HEPATOPROTECTIVE POTENTIAL OF ANTIOXIDANTS FROM *EUPHORBIA HIRTA* AND *AEGLE MARMELOS*

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

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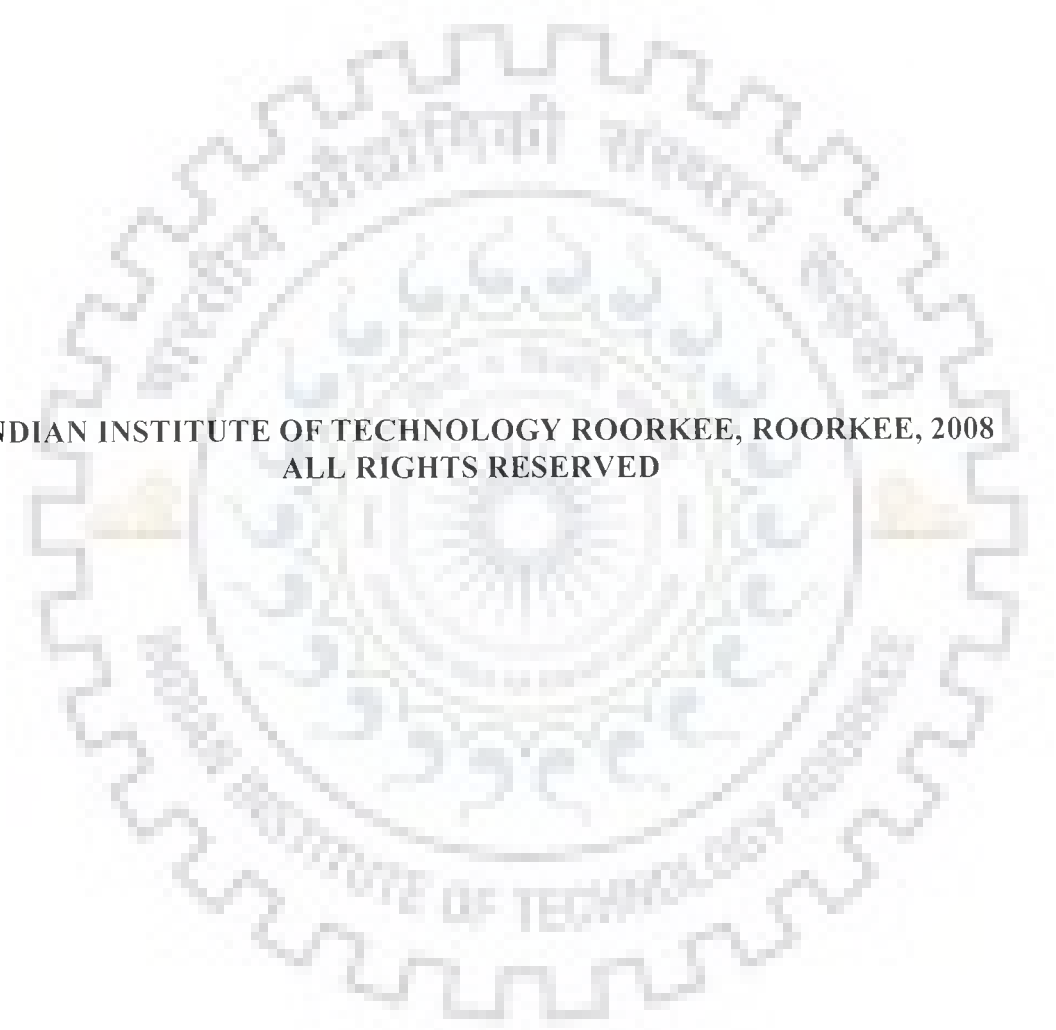

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"HEPATOPROTECTIVE POTENTIAL OF ANTIOXIDANTS FROM *EUPHORBIA HIRTA* AND *AEGLE MARMELOS*"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2004 to December 2008 under the supervision of **Dr. Ramasare Prasad**, Associate Professor and **Dr. Vikas Pruthi**, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

(NILESH KUMAR SHARMA)

This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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The Ph. D. Viva-Voce Examination of **Mr. Nilesh Kumar Sharma**, Research Scholar, has been held on.....

