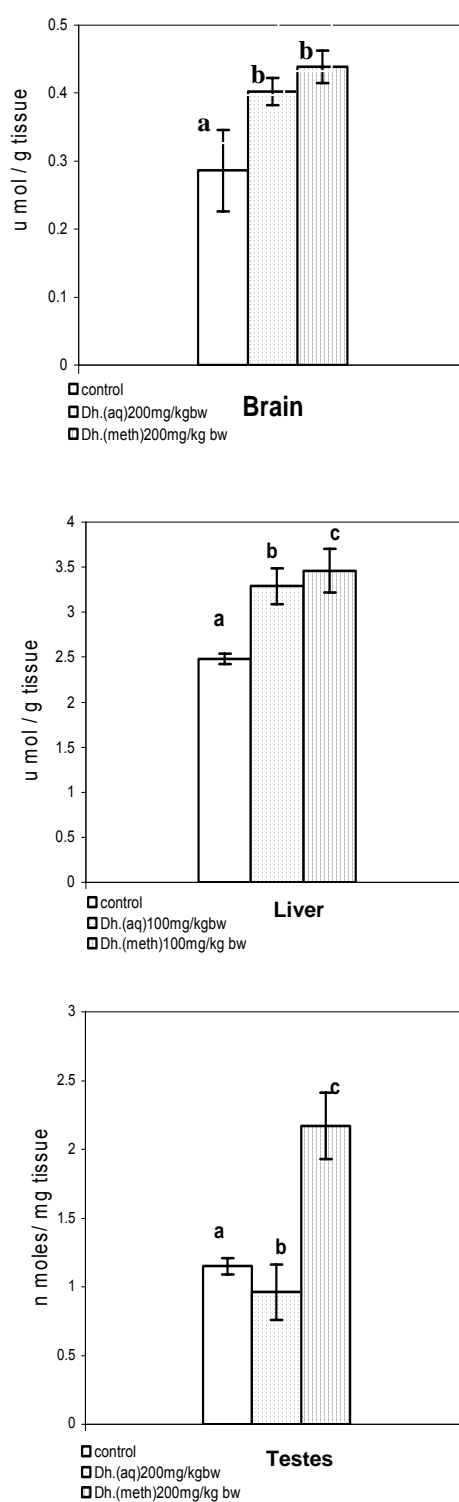
**Fig 2.12: Effect of Dh. root extract (multiple dose) on antioxidant enzymes Glutathione reductase**, each bar represents mean  $\pm$  SE, n=4; bars with different alphabets indicate statistically significant difference at  $p<0.05$  (DMRT)



**Fig 2.13: Effect of Dh. root extract (multiple dose) on antioxidant enzymes Glutathione -S-transferase**, Each bar represents mean  $\pm$ SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)



**Fig 2.14: Effect of Dh. root extract (multiple dose) on antioxidant enzymes: Catalase,** Each bar represents mean  $\pm$  SE, n=4; bars with different alphabets indicate statistically significant difference at  $p<0.05$  (DMRT)



**Fig 2.15: Effect of (multiple dose) Dh. extracts on GSH levels in tissues.** Each bar represents mean  $\pm$  SE, n=4; bars with different alphabets indicate statistically significant difference at  $p < 0.05$  (DMRT)



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## DISCUSSION

Dietary antioxidants are considered to be nutraceuticals in view of their health benefits. Numerous studies have shown that antioxidants present in fruits and vegetables prevent oxidative cellular damage and therefore implicated in the prevention or slowing down of degenerative diseases including aging (Prior, 2003). Besides fruits and vegetables, several medicinal plants contain antioxidants that contribute to their therapeutic properties (Catherine et. al., 1997; Ridgway et. al., 1997; Bors et. al., 1998; Miller et.al., 2001). A well known example is the *Ginseng*, *Ginko biloba* (Haramaki et. al., 1996). The ancient Indian system of medicine, *ayurveda* (ayur=life, veda=knowledge) mainly includes several hundred plants of the Indian subcontinent many of which are known for their “healing” properties.

Several polyherbal preparations from ayurveda are used for their rejuvenating potential and well-known among them is “Chayvanaprash”(Jose and Ramdas, 2000). Recent research has shown that plants of traditional Indian medicine show antioxidant activity (Scatezzini and Speroni, 2000) “Rasayana” is a rejuvenating recipe of several plants in ayurvedic preparations (Singh, 1992). Sharma et. al., (1992) have shown antioxidant activity of “rasayana”. Some of the plants that are used in these preparations showing antioxidant activity are *Embllica offinalis*, *Curcuma longa*, *Mangifera indica*, *Momordica charantia*, *Santalum album*, *Swertia chirata*, *Withania somnifera* (Scartezini and Speroni, 2000).

The antioxidant properties of the medicinal plants are due to the phytochemicals present in them which generally belong to phenolic and flavanoid groups. Numerous antioxidant molecules have been isolated from several plants including fruits and vegetables (Bors, 1996; Prior 2003).

*Decalepis hamiltonii*, (family: Asclepiadaceae), grows as a wild climber in the forests of western and eastern ghats of southern India (Nayar et. al., 1978). Although it is not mentioned in the ayurvedic preparations, the roots of *D. hamiltonii* are often used as a substitute to the medicinal plant *Hemidesmus indicus* in view of their similar aromatic properties (Nayar et. al., 1978). The tuberous roots are consumed by people in this region as pickles and the beverage is believed to have a “cooling effect”. However, no scientific evaluation of the alleged health promoting potential of *D.hamiltonii* has been done.

The present work has shown for the first time that the root extracts – both organic and aqueous-show potent antioxidant activity. The root extracts possessed a wide range of free radical scavenging activity such as inhibition of ROS and membrane lipid peroxidation in *in vitro* assays. The extracts also prevent LPO in tissues, such as liver, brain and erythrocyte showing their relevance to the possible role in preventing oxidative damage to the tissues.

The antioxidant compounds in the roots of *D. hamiltonii* have yet to be isolated. However, earlier work has shown that the roots of *D.hamiltonii* contain amyriins sterols, lupeols, resins etc. (Murti and Sheshadri 1941 a,b,c). Recently, composition of the essential oil from the roots of *D.hamiltonii* has been reported to contain 2-hydroxy,4-methoxy

benzaldehyde (HMBA) as the major flavour component (Nagarajan et. al., 2001). In the present work, we have shown that the methanolic extract of *D.hamiltonii* roots contain HMBA which is precipitated on storage at 4°C as revealed from the HPLC analysis of the precipitate and confirmed with the authentic compound, HMBA (Sigma). Retention points of both the peaks were similar and spiking the extract with the authentic HMBA was not distinguishable from the peak in unspiked samples in HPLC (Results). Further, HMBA showed potent antioxidant activity *in vitro* as it inhibited microsomal lipid peroxidation. These results suggest that HMBA present in the alcoholic extract of the roots of *D.hamiltonii* is one of the antioxidant molecules which is reported for the first time. These *in vitro* results clearly establish the antioxidant properties of the roots of *D.hamiltonii*.

Our study further demonstrates that the roots extracts of *D.hamiltonii* also show *in vivo* effects when orally administered to the laboratory rat. Either single or multiple dose of the root extracts administered orally, led to the enhancement of several biochemical antioxidants such as glutathione and the enzymes involved in the antioxidant defenses of the body. Glutathione (GSH), a tripeptide, is present in the tissues and acts as the first line of defense against the free radical oxidative insult (Chattopadhyay et. al., 1992; Manoj et. al., 2003). If GSH depleted in the tissues, by drugs or environmental agents, it leads to oxidative stress and damage to the cells (Wefers and Sies, 1983; Grattagliano 2002). Similarly the antioxidant enzymes such as SOD, GPx, GST, and GR, contribute the antioxidant detoxification potential in the tissues (Halliwell 1978; Sandhir et. al., 1999; Grattagliano 2002). Our results Show that the root extracts of *D.hamiltonii*

stimulate the GSH content as well as increase the activity of the antioxidant enzymes in the liver of the rats treated with the extracts. This study further confirms that the roots of *D.hamiltonii* not only contain free radical scavenging ability *in vitro* but also the potential to enhance the biochemical antioxidant defenses *in vivo*. The significance of this work lies in the fact that the roots of *D.hamiltonii* could be a novel source of nutraceuticals since it appears to be a cocktail of antioxidants. Since the edible roots of *D.hamiltonii* comprise ingredients that could contribute towards development of newer functional foods with disease prevention or health promoting potential.

## INTRODUCTION

Liver is the major organ of metabolic, secretory and excretory functions in the body. Hepatic dysfunction leads to liver diseases, a serious health problem. The principal causative factors for liver diseases include alcohol consumption, malnutrition, anemia and infections due to viral inflammations etc (Nadeem. et. al., 1997). Chronic alcoholic abuse results in hepatic steatosis, fibrosis, alcoholic hepatitis and cirrhosis (Visen et. al., 1996; Jesi et. al., 2001).

The role of free radicals in pathogenesis of liver injury is well studied (Poli 1993). Free radicals are implicated in hepatic fibrosis with loss of normal histoarchitecture (Slater, 1966; Irshad & Chaudhuri 2002). Liver is the main target for the toxicity of several chemical compounds including medicaments (Cameron et. al., 1996). Carbon tetrachloride- induced hepatic injury is an important model system for free radical-induced peroxidative chain reaction. CCl<sub>4</sub> causes tissue necrosis and cell death involving swelling of organelles, breakdown of plasma membrane, and release of cytosolic enzymes and spillage of cell contents that result in fatty degeneration typical of liver cirrhosis (Hays 1989).

Several Indian medicinal plants are being investigated for hepatoprotective potential (Jose and Kuttan 2000). In Ayurveda, the ancient Indian system of medicine, several medicinal herbs have been shown to be effective for various liver disorders. Handa and co-workers have extensively reviewed hepatoprotective plants and herbal formulations available in the Indian market. Indian medicinal plants belonging to about 40 families are

considered to be hepato protective agents (Handa et. al., 1986) (For details see chapter I literature review).

*Silybrum marianum* seeds containing flavolignan known as silymarin, possess hepatoprotective activity (Ramesh et. al., 1989). Roots of *Picrorhiza kurroa* that grows in the Himalayan regions of India, has been shown to possess hepatoprotective property (Dwivedi et. al., 1990). Bark of *Bauhinia vahlii* (family-*Caesalpinaceae*) was effective against the inflammation of liver (Gupta et. al., 1996). Ethno-medico botanical survey reports suggest that root extracts of *Cyperus rotundus* (family-*Cyperaceae*), roots of *Mimosa pudica* (family-*Mimosaceae*), seed powder and root powder of *Raphanus sativus* (family-*Brassicaceae*), whole plant powder of *Phyllanthus niruri* (family-*Euphorbiaceae*) were used in folk medicine for liver disorders (Iman, Gupta, Hussain 1997).

The tuberous roots of *Decalepis hamiltonii* possess a sarsaparilla like taste and widely used as pickles and beverage (George et. al, 1998). Pharmacognostical studies of *Decalepis hamiltonii* was compared with *Hemidesmus indicus* by Nayar et. al., (1978) since *D. hamiltonii* is often used as a substitute for *H. indicus* in ayurvedic preparations. Early work of Murti and Sheshadri (1941 a,b,c) has shown that the *D. hamiltonii* roots contains aldehydes, inositols, saponins, crystalline resin acid, ketonic substances of sterols, amyrins and lupeols.

Recently Nagarajan and Rao (2001) have reported that the volatile extract of *D. hamiltonii* contains several flavour compounds of which 2-hydroxy, 4-methoxy benzaldehyde constitutes 94%.

We have shown that the root extracts of *D. hamiltonii* show strong antioxidant activity (Chapter III). Since antioxidant rich extracts are promising material for hepatoprotective potential, we have investigated whether *D. hamiltonii* root extracts prevent liver injury induced by the well known hepatotoxicants, CCl<sub>4</sub> and ethanol, in the laboratory rat.

## MATERIALS AND METHODS

### Chemicals:

Bovine serum albumin, ethylene diamine tetraacetic acid, glutathione reductase, 1-chloro-2,4 dinitro benzene (CDNB), 5, 5-dithiobis (2-nitro benzoic acid) (DTNB), reduced glutathione, nicotinamide adenine dinucleotide (NAD),  $\alpha$ -ketoglutaric acid, oxaloacetic acid, 2,4 dinitro phenylhydrazine and 1-naphthyl phosphate, 6-benzamido-4-methoxy-m-toulidine diazonium chloride (BMTD) were purchased from Sigma-Aldrich Co. St. Louis, Missouri USA, Tris, aspartic acid, polyvinyl pyrrolidone, DL-alanine were purchased from Sisco research laboratories, Mumbai, and E-(Merck), India.

### Animals:

Adult rats male albino (3 months old) of CFT-Wistar strain in the weight range of 200-250 g were individually housed in polypropylene cages and given feed (laboratory rat chow, Brooke bond, Lipton, India) and water provided ad libitum and the animals were acclimatized.

**Preparation of the root extracts:** Preparation of the root extracts was done as described in chapter-II. The methanolic extract was suspended in oil (500mg/ml) for administration to animals. The aqueous extract was diluted with distilled water for administration to experimental animals.

Several experiments were carried out to evaluate the hepatoprotective action of *D.hamiltonii* extracts (aqueous and methanolic) against CCl<sub>4</sub> and ethanol induced toxicity. The details of the experimental protocol are given in table 3.1.



**Table 3.1: Experimental protocol for hepatoprotective study of *D. hamiltonii***

Experiment	Group n=4			
	I	II	III	IV
Single dose pretreatment with 200 mg/kg b.w. of the root extract (aqueous and methanolic)	Control (oil only)	CCl <sub>4</sub> ( 1ml/kg b.w) single dose or ethanol (4.3 ml/kg b.w)	<i>D.hamiltonii</i> extract only (single dose)	<i>D.hamiltonii</i> extract (single dose) + CCl <sub>4</sub> (single dose) or ethanol (4.3 ml /kg b.w)
Multiple dose pretreatment with 200 mg/kg b.w of the extract (aqueous and methanolic)	Control (oil only)	CCl <sub>4</sub> ( 1ml/kg b.w) or ethanol 4.3 ml/ kg b.w (single dose)	<i>D.hamiltonii</i> extract only (6 daily doses of 200 mg/kg.b.w)	<i>D.hamiltonii</i> extract 6 days (200 mg/kg b.w) + single dose CCl <sub>4</sub> (1 ml kg/b.w) or single dose of ethanol (4.3 ml /kg b.w)

### Biochemical assays

The animals were sacrificed by ether anesthesia and blood was collected by cardiac puncture in dry test tubes, allowed to clot at room temperature, centrifuged at 4° C at 1000 g for 15 min and serum separated was used for biochemical assays. Liver was excised, weighed and a portion of it was fixed in Bouin's fluid for histopathological studies and the rest was stored frozen to be used for biochemical assays.