Signature of Supervisors

Signature of External Examiner

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List of Abbreviations

$^1O_2$	Singlet oxygen
AAPH	2,2`-Azobis(2-amidinopropane) hydrochloride
ABTS	2,2`-Azinobis(3-ethylbenzthiazoline-sulphonic
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CYP450	Cytochrome P-450
DNPH	2,4-Dinitrophenylhydrazine
ESI-MS	Electrospray ionisation – mass spectrometry
GC-MS	Gas chromatography-mass spectroscopy
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
m/z	Mass to charge ratio
MDA	Malondialdehyde

NADPH (reduced form)	Nicotinamide adenine dinucleotide phosphate
NO•	Nitric oxide
$O_2^{\bullet}$	Superoxide anion
OH•	Hydroxyl radical
ORAC	Oxygen radical absorbant capacity
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TIC	Total ion chromatogram
TPTZ	Tripyridyltriazine
TRAP	Total peroxyl radical-trapping antioxidant

Chapter 1

Introduction

The reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot-}$) hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) are highly reactive molecules and have been the subject of much research interest in recent years (Sies, 1997; Arouma, 1998; Valko *et al.*, 2004). They are produced during cells biological processes *in vivo*, some of these ROS play positive roles in cell physiology; however, they may also cause great damage to cells as well organisms (Valko *et al.*, 2007). The role of free radical mediated oxidative stress toxicity has been considered to be one of the widely accepted mechanisms in the development of aging and various human diseases including liver pathophysiological condition (Halliwell, 2007). Under normal physiological condition generation of free radicals is unavoidable process and suggested to play some beneficial role in physiological function (Valko *et al.*, 2007). However, altered cellular metabolism and external factors which include radiation, diet, smoking and drugs/toxins enhance the production of excessive toxic free radical inside our body (Ames *et al.*, 1993; Forsberg, 2001). Our innate antioxidant defense system becomes helpless to neutralize the toxic free radicals which lead to redox imbalances and oxidative stress mediated damage to DNA, protein and lipid, leading to the development of many human diseases including degenerative diseases (Arouma, 1998; Perry *et al.*, 2006; Negre-Salvayre *et al.*, 2008), carcinogenesis (Andersen *et al.*, 2004), aging (Harman, 1994); cardiovascular diseases (Finkel and Holbrook, 2000), and liver chronic diseases (Cessarato *et al.*, 2004; Manibusan, 2007).

Furthermore, fats and oils are easily deteriorated by oxidation. The addition of antioxidant to food is found to be an effective way to prevent the development of various off-flavours and undesirable compounds that result in lipid peroxidation (Lovell *et al.*, 1995; Benzie, 1996). Besides, there are several drugs and toxins, which are reported to inflict damage to most of organ in our body. Liver is a vital organ playing pivotal role in detoxification and metabolic control of many toxins which are further excreted out of the body (Jaeschke *et al.*, 2002). In due course of such detoxification process, liver itself faces a load of free radicals that are generated from various sources like detoxification system, antioxidant enzymes and immune system (Britton and Bacon, 1994; Lykkesfeldt *et al.*, 2007). Several attempts are made to study on carbon tetrachloride (CCl_4) intoxicated rat liver toxicity model, because CCl_4 is a potent hepatotoxin and it leads to hepatic oxidative stress toxicity and liver damage (Recknagel *et al.*, 1989; Weber *et al.*, 2003; Manibusan *et al.*, 2007), which is reported to show great similarity with most of the chronic liver diseases (Cesaratto *et al.*, 2004). The role of CYP2E1 enzyme in the biotransformation of CCl_4 to more toxic free radical and their subsequent inflicting pathway to create free radical mediated damage to DNA, protein and lipid is manifested from several reports (Poli and Parola, 1997; Ingelman-Sundberg, 1988; Neve and Ingelman-Sundberg, 2008). In view of the above implications and importance of liver as an organ, there is a need to protect it from oxidative stress toxicity by external supply of antioxidative agents, when innate antioxidant defense is not able to cope up with excessive free radical generation and subsequent oxidative stress damage (Halliwell, 1997; Serafini, 2000; Valko *et al.*, 2007). The preventive role of antioxidants has been suggested to be one of the remedial alternatives against oxidative stress mediated several diseases

including liver disorders (Halliwell, 1999; Rice-Evans, 2000; Dragland, 2003; Prior, 2003; Proestos *et al.*, 2008).

Therefore, antioxidant with free radicals scavenging could have much relevance as prophylactic and therapeutic agents in diseases in which oxidants or free radicals are implicated. A number of synthetic antioxidants such as BHA (Butylated hydroxy anisole) and BHT (Butylated hydroxy toluene) have been developed but their use has been questioned because of their toxicity (Barlow, 1990; Rice-Evans, 1995). Consequently, there is considerable interest in preventive medicine and food industry in the development of natural antioxidants.

Among several sources of antioxidant and hepatoprotective agents, the importance of plants as a natural source have been well established due to its diversity and availability of wide array of natural compounds (Halvorsen *et al.*, 2002; Vitaglione *et al.*, 2004). These are found to be safer, reliable and compatible with human diet without any side effects as often reported from use of synthetic antioxidants. Therefore, search for natural antioxidants showing hepatoprotective role in liver oxidative stress toxicity has been gaining momentum and considered to be the thrust areas in biomedical sciences globally (Abalea *et al.*, 1999; Seef *et al.*, 2001; Lee *et al.*, 2008).

India due to its geographical location and climatic conditions are blessed with a widely diversified plant flora and are endowed with diversified classes of phytochemicals, which has been demonstrated to deliver preventive role in many traditional a well as therapeutic medicine (Dahanukar *et al.*, 2000). It is more likely that some of these may be valuable source of natural antioxidants. There are several reports of evaluation of antioxidant potential of various food and medicinal plants in India (Scartezzini and Speroni, 2001; Govindarajan *et al.*, 2005; Koleckar *et al.*, 2008,

Ali *et al.*, 2008) and many Indian medicinal plants are considered as potential sources of antioxidant compounds such as *Terminalia chebula*, *Terminalia bellerica*, *Terminalia muelleri* and *Phyllantthus emblica*. However, still a vast majority of dietary and medicinal plants endowed with antioxidant potential which are yet to be explored and in some cases it is reported but either it is not scientifically documented and validated or their active constituents are yet to be identified. Therefore, in the present work an attempt has been made to search a potential source of antioxidant and identify its active constituents.

In view of above scientific needs and mentioned problems, we have focused on the following objectives.

1. Screening of various Indian medicinal plants to find out most suitable source of antioxidant activity.
2. Extraction and bioactivity guided fractionation of active antioxidant constituents from *A. marmelos* and *E. hirta* employing liquid-liquid partitioning, TLC, column chromatography and suitable *in vitro* antioxidant assays.
3. Identification of components in active fraction from *A. marmelos* and *E. hirta* using spectrophotometric, HPLC, GC-MS and ESI-MS analytical techniques.
4. *In vitro* antioxidant activity determination and evaluation of protective potential against oxidative damage to biomolecules including DNA, protein and lipid using biochemical and molecular biology based assays.
5. To evaluate hepatoprotective role against CCl_4 intoxicated liver oxidative stress model employing biochemical, histological and molecular biology techniques.

Review Literature

2.1 Oxidative stress and human diseases

Life processes, especially under stress conditions, generate a wide range of potentially damaging by-products as well as useful metabolites. Oxidants produced in this way, often free radicals, can cause extensive damage to DNA, proteins and lipids (Halliwell, 2007; Valko, 2007; Pham-Huy *et al.,* 2008). Oxidative stress can therefore occur when the production of free radicals increases, when quenching of free radicals or repair of damaged macromolecules decreases, or when these changes occur simultaneously. Oxidative stress is defined as the process which helps to create disturbances disturbance in the pro-oxidant-antioxidant balance in favour of the former and leading to potential damage (Sies, 1997; Finkel and Holbrook, 2000; Davies and Pryor, 2005). This imbalance can be an effect of depletion of endogenous antioxidants, low dietary intake of antioxidants and/or increased formation of free radicals and other reactive species. Processes associated with oxidative stress can result in an elevated level of oxidatively modified or toxic molecules that can cause cellular malfunction and death (David *et al.,* 2000; Kohen and Nyaska, 2002; Andersen, 2004; Carnelio *et al.,* 2007). Oxidative stress and destruction caused by radicals have been studied coupling to many diseases including aging (Harman, 1994) and neurodegenerative diseases, such as Parkinson and Alzheimer, diabetes (Perry *et al.,* 2006), cardiovascular diseases (Finkel and Holbrook, 2000) and liver chronic diseases (Cessarato *et al.,* 2004; Manibusan *et al.,* 2007).