**Glutamate oxaloacetate transaminase (GOT; EC 2.6.1.2)** was assayed by the colorimetric assay method (Bergmeyer & Bernt 1974).

**Assay:** To 1 ml of buffer substrate solution (0.1 M phosphate buffer containing the substrates, L-aspartate & keto glutaric acid), the enzyme sample (0.1ml) was added to the reaction mixture and incubated for 30 minutes at 37° C. Then the chromogen solution (6-Benzamido-4-methoxy-m-toulidene diazonium chloride) of 1 ml was added and vortexed, incubated for

10 minutes at 37° C. The reaction was arrested by the addition of 5 ml of pyro-sulphite solution. The absorbance was read against the enzyme blank at 550 nm in a spectrophotometer. The enzyme activity was calculated using the oxaloacetic acid standard curve.

**Glutamate pyruvate transaminase (GPT; EC 2.6.1.1)** was assayed by the colorimetric assay method of Reitman and Frankel as given in Bergmayer and Bernt (1974).

**Assay:** To 1 ml of buffer substrate solution, (0.1 M phosphate buffer pH. 7.4 containing alanine and  $\alpha$ -ketoglutaric acid), enzyme sample of (0.1 ml) was added to this reaction mixture and incubated for 30 minutes at 37° C. The chromogen solution (1mM 2,4-dinitrophenylhydrazine) of 1 ml was then added to this reaction mix, vortexed and incubated at room temperature for 20 minutes. The reaction was stopped by the addition of NaOH (10 ml) and read against enzyme blank at 546 nm. The enzyme activity was calculated using sodium pyruvate standard curve.

**Lactate dehydrogenase (LDH; EC 1.1.2.3)** was assayed by the UV-method (Bergmeyer and Bernt 1974).

**Assay:** To 1 ml tris buffer (0.1 M, pH 8.5), the substrate sodium lactate solution, 0.5 ml (10 mM), the cofactor (NAD) 0.2 ml, and the enzyme sample (0.1 ml) were added and made up to a final volume of 2 ml, mixed well and the change of the absorbance was recorded against enzyme blank at 340 nm every 30 seconds for 2 min. The enzyme activity was calculated using the NADH extinction of co-efficient of  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

**Alkaline phosphate as (ALP; EC.3.1.3.1)** was assayed by the method of Walter and Schutt (1974).

**Assay:** To 1.8 ml of tris buffer (0.1 M pH 8.5) the substrate para nitro phenyl phosphate of 50  $\mu$ l (final concentration 1.25 mM) and the enzyme sample of 50  $\mu$ l was added and incubated for 30 minutes at 37°C. The reaction was stopped by adding 0.02M NaOH (5 ml) mixed and the absorbance read against enzyme blank at 405 nm. The enzyme activity was calculated using the extinction co efficient  $1.85 \times 10^{-3} \text{ M}^{-1}$  for P-nitrophenol.

**Lipid peroxidation assay:** 0.5 g tissue was homogenized in 2.5 ml of 20% TCA and 2.5 ml of thiobarbutric acid was added (TBA-0.67 % w/v) mixed well, boiled for 20 minutes, cooled and centrifuged. The colour developed was measured at 535 nm. The levels of MDA formed was calculated using the extinction co-efficient of  $\Sigma = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  (Beuege and Aust, 1978).

GSH level and the glutathione dependent enzymes, GST, GRT, GPX, were assayed and the details of the assay are described in detail (See chapter III).

**Histopathology:** A small piece of liver tissue from the central liver was dissected, fixed in Bouin's solution for 24 hours and then washed with 70 % alcohol thoroughly for paraffin embedding and sectioning and then processed 6  $\mu$ m thick sections stained with haemotoxylin-eosin and examined under microscope.

**Statistical analysis:** The data were statistically analysed by ANOVA and Duncan's multiple range test (DMRT) and the significance was estimated at  $P < 0.05$  level, using the statistical package computer software programme from computer center of CFTRI.

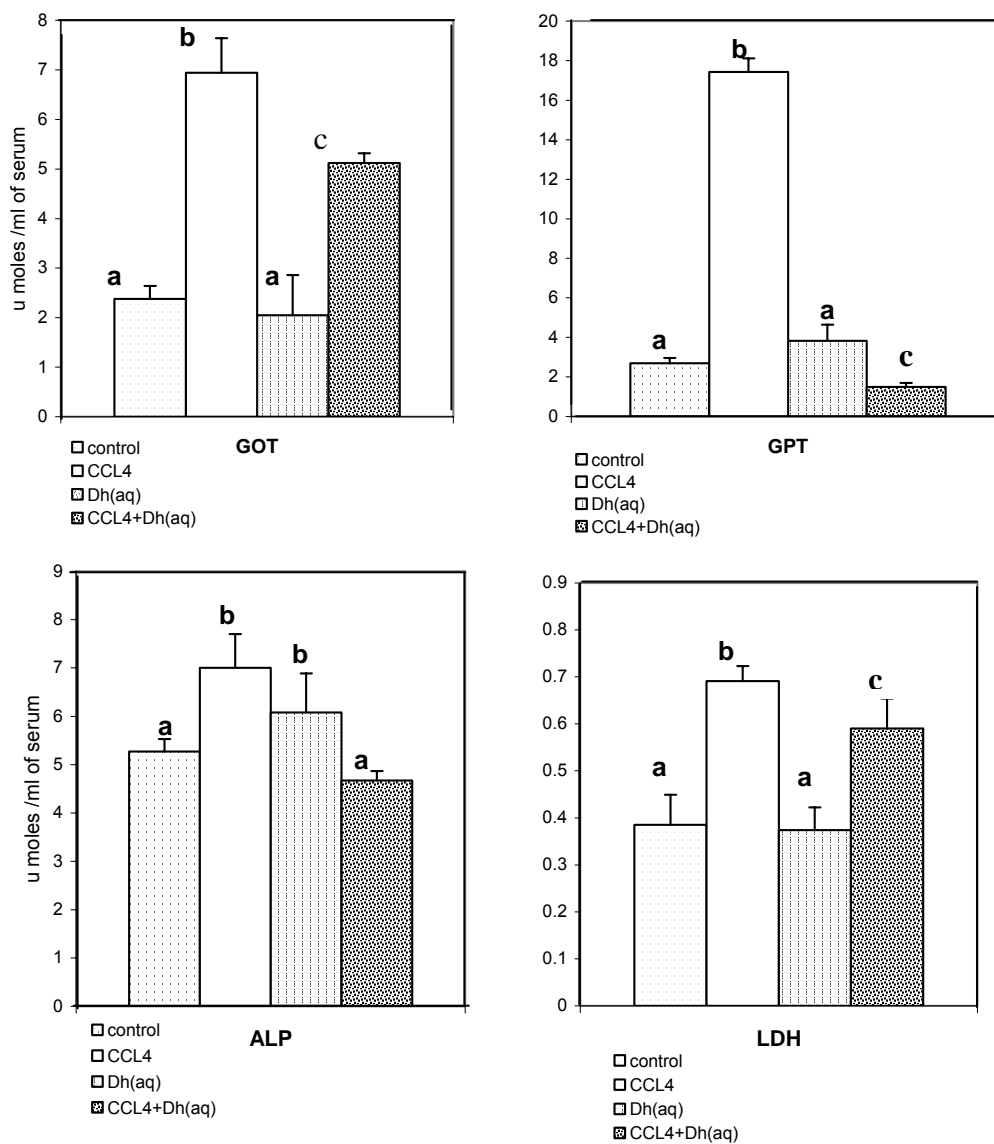
## RESULTS

**CCl<sub>4</sub> hepatotoxicity:** Rats treated with CCl<sub>4</sub> and ethanol, were dull and sluggish compared to the control group. Rats treated with *D. hamiltonii* extracts (both aqueous and methanolic) alone appeared normal. Histopathological examination of the liver showed extensive damage showing necrosis (Plate-I). Rats pretreated with *D. hamiltonii* extracts alone did not show any damage. The serum enzymes, GOT, GPT, LDH, ALP were elevated in CCl<sub>4</sub>-treated rats (fig 3.1, 3.2, 3.7 and 3.8). Elevated levels of TBARS (LPO) decrease in GSH and GSH dependent enzymes in the liver were observed in CCl<sub>4</sub> treated animals (fig. 3.11)

In rats pretreated with *D. hamiltonii* extracts followed by CCl<sub>4</sub> administration, liver damage was not discernible in histopathological examinations (Plate-I) and the serum enzymes, GOT, GPT, LDH and ALP (fig 3.1 to 3.8) were not as in the CCl<sub>4</sub> treated groups. In these animals, GSH and GSH dependent enzymes (GST, GRT and GPX) in the liver were comparable to the control group (fig 3.11, 3.12, 3.13, 3.14). These results show marked protective action of *D. hamiltonii* pretreatment on CCl<sub>4</sub> hepatotoxicity. Both aqueous and methanolic extracts showed hepatorotective property against CCl<sub>4</sub> toxicity.

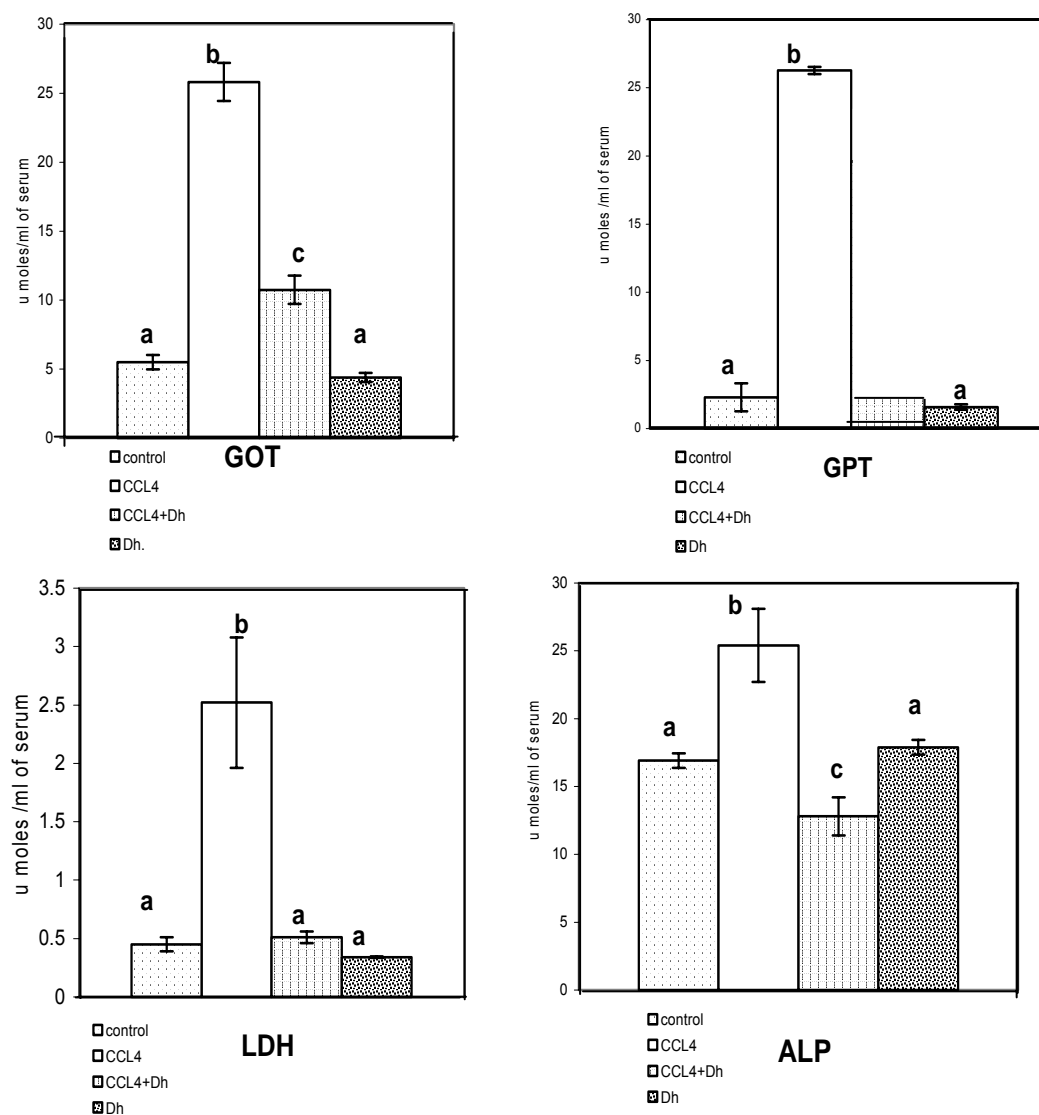
**Ethanol hepatotoxicity:** Ethanol induced damage to the liver, although to a lesser degree than CCl<sub>4</sub> as shown by histopathological observations (Plate-I). Ethanol treatment led to elevated serum levels of GOT, GPT, LDH and ALP, increased lipid peroxidation (TBARS) and decreased glutathione levels and the glutathione dependent enzymes in the liver (fig 3.9 to 3.14). Pretreatment of *D. hamiltonii* extracts (both single and multiple doses)

showed protection as evident from all the parameters. Ethanol elevation in the serum marker enzymes was prevented by the *D.hamiltonii* root extract pretreatment (fig 3.3, 3.4, 3.5 and 3.6). Other biochemical parameters also showed similar trend. Histopathological examinations (Plate- I) confirmed the hepatoprotective action of the *D.hamiltonii* extract pretreatment (fig 3.1 to 2.14). CCl<sub>4</sub> and ethanol treatment led to decreased GSH levels in the liver which was prevented by pretreatment with the *D.hamiltonii* root extracts (fig 3.11, 3.12). Further, treatment with the root extracts alone, led to elevated GSH levels above the control group (fig 3.11, 3.12). Although GSH dependent enzymes such as GPx, GR and GST were marginally affected by the hepatotoxicants, pretreatment with *D.hamiltonii* extracts restored the normal levels. Further, the GSH-dependent enzymes showed significantly increased activities in rats treated with root extracts alone (fig.3.13, 3.14).