2.2 Liver disease and oxidative stress

The liver is one of the body's most vital organs, facilitating hundreds of functions. The liver is subjected to toxic injury more often than any other organ (Ljubuncic *et al.*, 2000; Adams and Linder, 2007; Abd Ellah *et al.*, 2007). Thus, the liver is exposed to all ingested substances that are absorbed into the portal blood. Its complexity makes it vulnerable to almost as many different diseases. As our most important detoxifying organ, it cleanses the blood of toxins either ingested or produced by the body itself (Lee and Senior, 2005; Bleibel *et al.*, 2007). When the liver is not functioning optimally, the body begins to store toxins in the tissues leading to altered physiological functions. Drug-induced hepatotoxicity has now become a significant cause of acute liver failure, accounting for 50% of cases. It has been concluded that oxidant stress contributes to the deterioration of the liver disease (Shimizu *et al.*, 2001; Cullen, 2005; Tanikawa and Torimura, 2006). The evidence is clear that oxygen and other toxic free radical species resulting from oxidative stress occur in chronic liver disease and contribute to liver damage in various common types of chronic liver disorders (Watkins and Seeff , 2006; Jones and Czaza, 2008).

2.2.1 Free radical mechanism in CCl_4 toxicity

Carbon tetrachloride induces functional and morphological changes in the cell membrane hydropic degeneration, centrilobular necrosis, fatty changes, cirrhosis and hepatoma (Slater, 1984; Castro *et al.*, 1997). The centrilobular region of the hepatic lobule is by far the most common site of acute carbon tetrachloride toxic injury (Wilson, 1988). The oxidative stress mediated ROS generations during CCl_4 intoxication in the liver are represented by mitochondria and cytochrome P450 system in hepatocytes, Kupffer cells and neutrophils. Reactive

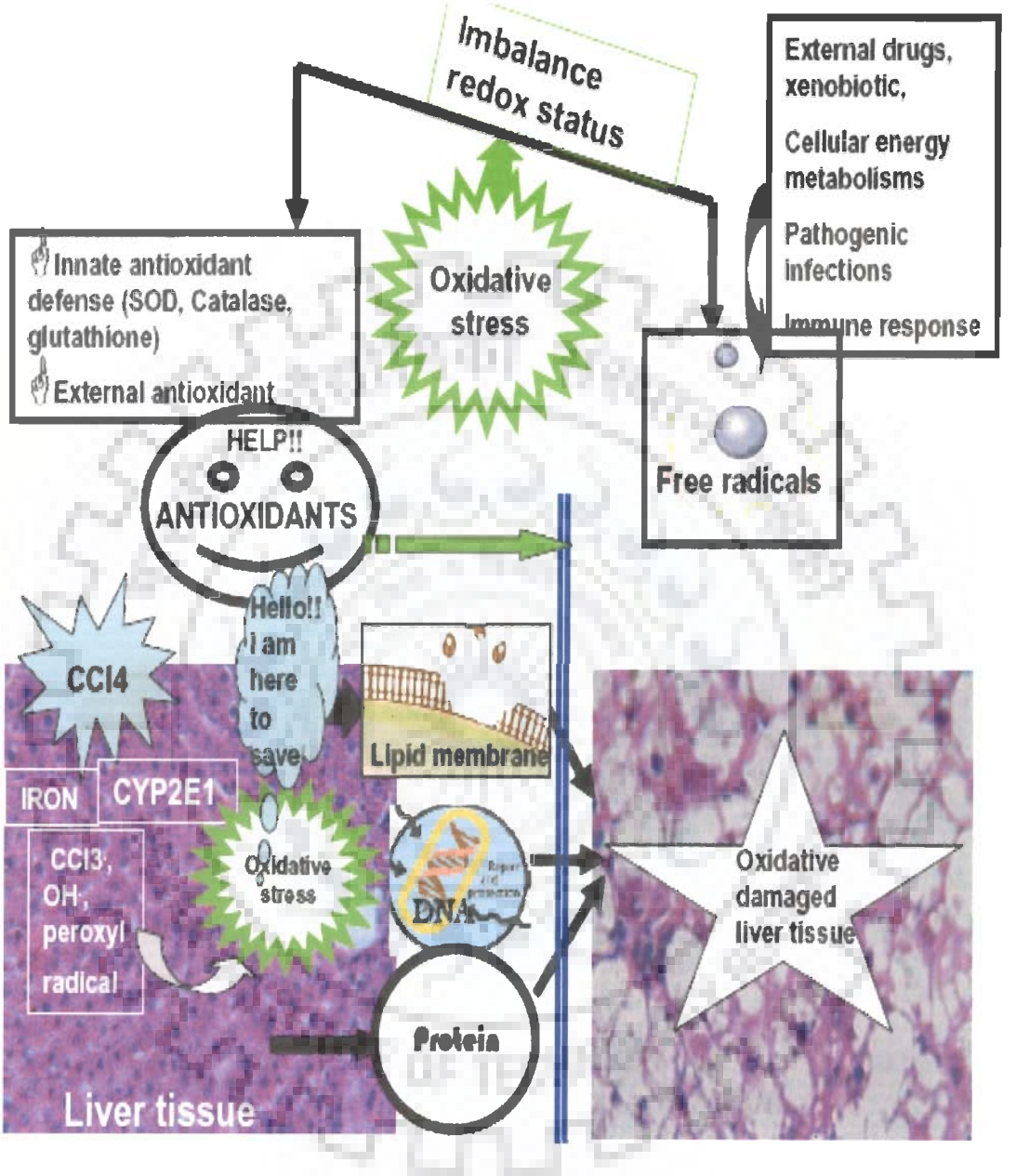

Fig 2.1 Schematic diagram for free radical mediated oxidative stress toxicity induced by CCl_4 in rat liver model system.

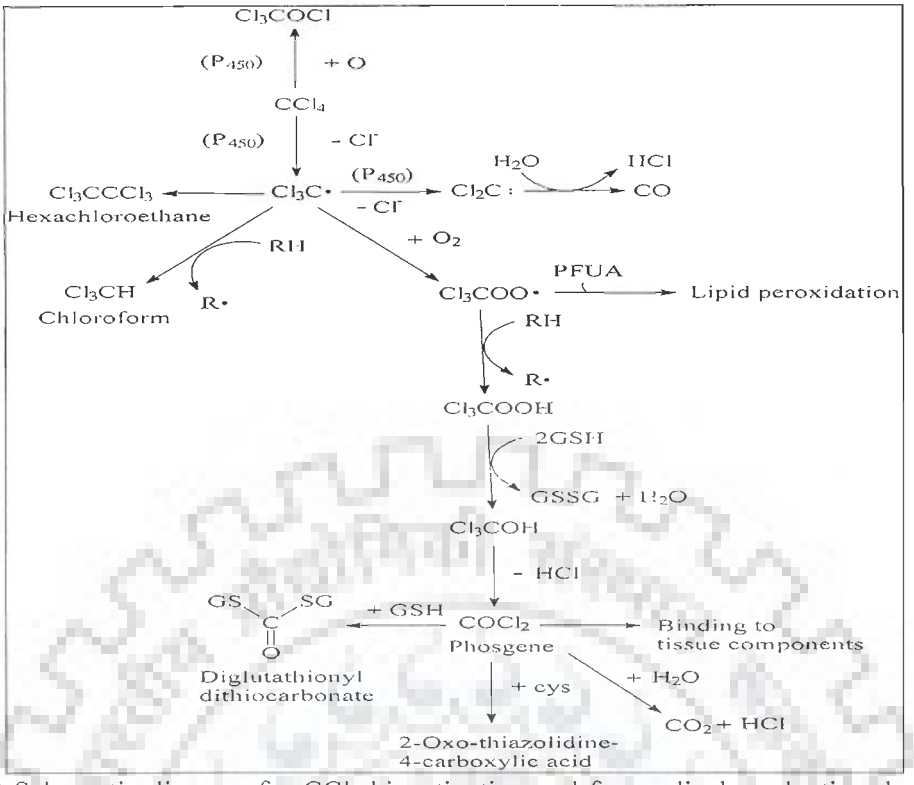

Fig 2.2 Schematic diagram for CCl_4 bioactivation and free radical production during liver toxicity (Jaschke, 2004).