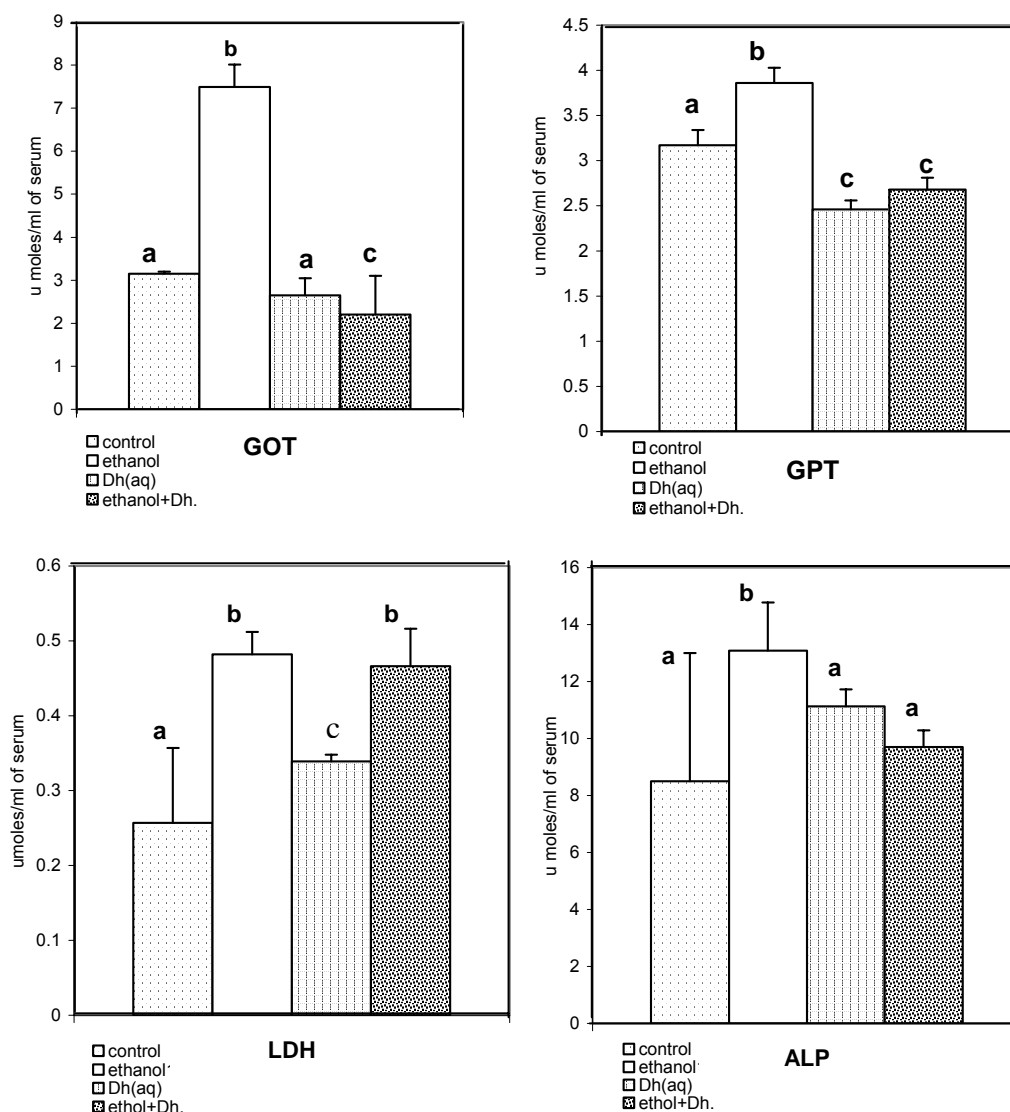
**Fig 3.1: Protective effect of a single dose pretreatment with the *D. hamiltonii* root extract (aq) on CCl<sub>4</sub> hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase (GPT); Lactate dehydrogenase (LDH); Alkaline phosphate (ALP)

Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at p<0.05 level (DMRT)



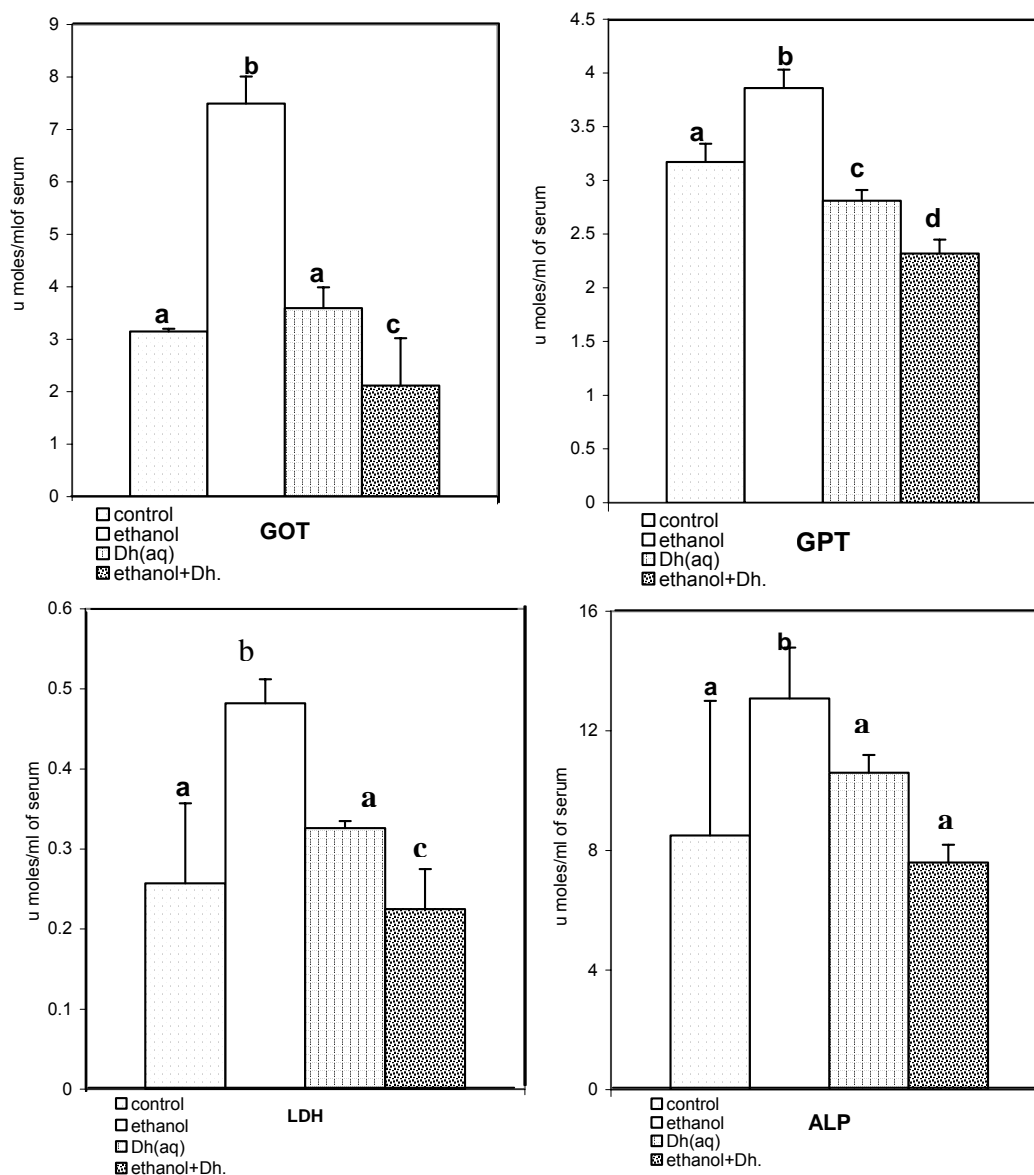
**Fig 3.2: Protective effect of a multiple dose pretreatment with the *D. hamiltonii* root extract (aq) on  $\text{CCl}_4$  hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase (GPT); Lactate dehydrogenase (LDH); Alkaline phosphate (ALP)

Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)

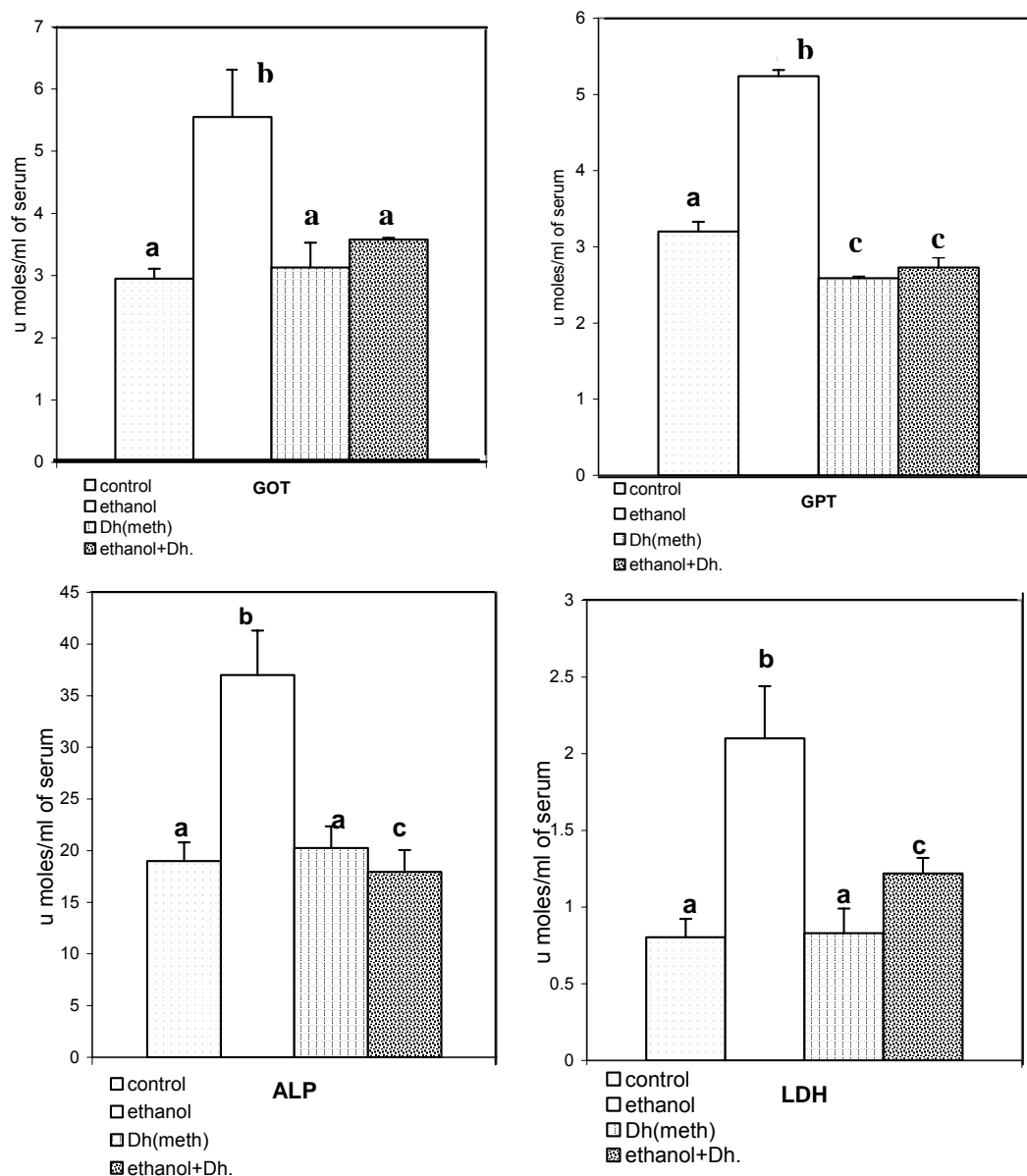


**Fig 3.3: Protective effect of a single dose pretreatment with the *D. hamiltonii* root extract (aq) on ethanol hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase (GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP)  
 Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)