oxygen species (ROS) cause extensive damage to DNA, proteins and lipids and have been implicated in the initiation of various liver pathological processes, such as fibrogenesis, cirrhosis and steatosis (Poli and Parola, 1997; Cesaratto *et al,* 2004). The role of oxidative stress and inflammation in hepatic disorders has been well established (Cesaratto *et al.,* 2004) and changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis (Cullen *et al.,* 2005; Halliwell, 2007).

Carbon tetrachloride (CCl_4) is widely used as a hepatotoxic compound for screening the anti-hepatotoxic/hepatoprotective activity of drugs in experimental model systems, because CCl_4 induced hepatotoxicity is regarded as an analogue of hepatotoxic effects such as hepatic cirrhosis, fatty degeneration, fibrosis, hepatocellular death and carcinogenicity in liver diseases (Recknagel and Glende, 1973; Slater, 1984; Recknagel *et al.,* 1989; Lee *et al.,* 2004). The leading theory for

the mechanism of cellular damage caused by CCl_4 is that the compound is bioactivated by the P450 enzyme system (CYP2E1) in smooth endoplasmic reticulum to trichloromethyl free radical (Recknagel, 1967; Slater, 1984). Once the trichloromethyl radical is formed, it reacts with molecular oxygen to form the highly toxic trichloromethyl peroxy radical (Slater, 1984; Albano *et al.*, 1987; Packer *et al.*, 1978). This free radical can react with sulfhydryl groups, such as glutathione (GSH) and thiol-groups in the protein side chain. Also it covalently binds with cell proteins, and then initiates the lipid peroxidation process in the cellular membrane, which eventually leads to various liver pathological processes (Williams and Burk, 1990). The free radicals accumulation inside liver inflicts on fatty acids of membrane lipids to propagate a chain reaction leading to lipid peroxidation. These chains of events result in the breakdown of membrane structure and disrupting cell energy processes and protein synthesis (Recknagel *et al.*, 1989). The schematic diagram for free radical injury during CCl_4 intoxication is presented in Fig 2.1 and Fig 2.2. The elevation of MDA levels, which is one of the end products of lipid peroxidation in the liver tissue and the reduction of hepatic GSH levels are important indicators of generation of oxidative stress condition in CCl_4 intoxicated rats. CCl_4 is an extensively used xenobiotic to induce lipid peroxidation and toxicity (Parola and Robin, 2000). It is widely accepted view that CCl_4 induced damage also produces alteration in the antioxidant status of the tissues, which is manifested by abnormal level of antioxidant marker enzymes. It has been suggested that during CCl_4 hepatotoxicity, lipid peroxidation seems to be at least one of its toxic principles. The further insights implies that Fe^{2+} ions play a role as mediators of CCl_4 induced hepatotoxicity due to their ability to produce free radicals *in vivo* and *in vitro* condition Iron is a potent catalyst of oxidative stress and may act synergistically with other promoters of lipid

peroxidation by catalyzing these reactions. Iron overload can also directly cause lipid peroxidation, and one of the subsequent products, malondialdehyde, has been shown to activate oxidative stress liver injury (Wilson, 1988).

Previous studies on the mechanism of CCl_4-induced hepatotoxicity reported that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl_4 and that liver necrosis begins when the GSH stores are depleted (William and Burk, 1990). It has been observed that none of these oxidative stress mediated damage processes *per se* is considered the ultimate cause of CCl_4 induced cell death; it is by cooperation that they achieve a fatal outcome (Manibusan, 2007; Jones and Czaza, 2008).