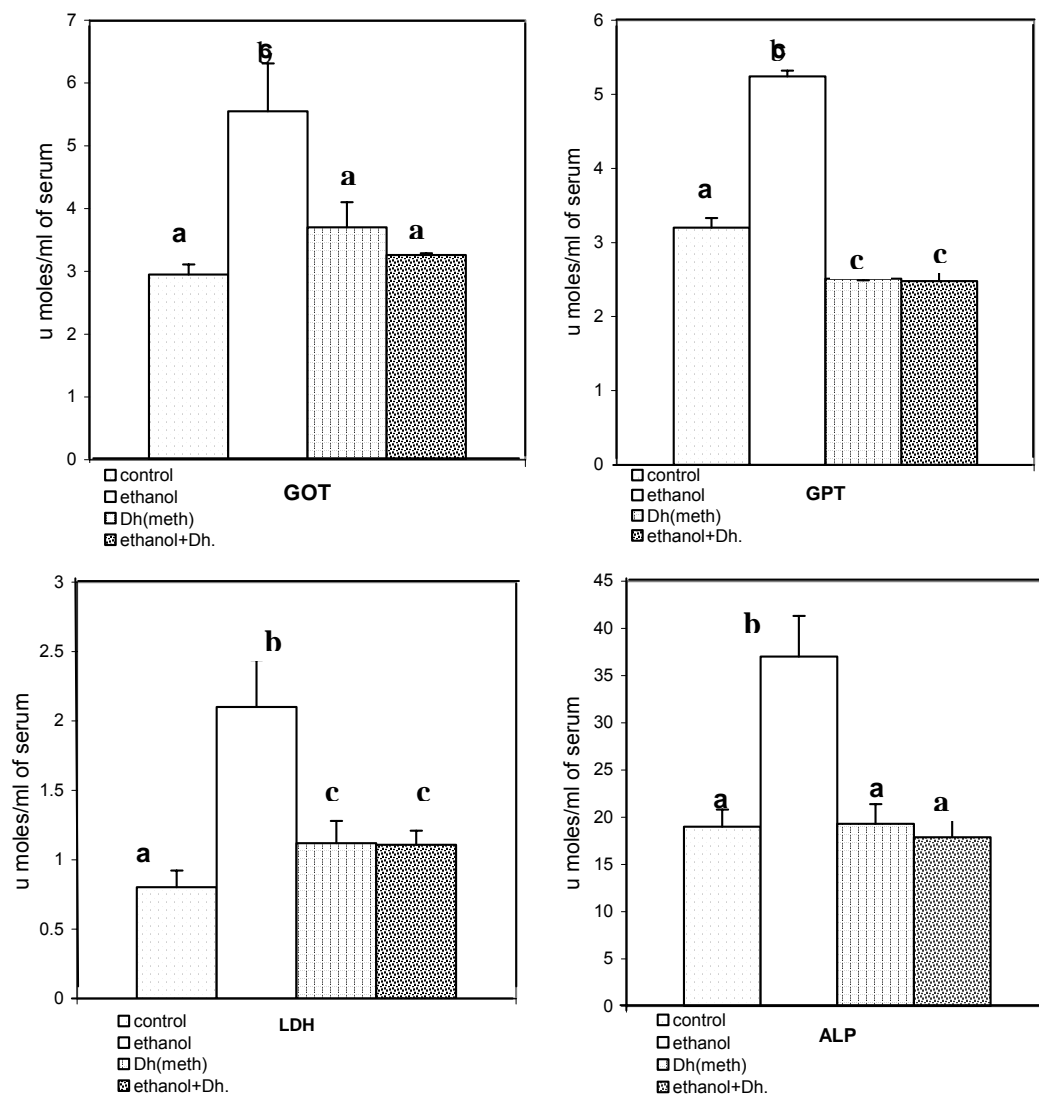




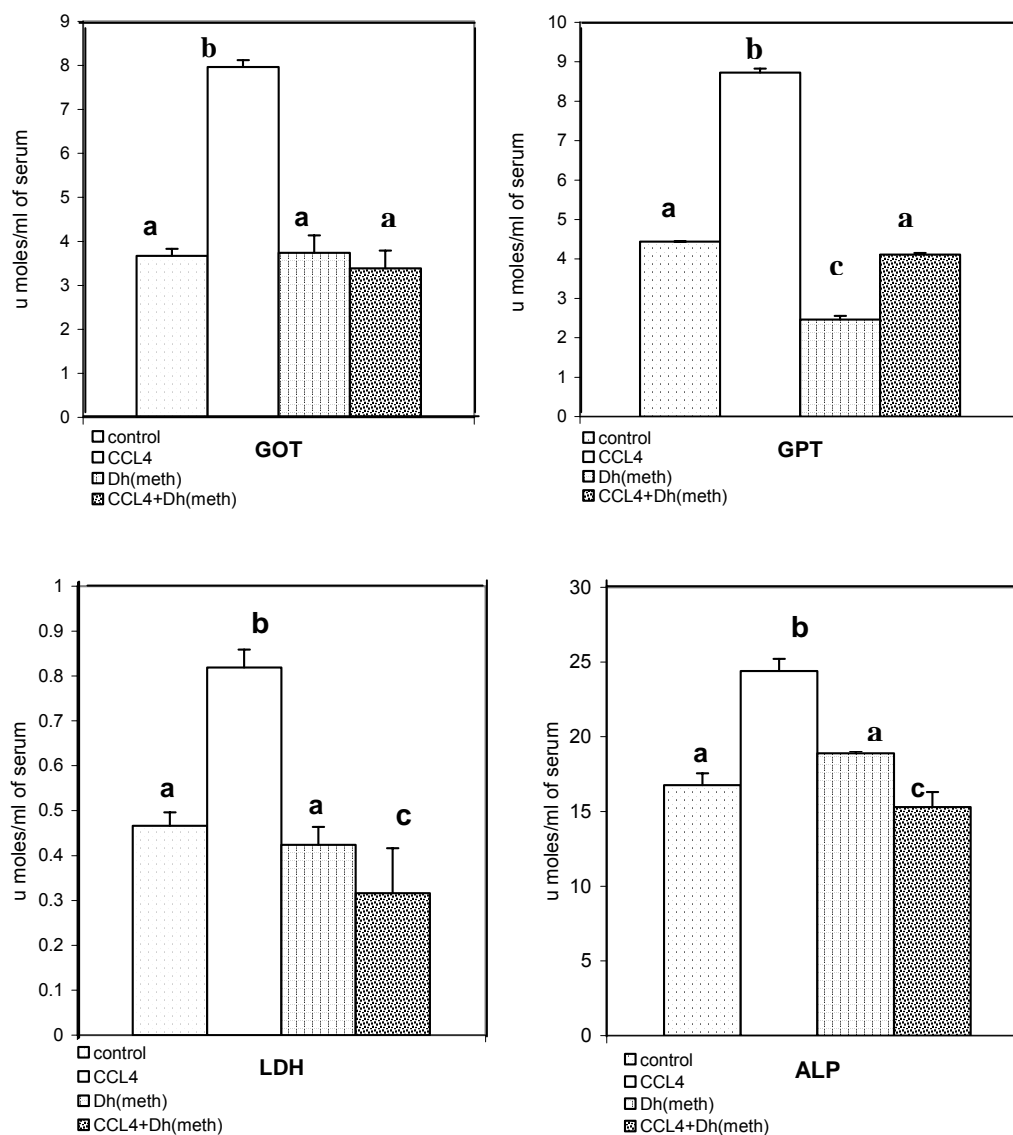
**Fig 3.4 : Protective effect of a multiple dose pretreatment with the *D. hamiltonii* root extract (aq) on ethanol hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase(GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP)  
 Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)



**Fig 3.5: Protective effect of a single dose pretreatment with the *D. hamiltonii* root extract (meth) on ethanol hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase(GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP)  
 Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)

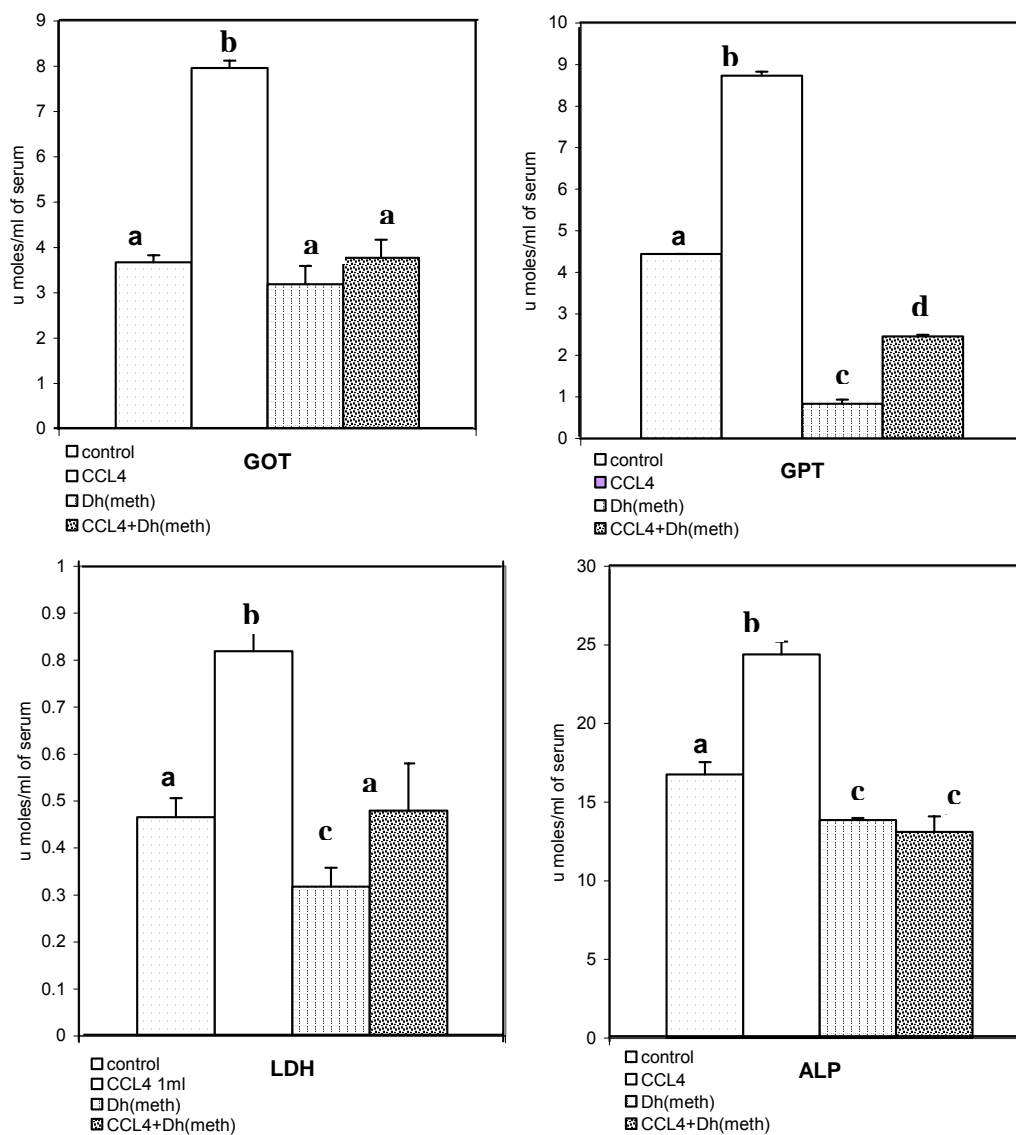


**Fig 3.6: Protective effect of a multiple dose pretreatment with the *D. hamiltonii* root extract (meth) on ethanol hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase(GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP)  
 Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)

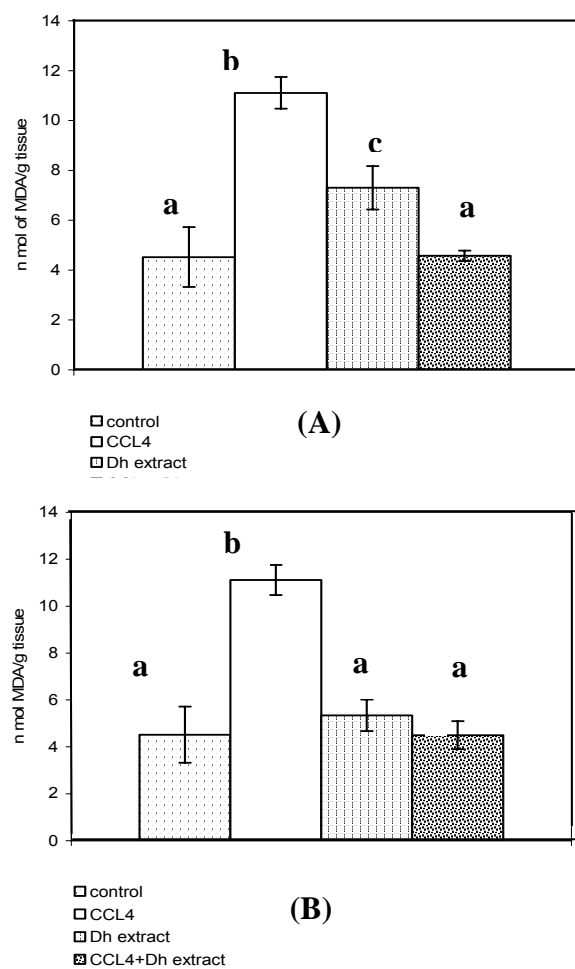


**Fig 3.7: Protective effect of a single dose pretreatment with the *D. hamiltonii* root extract (meth) on CCl<sub>4</sub> hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase(GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP)

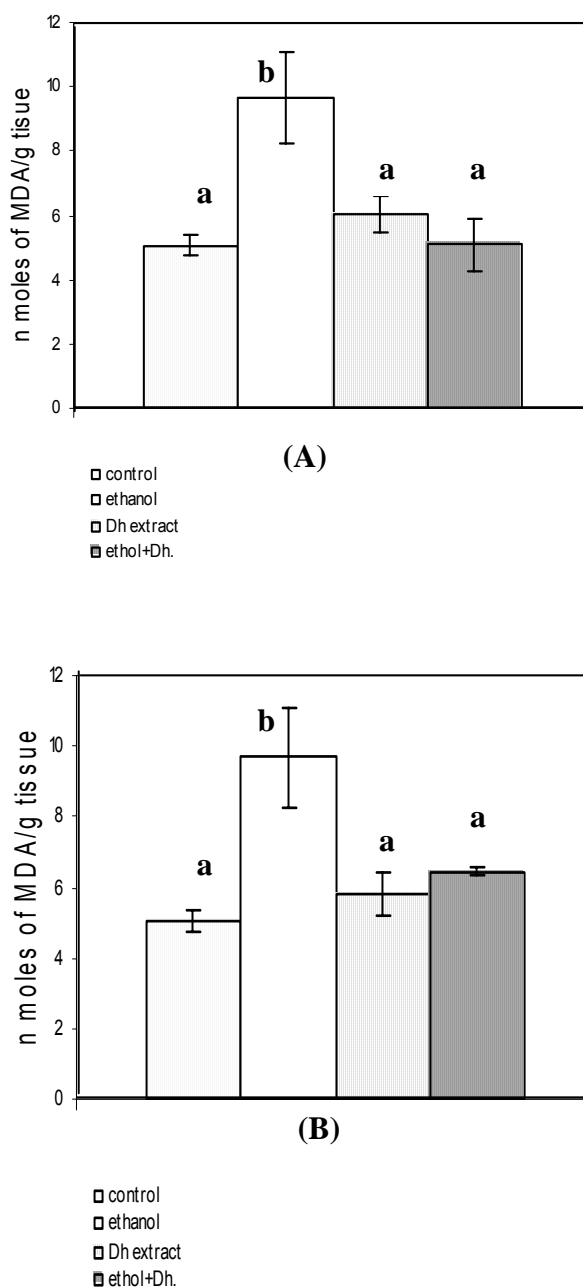
Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)