2.2.2 Role of CYP2E1 enzyme in CCl_4 induced hepatotoxicity

Of the multiple forms of P450s present in liver endoplasmic reticulum, cytochrome P450 2E1 (CYP2E1) has been implicated as a key metabolizing enzyme for CCl_4 bioactivation and CCl_4-mediated liver injury in animal studies with CYP2E1 inducers. The enhancement of CCl_4 induced hepatotoxicity by ethanol pretreatment suggests the important role played by CYP2E1 as it is the P450 isozyme that is ethanol inducible and able to metabolize many small hepatotoxic and carcinogenic compounds (Neve and Ingelman-Sundberg, 2008). It is well known today that the inhibition of CYP2E1 decreases CCl_4 hepatotoxicity. On the other hand, the induction of this cytochrome increases the drug's hepatotoxicity (Weber *et al.*, 2003). The increased expression of the cytochrome P-450(CYP)2E1 during CCl_4 intoxication oxidative stress enhances generation of superoxide, hydroxyl, and hydroxyethyl radicals, and peroxisomal β-oxidation of free fatty acids, which generates hydrogen peroxide. In hepatic steatosis, excess triglyceride levels may be present as substrate for peroxidation. Hepatic lipid peroxidation may lead to formation of cytotoxic

intermediates, which may cause cellular injury directly or by initiating an inflammatory response (Jaschke, 2004).

2.3 Free radical generation and their mechanism

2.3.1 The role of free radicals and their generation

Free radicals are highly reactive substances formed in the body's cells as a result of metabolic processes. A free radical is a molecule with one or more unpaired electrons in its outer orbital. Many of these molecular species are oxygen (and sometimes nitrogen) centered. Oxygen free radicals and its non radical products are associated with reactive oxygen species (Mc Cord, 2000). Free radicals are unstable, highly reactive molecules characterized by the presence of unpaired electrons in their outermost shells (David *et al.*, 2000).

Free radicals and various reactive oxygen species are continuously produced in the body (Ames *et al.*, 1993; Halliwell, 1999; Valko *et al.*, 2006). They can be formed as a by-product in the mitochondrial respiratory chain due to leakage of electrons from the electron transport chain (Pryor *et al.*, 2003), by reactions catalyzed by transition metal ions such as iron and copper ions, by an array of enzymatic system like CYP-450, xanthine oxidase, lipoxygenase etc. They may also be derived from external sources such as cigarette smoke, radiation, UV light, pollution and from the metabolism of certain drugs. Free radicals are not only produced as an unwanted product; they are also formed deliberately in the body for useful purposes and have important physiological functions (Arouma, 1998). Free radicals are not always harmful metabolic byproducts; when tightly regulated, ROS can act as intracellular signaling molecules and activation of phagocytic cells (neutrophils, monocytes, macrophages and eosinophils) produce superoxide anion radicals and hydrogen

peroxide as one mechanism to kill bacteria and fungi and to inactivate viruses (Sies, 1999).

Xanthine oxidase is a molybdenum and iron containing hydroxylating enzyme involved in the degradation of purine like nucleotides. This enzyme catalyzes the reaction of hypoxanthine to xanthine, forming superoxide anions, and of xanthine to uric acid, forming hydrogen peroxide (Jaschke, 2002). Cytochrome P450 enzymes metabolize a variety of substrates such as fatty acids, cholesterol or bile acids and exogenous compounds like drugs (e.g. acetaminophen, CCl_4) and alcohol. The underlying biochemical reactions consume oxygen whereby small amounts of ROS are generated. One type of cytochrome molecule that is especially active in producing ROS is known as CYP2E1, which is ethanol inducible and involved in biotrasformation of many drugs and toxin to more toxic substances (Cessarato *et al.*, 2004). Peroxisomes under physiologic condition produce hydrogen peroxide and not superoxide anions. Peroxisomal oxidation of fatty acids has recently been recognized as a potentially important source of hydrogen peroxide production during prolonged starvation. Macrophages and neutrophils contain a group of enzymes called the NADPH oxidase complex (Mc Cord, 2000). Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of ROS, including superoxide anions, nitric oxide and hydrogen peroxide ROS can also be produced by exogenous substances, including environmental toxins, xenobiotics, radiation, ultraviolet light, metal ions and barbiturates. The mitochondrial respiratory chain, located in the inner membrane, is a powerful source of ROS (Halliwell, 2007).