**Fig 3.8: Protective effect of a multiple dose pretreatment with the *D. hamiltonii* root extract (meth) on CCl<sub>4</sub> hepatotoxicity: serum marker enzymes.** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase(GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP)  
Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)



**Fig 3.9: Protective effect of *D. hamiltonii* root extract (meth) on  $\text{CCl}_4$  hepatotoxicity: Lipidperoxidation,**  
**(A) Single dose pretreatment of *D.hamiltonii*.**  
**(B) Multiple dose pretreatment of *D.hamiltonii***  
 Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at  $p < 0.05$  level (DMRT)

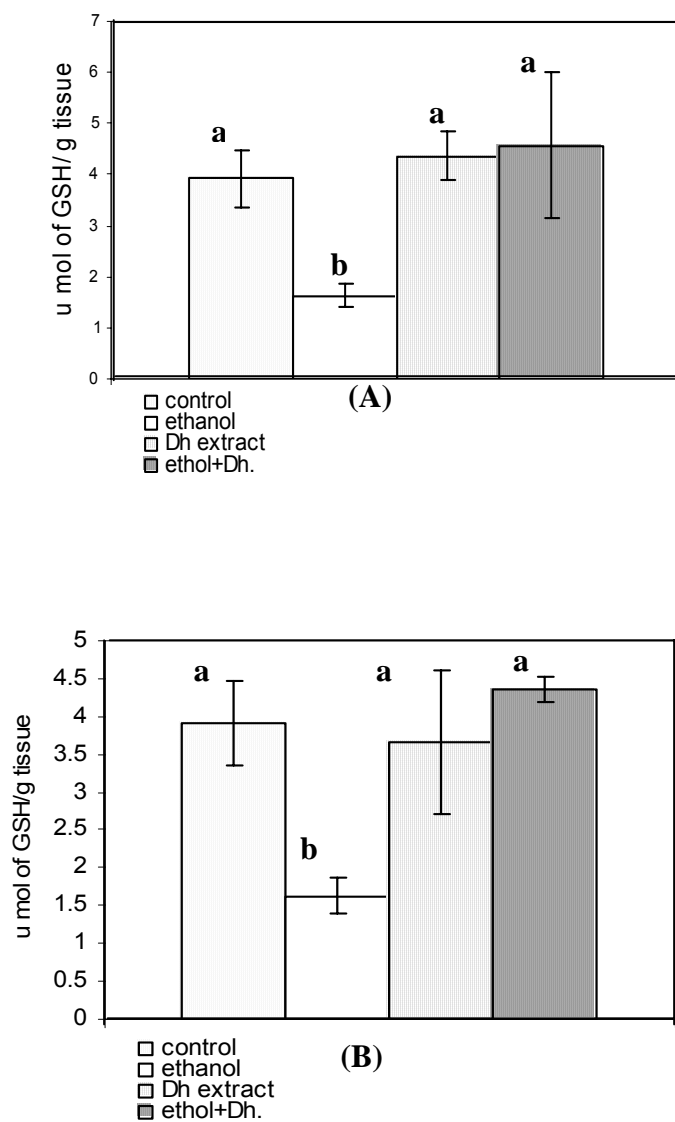


**Fig 3.10: Protective effect of *D. hamiltonii* root extract (meth) on ethanol hepatotoxicity: Lipid peroxidation,**

**(A) Single dose pretreatment of *D.hamiltonii* .**

**(B) Multiple dose pretreatment of *D.hamiltonii***

Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at p<0.05 level (DMRT)



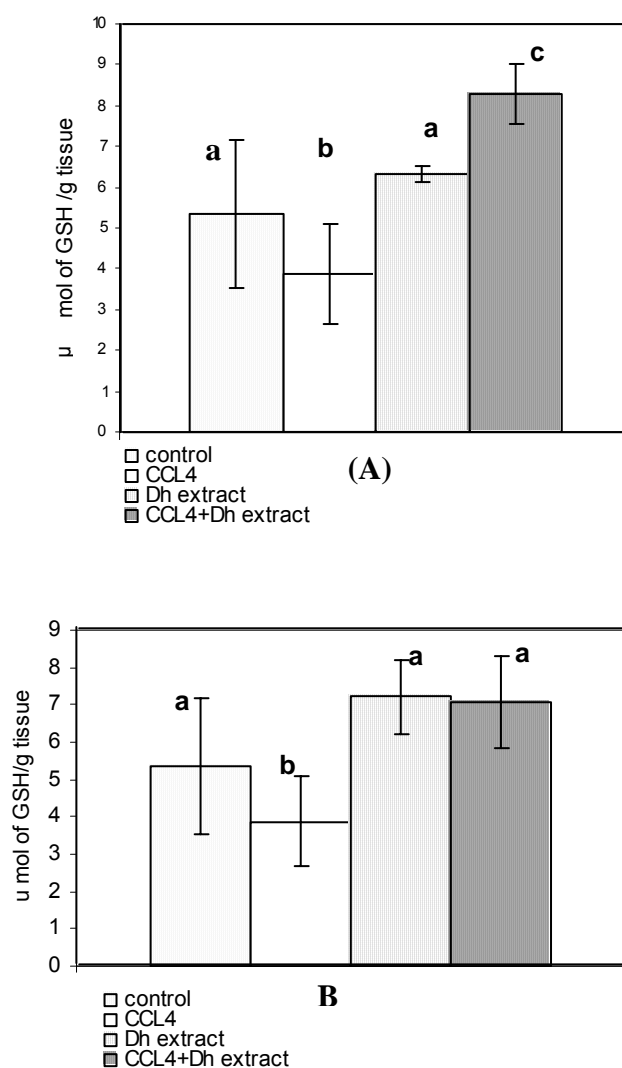
**Fig 3.11: Protective effect of *D. hamiltonii* root extract (meth) on ethanol hepatotoxicity: Glutathione levels**

**(A) Single dose pretreatment of *D.hamiltonii***

**(B) Multiple dose pretreatment of *D.hamiltonii***

Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at p<0.05 level (DMRT)



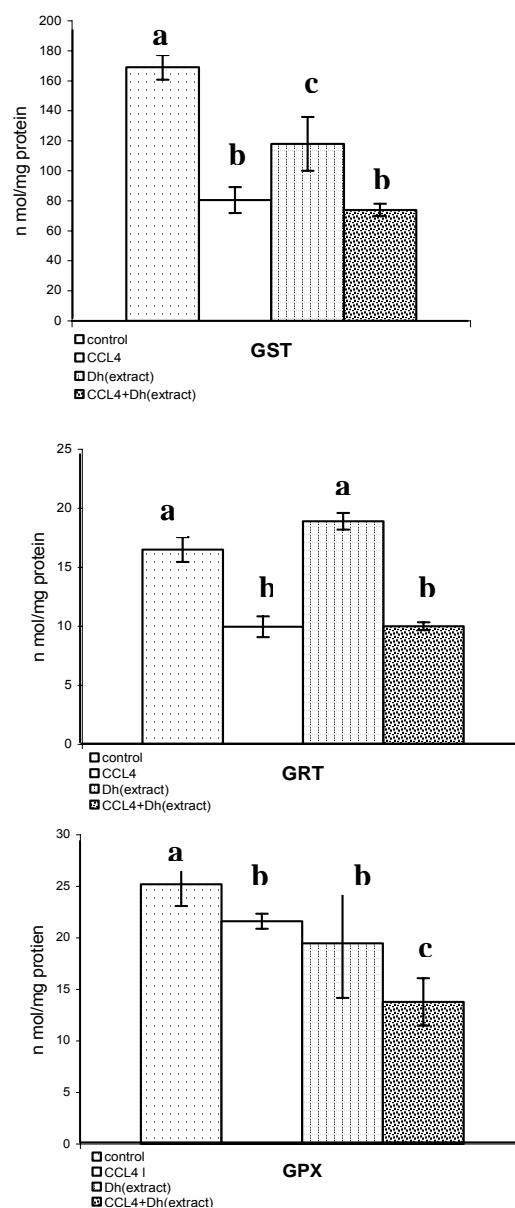


**Fig 3.12: Protective effect of *D. hamiltonii* root extract (meth) on CCl<sub>4</sub> hepatotoxicity: Glutathione levels**

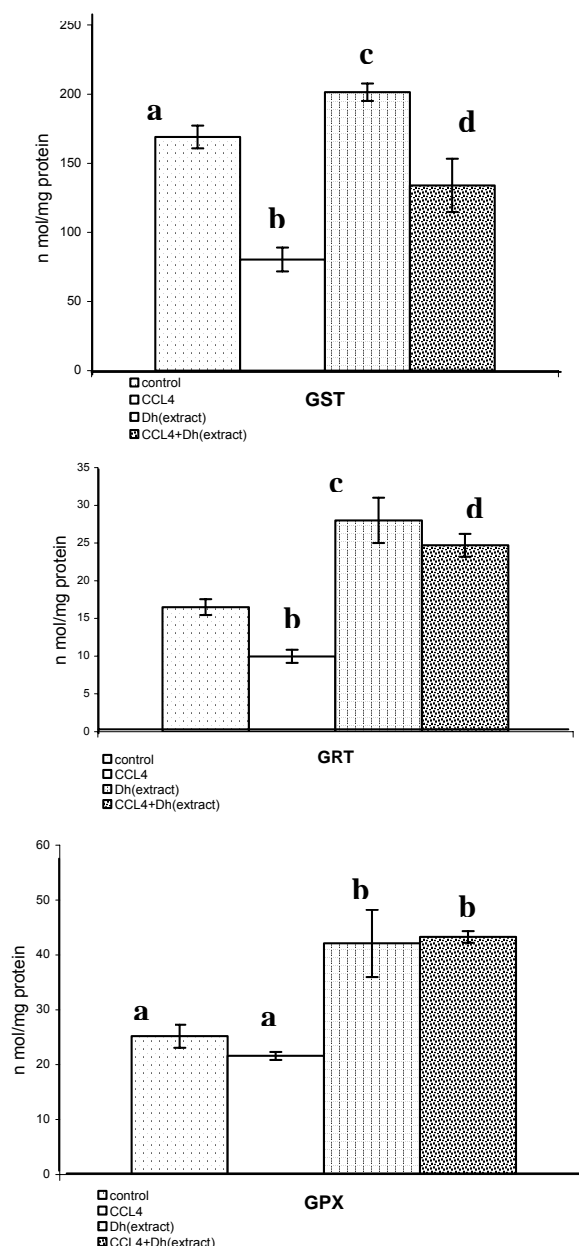
**(B) Single dose pretreatment of *D.hamiltonii***

**(B) Multiple dose pretreatment of *D.hamiltonii***

Values are shown as Mean  $\pm$  SE, n=4 values denoted by different alphabets differ significantly at p<0.05 level (DMRT)



**Fig 3.13: Protective effect of a single dose pretreatment with the *D. hamiltonii* root extract (meth) on CCl<sub>4</sub> hepatotoxicity: GSH dependent enzymes.** Glutathione-s-transferase (GST); Glutathione reductase (GRT); Glutathione peroxidase (GPX)  
Values are shown as Mean ± SE, n=4 values denoted by different alphabets differ significantly at p<0.05 level (DMRT)



**Fig 3.14: Protective effect of a multiple dose pretreatment with the *D. hamiltonii* root extract (meth) on CCl<sub>4</sub> hepatotoxicity: GSH dependent enzymes.** Glutathione-s-transferase (GST); Glutathione reductase (GRT); Glutathione peroxidase (GPX) Values are shown as Mean ± SE, n=4 values denoted by different alphabets differ significantly at p<0.05 level (DMRT)

## DISCUSSION

Several polyherbal formulations are used in ayurvedic preparations as hepatoprotective agents (Handa et. al., 1986; Vijayapadma et. al., 1998; Bhadauria et. al., 2002; Satturwar 2003) Well known Ayurvedic formulations used are *Jigrine*, (Kapur et. al., 1994), *Haridradighrita*, (Satturwar 2003) *Liv-52*, (Vijaya et. al., 1998) *Hepatomed* (Sharma et. al., 2002) which contain poly herbal extracts.

A few examples of the plants used for hepatoprotective preparations are leaves of *Ocimum sanctum* (Mukesh et. al., 2002), roots of *Irula racemosa* ( Rao and Mishra, 1997), leaves of *Parderia foetida*, (De, and Shukla et. al., 1996) rhizome of *Picrorhiza kurrooa*, (Ansari et. al., 1988) leaves of *Wedelia calendulacea*, (Emmanuel et. al., 2001) fruits of *Solanum nigrum*, (Nadeem et. al., 1996) *Sarcostemma brevistigma*, (Sethuraman et. al., 2003) *Lawsonia alba*, (Bhandarkar et al., 2003) *Andrographis paniculata*, (Handa & Sharma 1990) and *Swertia chirata* (Mukerjee et. al., 1997).

Important plants species which have been extensively studied as hepatoprotectants against the hepatotoxic agents, CCl<sub>4</sub>, glucosamine, *Plasmodium berghei* and ethanol, are Picroliv & Kutkin from *Picrorhiza kurrooa*, (Vinita et al., 1992; Das et al., 1976; Visen et al., 1996) Silymarin from *Silymarin marianum* (Ramesh et. al., 1989) and andrographolide from *Andrographis paniculata* (Handa and Sharma 1990).

Picroliv is a standardised mixture of the iridoid glycosides picroside and kutkoside from the rhizomes of *Picrorhiza kurrooa*. Picroliv 50, Picroliv 100, Picroliv TM, Picroliv R are some of the commercially marketed products. Hepatoprotective effect of this plant has been shown (Vinita et. al.,

1992). The biological activity of Picroliv is attributed to protection against cellular damage by enhancement of nucleic acid and ATP synthesis. (Vinita, Kapoor and Dhawan 1992; Visen et. al., 1996 ; Ansari et. al., 1988; Ali et. al., 2000). It is also known to possess cholerectic and laxative properties (Das et. al., 1976). *In vitro* studies have shown protective effects against hepatitis B virus and CCl<sub>4</sub> induced damage (Raj 1990; Dwivedi et. al., 1990).

Our study on *D. hamiltonii* has shown potent hepatoprotective property of both aqueous and methanolic extracts against CCl<sub>4</sub> and ethanol induced hepatotoxicity in rats.

CCl<sub>4</sub> and ethanol cause damage to cytoarchitecture of liver. CCl<sub>4</sub> a known hepatotoxin, and its metabolite trichloromethyl peroxy radical are involved in the pathogenesis of liver. CCl<sub>3</sub>O<sub>2</sub><sup>-</sup> free radical disrupts the structure and function of the cell membranes and cell organelles and induces lipid peroxidation leading to fatty liver and necrosis. (Slater 1984) Liver injury by CCl<sub>4</sub> leads to elevated serum enzyme levels of GOT, GPT, ALP, LDH due to the damaged structural integrity of liver, and as these cytoplasmic enzymes are released into the blood circulation as a consequence of the damage.

In our study, pretreated groups of rats with *D. hamiltonii* extracts (both single and multiple doses), the serum enzyme levels GOT, GPT, ALP, LDH were similar to the control (untreated) animals indicating that the pretreatment by itself was not toxic.

Ethanol is a well known hepatotoxic chemical (Kurose et. al., 1996). Ethanol is metabolized by the liver to acetaldehyde which is also a potent

hepatotoxic metabolite (Mauch et. al., 1986). Ethanol is also known to induce oxidative stress by its ability to induce free radicals and membrane lipid peroxidation (Luczaj et. al., 2004). In our study ethanol induced liver damage was relatively mild when compared to that of CCl<sub>4</sub> histopathologically. However, elevated levels of serum enzymes GOT, GPT, ALP, & LDH were seen due to the hepatotoxic effect of ethanol. Increase LPO in the liver is also indicative of oxidative stress caused by ethanol. *D. hamiltonii* pretreatment effectively prevented hepatotoxicity of ethanol. In the pretreated rats with *D.hamiltonii* extract ethanol administration did not lead to rise of the serum enzyme levels which were comparable to the control group. Ethanol-induced LPO in the liver was also prevented by *D.hamiltonii* pretreatment indicating the antioxidant action which could be important in the protective action. The antioxidant activity of *D.hamiltonii* has been demonstrated by us earlier. (chapter-II)

GSH, an important antioxidant molecule in the non-enzymic mode of defense in the cell (Halliwell, 1978). Necrosis occurs when GSH levels fall drastically in hepatic tissue from normal levels as observed in ethanol and CCl<sub>4</sub> toxicity (Grattagliano et. al., 2002). Depletion of GSH is an initiator of oxidative stress (Wefers and Sies 1983; Grattagliano 2002). Oxidative stress, xenobiotic toxicity and hypoxia ultimately results in membrane damage and leads to hepatocellular necrosis.

In our study, *D. hamiltonii* pretreatment has been shown to ameliorate hepatotoxic effects of CCl<sub>4</sub> and ethanol administration. The antioxidant enzymes such as GST, GRT and GPX are affected by CCl<sub>4</sub> and ethanol in

treated animals but the antioxidant enzyme defenses are restored in the animals pretreated with *D. hamiltonii* extracts.

Further, *D. hamiltonii* extracts induced GSH and GSH dependent enzymes in the liver which act as antioxidant defenses and contribute to the hepatoprotective action (Chattopadhyay et. al., 1992; Nadeem et. al., 1997; Khanna and Ramesh Chander 1995; Neha and Rawal 2001; Manoj and Aqeel 2003)

The biochemical basis for hepatoprotective action of *D. hamiltonii* extracts is due to its antioxidant constituents with free radical scavenging property and the ability to enhance body's own antioxidant defense as evident from the elevated levels of GSH and GSH dependent enzymes.

## INTRODUCTION

In recent years there has been a growing interest in health products derived from natural sources like natural foods, herbal medicines, since they are rich in antioxidants. Antioxidants, by virtue of scavenging free radicals, prevent or slow down the progression of several degenerative diseases (Halliwell and Gutteridge 1992). Natural antioxidants present in are a wide array of sources viz., foods, fruits, vegetables, spices, culinary herbs, and traditional medicinal plants (reviewed in chapter I).

Some of the commonly used botanicals such as *ginko biloba*, *St. John's wort* (*Hypericum perforatum*), *Kava*, (*Piper methysticum*), *Garlic*, *Echinea*, *Ephendra*, etc are rich sources of antioxidants and are being marketed as herbal health products (Vale 1988; Jamieson and Duffield 1990; Mathews 1998; Ernst 1999; Piscitelhi et. al., 2000). Natural antioxidants present in herbal health products are a complex chemical compounds, comprising polyphenols, flavonoids and other secondary metabolites. Studies to evaluate their adverse effects, if any, are necessary in order to ensure their safety to man (Kruger and Mann 2003).

In view of the recent global trend towards the revival of interest in traditional system of medicine, screening of herbal products for their mammalian safety is of paramount importance. Among the several herbal products only a handful of them have been tested for their toxicity and safety viz; applenphon, (Shoji et. al., 2004.) salacinol, tarralin (Kowsalya et. al., 1995 Krishnakumar et. al., 1999; Wolf and Weisbrode 2003; David et. al., 2004), gum karaya, a food additive (Janaki and Sashidhar 2000; Geoffrey and Robert 2000).



*Decalepis hamiltonii*, a climber, grows in the forests of peninsular India. The tuberous roots are consumed as pickle and beverage; a debittered-health drink has also been developed (Chauhan et. al., 2000). The roots of *D. hamiltonii* have also been reported to protect stored grain from insects and a biopesticide has been formulated (George et. al., 1998).

Earlier work has shown that the roots of *D.hamiltonii* contain sterols, amyryns and lupeols (Murti and Sheshadri 1941 a, b, c; 1943 a, b.). However complete composition of the roots is not known. Our work has shown that the root extract of *D. hamiltonii* possess strong antioxidant properties (Shereen et. al., 2001) (chapter II) and hepatoprotective potential (chapter III). Since the root is consumed by man, it is essential to evaluate its mammalian safety. The following investigation were undertaken to study acute, subacute toxicity /safety in the laboratory rat.

## MATERIALS AND METHODS

Fresh roots (tubers) of *Decalepis hamiltonii* were procured from Biligiri Rangana hills, Karnataka state, India. Root powder and the extracts were prepared as described in chapter II.

### Chemicals:

Bovine serum albumin, ethylene diaminetetraacetic acid,  $\alpha$ -keto glutaric acid, 6-Benzamido-4-methoxy-m-toluidine diazonium chloride (fast blue- salt), oxalo acetic acid, 2-4, dinitrophenyl hydrazine and 1-naphthyl phosphate were purchased from Sigma Aldrich Co. St. Louis. U.S.A. Tris, aspartic acid, polyvinyl pyrrolidone, D L- alanine, vitamins, salt mixtures (HMW), casein, were purchased from Sisco research laboratories, Mumbai, India. Pyruvate, inositol, pyridoxal phosphate, calcium pantothenate were purchased from Hi-media laboratories, Mumbai, India. Rest of the chemicals were purchased from Qualigens fine chemicals, Mumbai, India.

**Animals:** Albino adult male rats (CFT-wistar strain) in the weight range of 180-250 g (3 months old) were individually housed in polypropylene cages with feed (Lipton, India) and water provided *ad libitum*.

**Experimental diet:** Basal diet containing wheat flour, ragi flour, bengal gram flour, refined oil, casein, calcium carbonate shark liver oil common salt were thoroughly mixed along with minerals (salt mixture). The protein content of the basal diet was 17 % (National Institute of Nutrition, 1989).

For subchronic study (90 day), the basal diet was mixed with 0.5%, 2.5% of *D. hamiltonii* root powder and the control diet was without the root powder.

**Acute toxicity:** Animals were divided into four groups of four rats each. Group I served as the control; group II, III and IV constituted the *D. hamiltonii* treated groups. The animals were administered orally with a single dose of *D.hamiltonii* root extract (250, 500 and 1000 mg/Kg b.w.) to group II, III and IV respectively. The control group was treated with the carrier (groundnut oil) only.

**Subchronic (90 day) study:** Weanling albino rats (CFT-wistar strain) were placed in individual cages and grouped into three groups. The animals of group II and III were fed with diet containing 0.5 % and 2.5 % of *D. hamiltonii* root powder respectively and the control group (Group I) on the control diet. Diet and water were provided *ad libitum*. The feed intake was monitored daily. The body weights of the animals were recorded every week.

At the end of 90 days, the animals were sacrificed by ether anesthesia and blood was collected in dry test tubes containing sodium citrate –EDTA for hematological analysis. The blood was also collected without anticoagulant and then allowed to clot at room temperature, centrifuged at 1000 g for 15 minutes at 4°C; serum separated was used for biochemical assays. Vital organs (liver, kidney, brain, adrenal and testes) were removed and portions were fixed in Bouins' fluid and processed for paraffin – embedding and histopathological analysis.

**Protocol for reproduction study:** Adult male rats fed with *D. hamiltonii* root powder at 0.5% and 2.5% for 90 days from weanling stage were used for fertility study. Each male rat was allowed to mate with two untreated (fed on control diet) female normally cycling virgin rats. At the end of two weeks mating period, the female rats were separated and housed individually and

their vaginal smears were examined for pregnancy. Pregnant rats were allowed to litter, the litters were recorded and the survivals of the pups were observed for two weeks.

### **Biochemical Assays**

**Serum enzymes:** Glutamate oxaloacetate transaminase (GOT; EC 2.6.1.2) was assayed by the colorimetric assay method of Bergmeyer and Bernt (1974). Glutamate pyruvate transaminase (GPT; EC 2.6.1.1) was assayed by colorimetric assay method of Reitman and Frankel as given in Bergmeyer and Bernt (1974). Alkaline phosphatase (ALP; EC 3.1.3.1) was assayed by Walter and Schutt (1974) method. Lactate dehydrogenase (LDH; EC. 1.1.2.3) was assayed by uv method (Bergmeyer and Bernt 1974).

**Total serum cholesterol:** Total serum cholesterol was assayed by the method of Searcy and Berquist (1960). 100  $\mu$ l serum was mixed with 1.5ml of ferric chloride (stock 504mg/10ml), then 1 ml of concentrated sulphuric acid was added to this reaction mixture and kept in dark for 45 minutes and the absorbance was recorded at 540 nm in a spectrophotometer. Cholesterol content was calculated using a standard curve.

**Statistical analysis:** Data were analysed statistically by Duncan's multiple range test to evaluate the differences among the groups at 5% ( $p < 0.05$ ) significance level using statistical package a software programme developed at the computer center of C.F.T.R.I.

## RESULTS

*Acute toxicity:* The treated animals were active and showed no signs of toxicity. The serum marker enzymes levels of GOT, GPT, LDH and ALP were not altered when compared to the control group (fig 4.1, 4.2).

*Subchronic (90 day) study:*

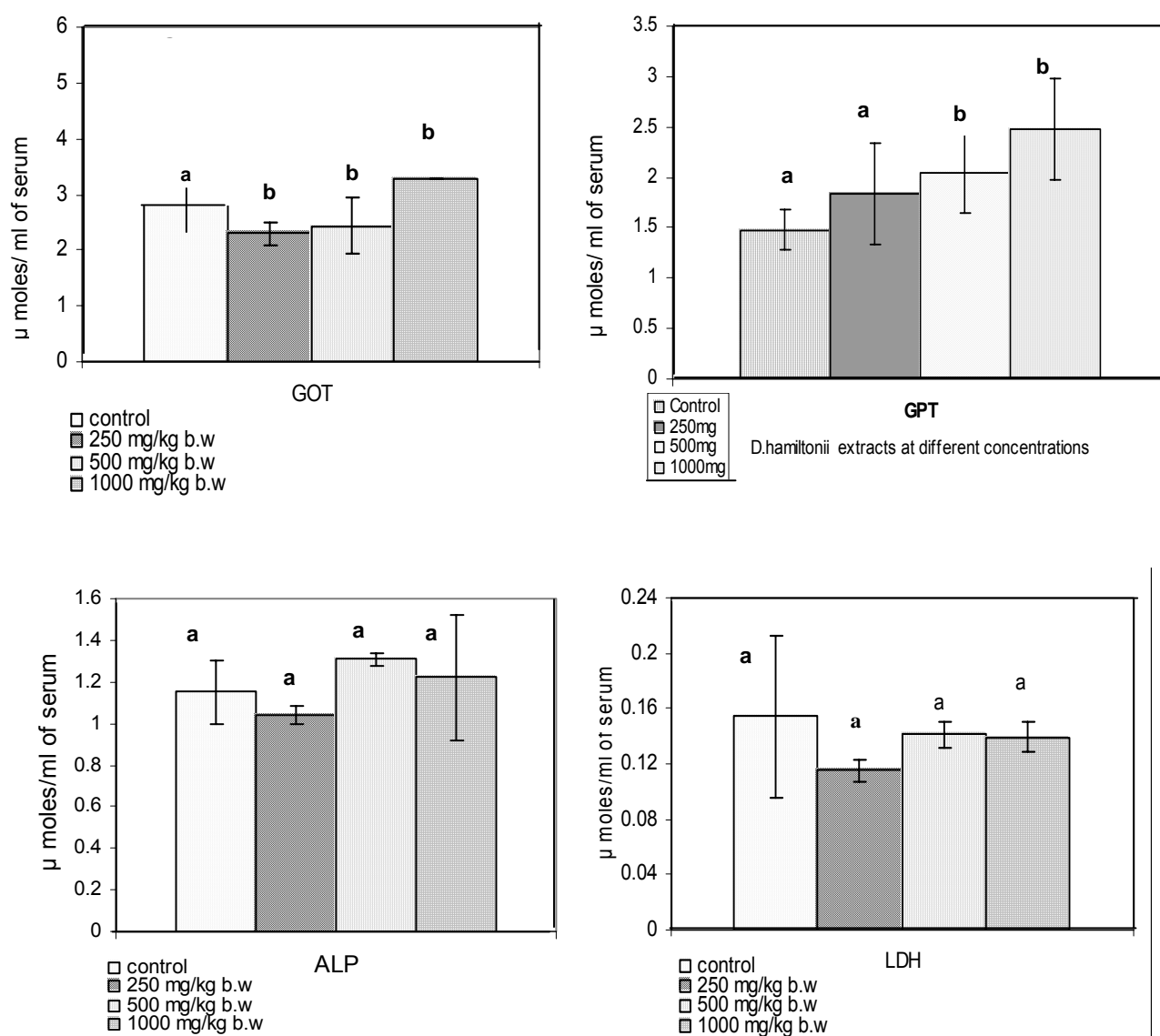
Body weights and food intake: *D.hamiltonii* fed animals at a dosage level of 0.5 % and 2.5 % appeared normal. Feed intake in the treated group was comparable to the control group (fig 4.2). Body weight gains in the treated groups were comparable to the control. (fig 4.3) and were not significantly different. Organ weights of treated animals were comparable to control group (table 4.1).

Hematology: Haemoglobin content, total erythrocyte count (RBC) and leucocyte count, packed cell volume of the treated animals were not significantly different from those of the control group (table 4.2).

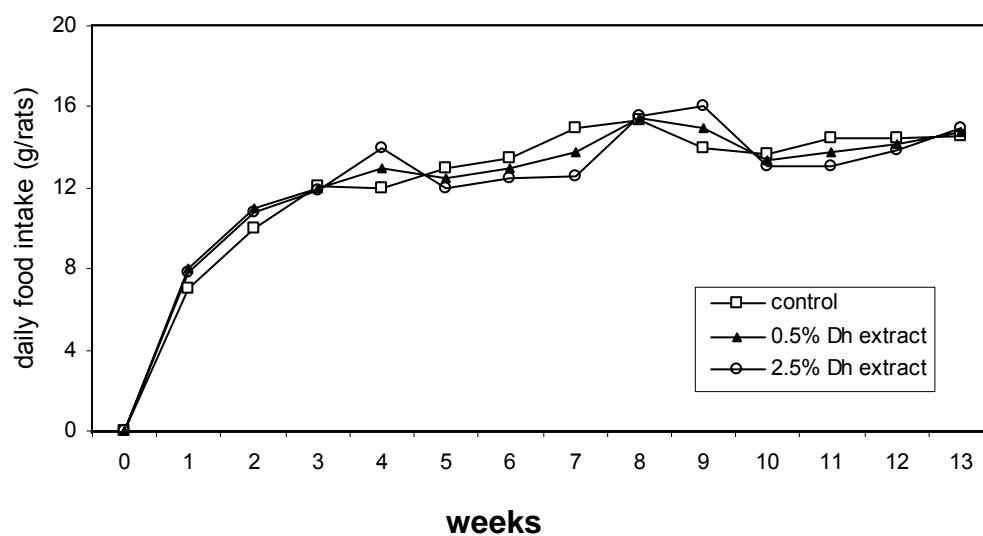
Histopathology: In animals of group II and III fed with 0.5% and 2.5% *D.hamiltonii* powder, the tissue sections did not show any lesions in the vital organs and were comparable with the histopathological profile: Histological examination of the vital organs (liver, kidney, intestine, brain, adrenal and testes) of rats fed with *D.hamiltonii* root powder in the diet, did not show alterations indicative of toxicity. The histological profile was similar to that of control group (Plate II).

The serum marker enzymes GOT, GPT, LDH and ALP in the treated groups were not significantly altered in the treated groups (fig 4.4). Interestingly serum total cholesterol was 30 % lower in the treated groups over that of control (fig 4.5).

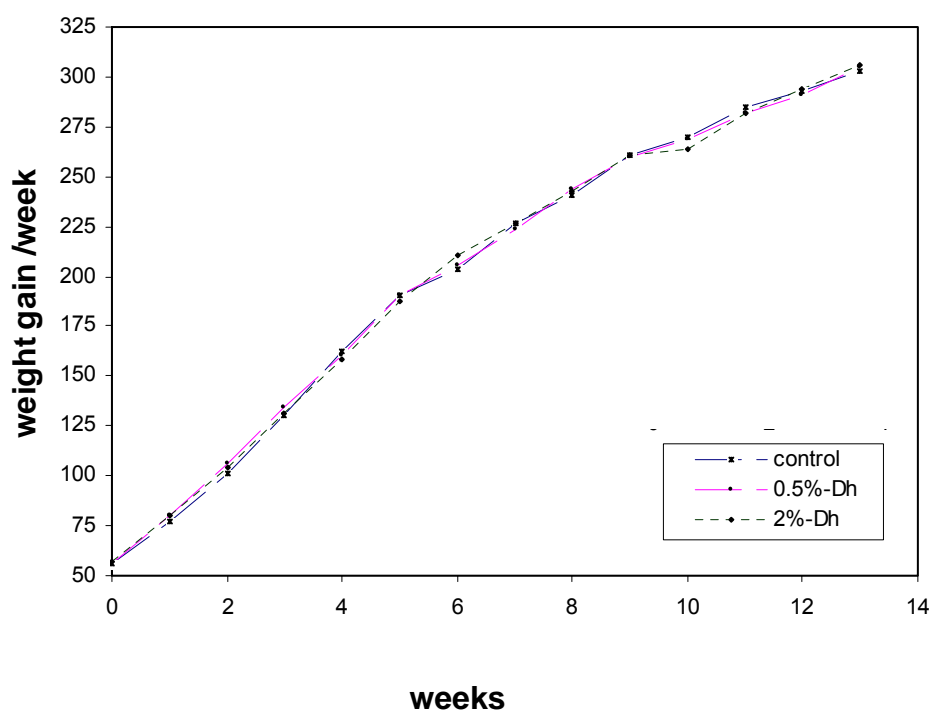
Reproductive potential: The numbers of pregnant rats were comparable between the control and treated groups. The litter size and the survival of the pups of the control and treated groups were not significantly different (table 4.3). The pups born to the rats fed with *D.hamiltonii* root powder in the diet did not show any deformities.



**Fig 4.1: Acute toxicity of *D.hamiltonii* extract to rats: serum enzymes** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase (GPT); Lactate dehydrogenase (LDH); Alkaline phosphate (ALP). Each bar represents the value or activity with S.E, n=4. Values denoted by different alphabets differ significantly at  $p < 0.05$  (DMRT)

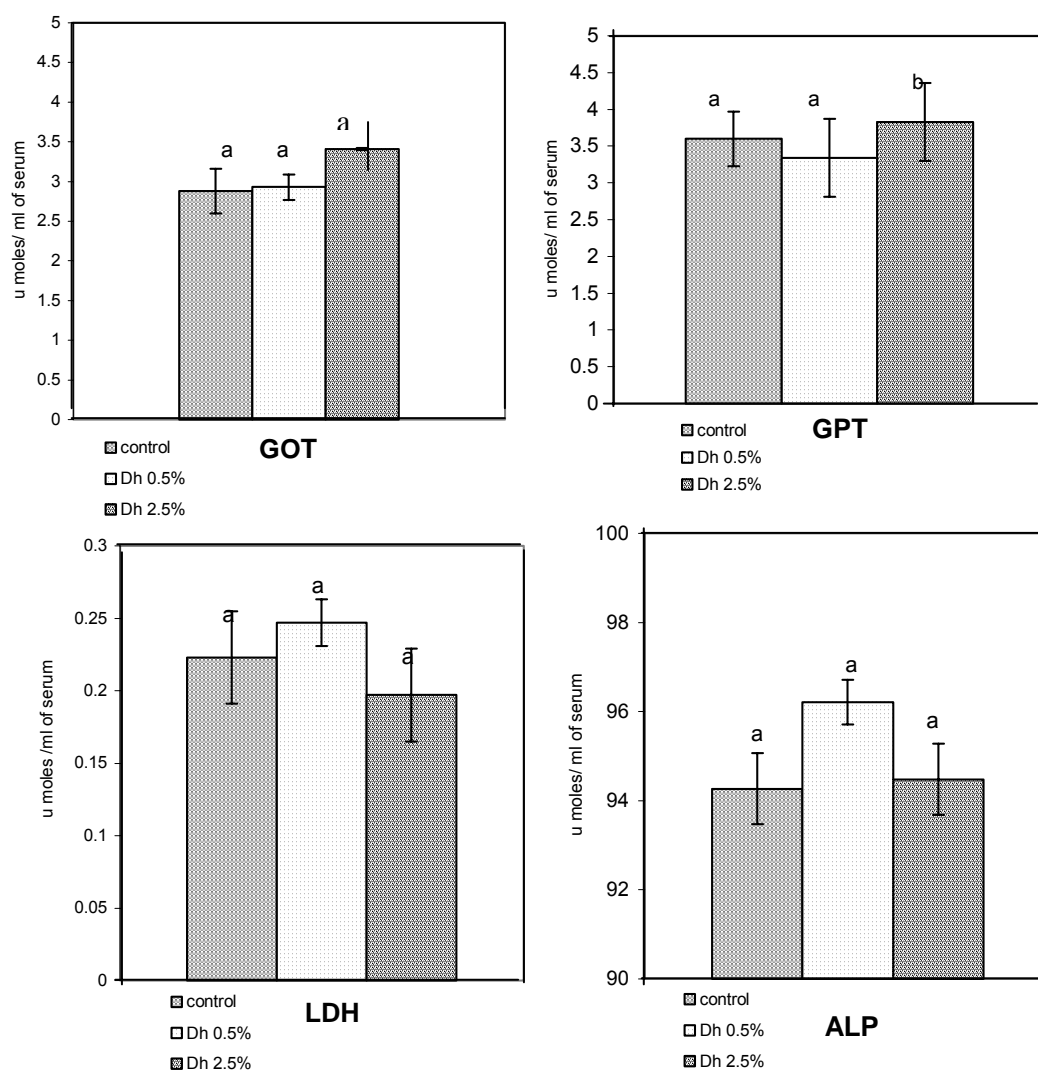


**Fig 4.2 : Daily food intake of weanling rats fed diets containing *D. hamiltonii* (0.5% & 2.5%) root powder for 90 days.**



**Fig 4.3 : Growth (body weight gain) of weanling rats fed diet containing *D. hamiltonii* root powder for 90 days.**





**Fig 4.4: Subchronic (90 day) toxicity effects of *D.hamiltonii* root powder 0.5% and 2.5%); serum marker enzymes** Glutamate oxaloacetate transaminase (GOT); Glutamate pyruvate transaminase(GPT); Lactate dehydrogenase(LDH); Alkaline phosphate(ALP). Each bar represents the value or activity with S.E, n=4. Values denoted by different alphabets differ significantly at p< 0.05 (DMRT)

**TABLE 4.1: Subchronic (90day) toxicity study of dietary *D. hamiltonii* root powder to male rats: Organ weights\***

ORGAN	CONTROL	Dh TREATED (0.5%)	Dh TREATED (2.5%)
Liver	3.50±0.21	3.31±0.123	3.402±0.20
Kidney	0.596±0.15	0.584±0.009	0.607±0.014
Brain	0.172±0.01	0.171±0.0066	0.175±0.015
Spleen	0.541±0.020	0.556±0.009	0.567±0.0033
Adrenal	0.035±0.003	0.034±0.0005	0.036±0.004
Testis	0.121±0.019 <sup>a</sup>	0.159±0.012 <sup>b</sup>	0.155±0.008 <sup>b</sup>
Epididymys	0.307±0.004	0.303±0.006	0.301±0.002

\*g /100 g bw; M±SE

The organs weights in treated groups were not significantly different from the control group at 5% level marginal increase except in the case of testes (DMRT).

**TABLE 4.2: Subchronic (90-day) toxicity study of *D. hamiltonii* root powder to rats: Hematological profile**

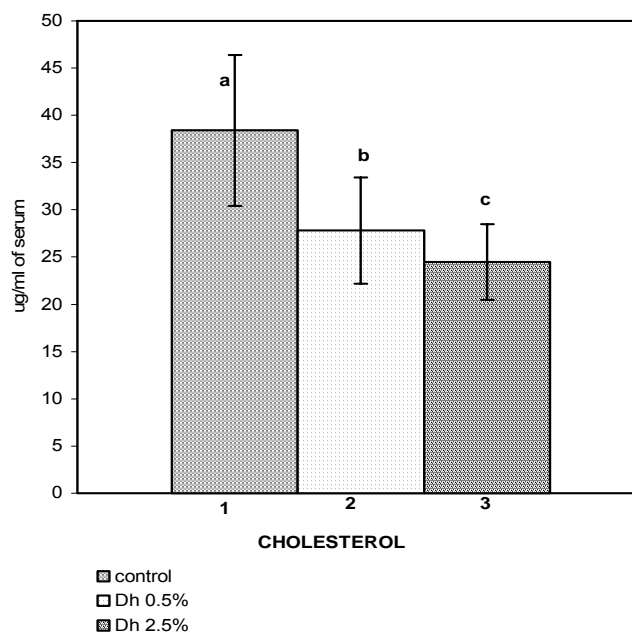
<b>Parameter</b>	<b>Control</b>	<b>Dh Treated (0.5%)</b>	<b>Dh Treated (2.5%)</b>
Hemoglobin (g/dl)	13.8	14.3	14
RBC ( $10^6$ /ml)	11.5	12.02	11.7
WBC ( $10^6$ /ml)	13100	12400	14000
PCV (%)	42.5	40.5	42.5

\* The values are mean of 5 animals.

**TABLE 4.3: Reproductive potential of male rats fed with *D. hamiltonii***

<b>Treatments*</b>	<b>No. of rats pregnant</b>	<b>Mean no. of pups</b>	<b>Mean no. of litter/male</b>	<b>Pup survival %</b>
Control	5/6	9±0.31	3/9	100
Dh-0.5%	4/6	13±0	3/11.9	100
Dh-2.5%	5/6	9.6±0.9	3/9.6	100

(\*Treated male rats were mated with untreated females; See materials and methods for details)



**Fig: 4.5:** Serum cholesterol content in rats fed with *Dh* root powder for 90 days. Values are Mean  $\pm$ SE (n=4) values denoted by different letters are significantly different at P< 0.05 (DMRT)

## DISCUSSION

Global market trend towards health products is increasingly concentrated on products derived from natural sources. As these herbal or plant extracts contain one or more complex chemical compounds, the safety evaluation of the herbal products becomes necessary in order to ensure safety to man. Many of the herbal products including hepatoprotective formulations have not been thoroughly investigated for their toxicity on experimental animals or man. Recently, some of the plant derived products have been evaluated for safety. Viz; applenphon, (Shoji et. al., 2004.) salacinol, tarralin (Kowsalya et. al., 1995 Krishnakumar et. al., 1999; Wolf & Weisbrode 2003; David et. al., 2004) gum karaya, a food additive (Janaki and Sashidhar 2000), these are few examples of nutritional, medicinal, dietary or functional foods.

The tubers of *D.hamiltonii* are consumed as pickles and as a beverage for its alleged health benefits. Earlier work has shown that the roots of *D. hamiltonii* contain sterols amyryns, lupeols (Murti and Sheshadri 1941 a, b, c; 1945 a, b). Some of the flavour compounds in the volatile extract of *D. hamiltonii* have been reported (Nagarajan et. al., 2001). Many of the constituents of the roots of *D hamiltonii* remain to be identified. We have shown that *D. hamiltonii* extracts possess antioxidant property (Shereen et. al, 2001) and hepatoprotective properties (chapter I and II).

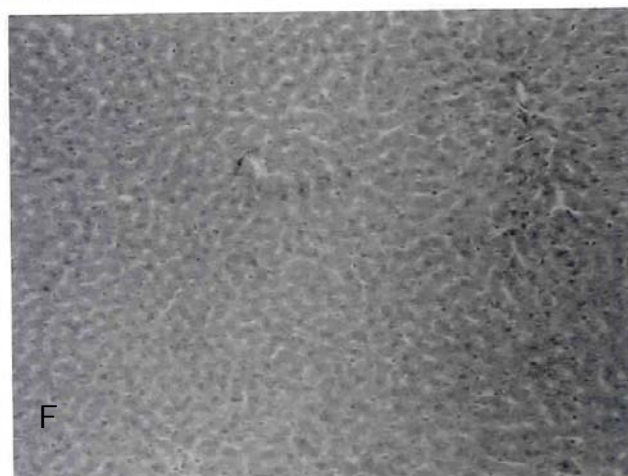
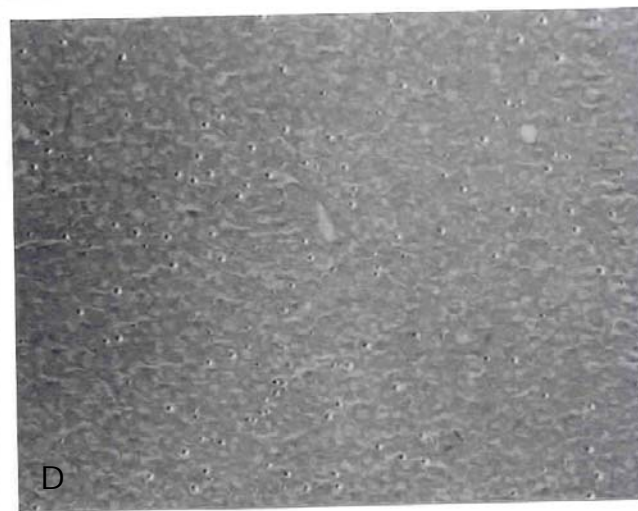
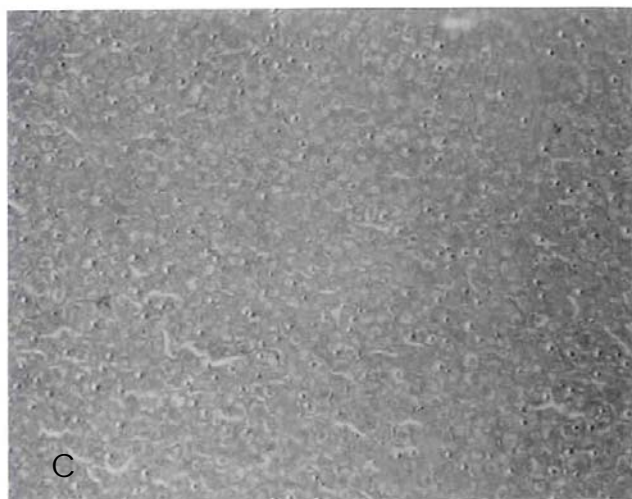
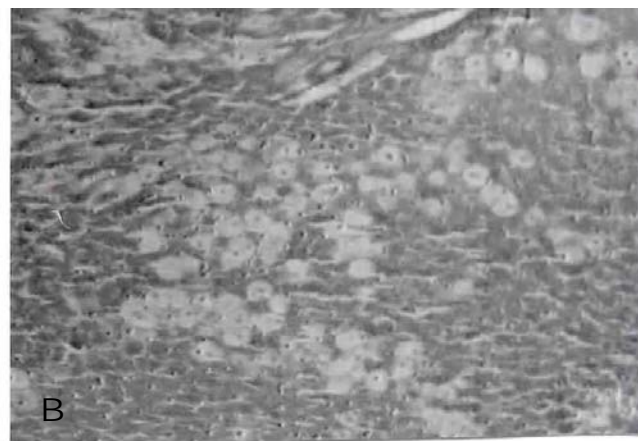
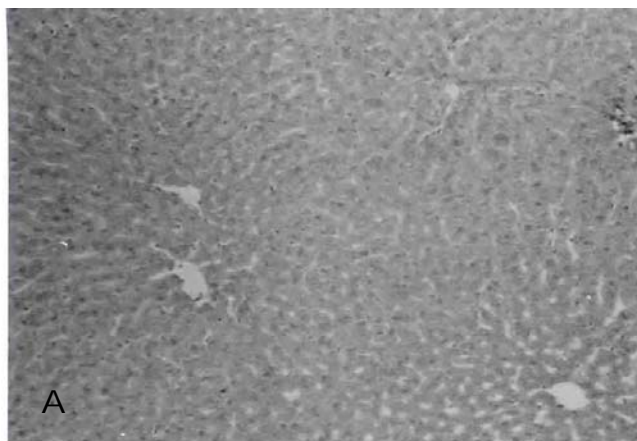
In this study we have evaluated *D. hamiltonii* roots for the mammalian safety. Our results on acute and subchronic toxicity study suggest that *D. hamiltonii* is not toxic to the laboratory rat. In acute study, the root extract

was not toxic upto 1000mg/kg b.wt. In the Subchronic study the *D. hamiltonii* root powder did not show toxicity upto 2.5% in the diet for 90 days

Our results showed no changes indicative of liver injury due to ingestion of *D.hamiltonii*. Mammalian safety assessment by subchronic toxicity study of *Decalepis hamiltonii* showed no effects on growth and no histopathological alterations in the vital organs were seen. Hematological profile was unaltered. Serum enzyme profile was not significantly changed. Cholesterol level in the *D. hamiltonii* – fed rats was significantly decreased which is a desirable health effect. The results indicate that *D. hamiltonii* is not toxic to rats' upto 2.5% in the diet for 90days. In the reproductive study, analysis of the result shows no adverse effects of *D. hamiltonii* on the testis as reflected in to number of pregnancy. Litter did not affect the reproductive potential of male rats.

Although the tuberous roots of *D. hamiltonii* have been consumed by man for centuries, there is no report of adverse effects on human health. However, this is the first study to evaluate the mammalian safety of the roots scientifically. This study along with our work on the antioxidant activity (chapter II) and hepatoprotective potential (chapter III) goes to establish the health promoting potential of *D. hamiltonii* roots. *D. hamiltonii* could well be considered the “Ginseng” of India.

## PLATE - ONE



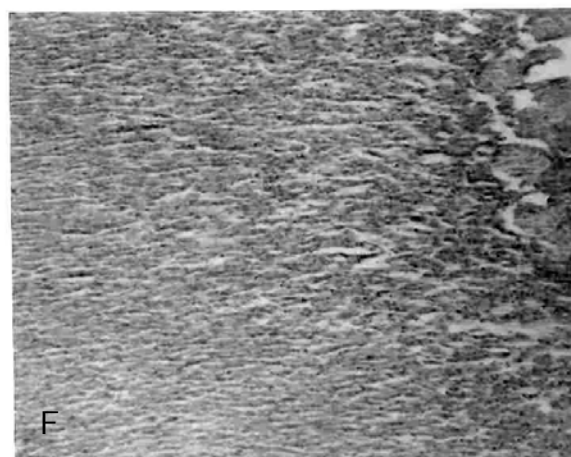
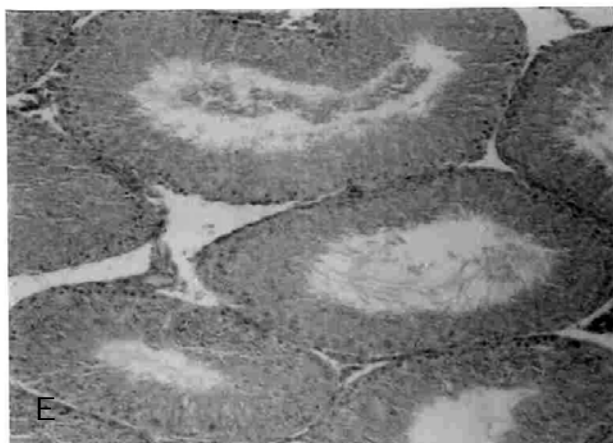
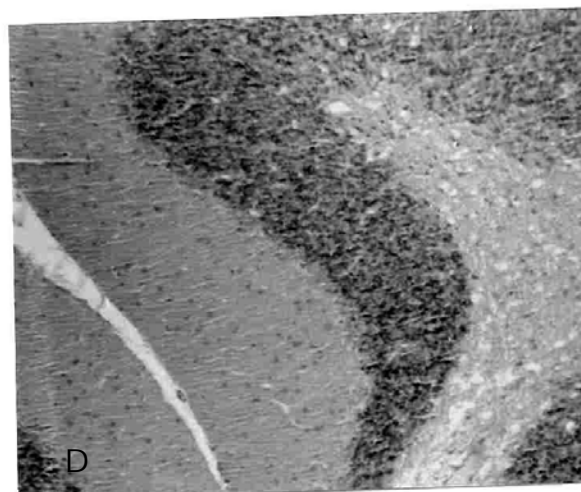
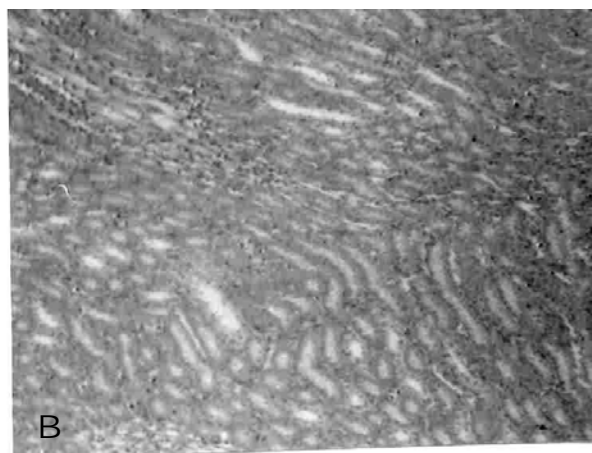
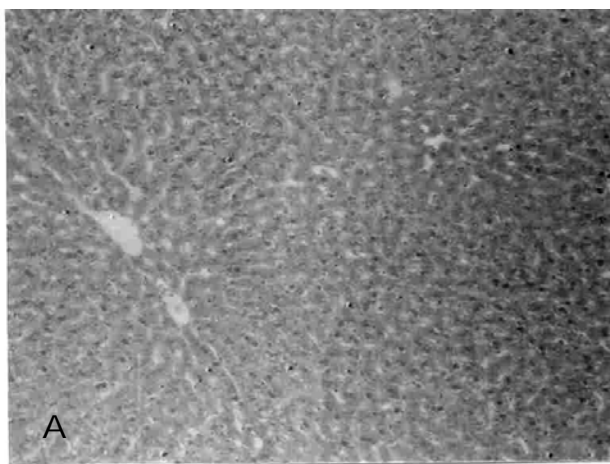


**PLATE-I: Hepatoprotective action of *D.hamiltonii* root extract on CCl<sub>4</sub> hepatotoxicity: Liver histopathology**

- A. Liver of control rats showing normal histoarchitecture.
- B. Liver of CCl<sub>4</sub> treated rats showing extensive necrosis.
- C. Liver of rats pretreated with aqueous extract of *D.hamiltonii* (multiple dose) followed by a single dose of CCl<sub>4</sub>. Note the absence of necrosis.
- D. Liver of rats pretreated with methanolic extract of *D.hamiltonii* (multiple dose) followed by single dose of CCl<sub>4</sub>. Note the absence of necrosis.
- E. Liver of rats pretreated with multiple doses of aqueous extract of *D.hamiltonii* roots alone. Histological picture shows normal histoarchitecture.
- F. Liver of rats pretreated with multiple doses of methanolic extract of *D.hamiltonii* roots alone. Histological picture is normal.

[Stain: haemotoxylin and eosin, magnification: 40X10]

## PLATE - TWO



**PLATE-II:** Subchronic (90 day) toxicity evaluation of the root powder of *D.hamiltonii* to the laboratory rat: Histology of vital organs of rats fed dietary (2.5%) root powder for 90 days.

**(A) LIVER**

**(B) KIDNEY**

**(C) INTESTINE**

**(D) BRAIN**

**(E) TESTIS    AND**

**(F) ADRENAL**

[Stain: haemotoxylin and eosin; magnification 40X10]

## CONCLUSIONS

- ❖ Roots of *Decalepis hamiltonii* possess strong antioxidant properties both *in vitro* and *in vivo*
- ❖ 2-hydroxy-4-methoxy benzaldehyde, a constituent of the root extract, is one of the natural antioxidant molecules.
- ❖ The root extracts of *D.hamiltonii* showed marked hepatoprotective potential against carbon tetrachloride and ethanol hepatotoxicity *in vivo*
- ❖ The root extracts as well as the root powder were not toxic at acute and subchronic level to rats.
- ❖ The roots of *D.hamiltonii* are a source of antioxidant nutraceuticals for better health.
- ❖ This is the first scientific evaluation of the roots of *D.hamiltonii* for its health promoting potential.

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## PATENTS

### National

A process for the preparation of novel cholinesterase inhibitor (NF/-95/02)

**Shereen.** Shivanandappa T., Sattur Avinash P., Divakar S. and Karanth N.G.

### International (WTO)

A novel cholinesterase inhibitor. Submitted, 2001 (NF/238/02).

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### U.S. (Patent)

A compound as cholinesterase inhibitor and its isolation from the fungus, *Sporotrichum* Sp. Filed. Under Process.

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