Superoxide ($O_2^{\cdot-}$) is generated by multiple enzymatic and non-enzymatic pathways and is often at the start of the oxidative stress cascade. A major source is via the cellular electron transport chains, such as those of mitochondria, chloroplasts and

the endoplasmic reticulum (Halliwell and Gutteridge, 2000). Superoxide anions are generated enzymatically by a number of oxidases, such as xanthine oxidase and the oxidase that is found in the plasmalemma of phagocytic cells. Superoxide anion radical $O_2^{\cdot-}$ can be formed by one electron transfer or by one electron oxidation of hydrogen peroxide (Rielski, 1978). It is less reactive than hydroxyl radical but potent damaging due to its ability to diffuse and reach to possible target. Superoxide anion itself is not a strong oxidant. Superoxide anion reacts with protons in water solution to form hydrogen peroxide, which can serve as a substrate for the generation of hydroxyl radicals and singlet oxygen. The superoxide anion can react with nitric oxide ($NO^{\cdot}$) and form peroxynitrite ($ONOO^{\cdot}$).

The hydroxyl radical OH• radical is probably the most reactive of the ROS species as it will react with almost all molecules in living cells (Fridovich, 1993). Hydroxyl radicals are short-lived and can be formed from $O^{2-\cdot}$ and H_2O_2 through the Haber-Weiss reaction or through the interaction of metals such as iron or copper and H_2O_2, through the Fenton reaction (Halliwell and Gutteridge, 2000). It has been reported that iron participates in the Fenton reaction, in which the very reactive hydroxyl radical is generated. It can be generated in the course of ionizing radiation on water (Reley, 1994) and also by reduction of H_2O_2 by metal ions as Fe^{2+} and Cu^{2+} or due to xanthine oxidase and NADPH oxidase activity. The hydroxyl radical has been implicated in damage to proteins, carbohydrates, DNA, and lipids (Volterra *et al.*, 1994; Reiter *et al.*, 1995; Dawson and Dawson, 1996).

Hydrogen peroxide is considered as key oxygen centered non-radical because of its high relatively high stability, diffusion and involvement in cell signaling cascades. It can be firmed by direct two-electron reduction of molecular oxygen, or by one electron reduction of superoxide anion (Chance *et al.,* 1979). Toxic potency of

H_2O_2 can be enhanced by Haber-Weiss that involves the direct reduction of H_2O_2 by $O_2^{\cdot-}$ with the formation of hydroxyl radical and hydroxyl ion. Monomeric oxidases located in the outer mitochondrial membrane such as amino acid oxidase and xanthine oxidase also produce H_2O_2 from superoxide anion (Mates and Sanchez, 2000). Hydrogen peroxide is highly diffusible and crosses the plasma membrane easily. Once generated H_2O_2 is metabolized by catalase or glutathione peroxidase to generate water and oxygen. Although H_2O_2 is the least reactive molecule among reactive oxygen species it is very damaging because it can be converted to hydroxyl radical. In the presence of metals such as Fe^{2+} or Cu^{2+}, H_2O_2 can generate hydroxyl radical through Fenton type reactions in which Fe^{2+} is oxidized to Fe^{3+} and H_2O_2 is converted to OH• and OH- species (Ames, 1993).

Peroxyl radicals (ROO•) are formed by a direct reaction of oxygen with alkyl radicals (R•), for example, the reaction between lipid radicals and oxygen. Decomposition of alkyl peroxides (ROOH) also results in peroxyl (ROO•) and alkoxyl (RO•) radicals. Peroxyl and alkoxyl radicals are good oxidizing agents which are involved in the propagation stage of lipid peroxidation.

Nitric oxide (NO•) is formed *in vivo* from L-arginine by nitric oxide synthase. Nitric oxide itself is not a very reactive free radical, but overproduction of NO can lead to protein oxidation in ischemia/reperfusion and neurodegenerative and chronic inflammatory diseases. Nitric dioxide (NO_2•) is formed from the reaction of peroxyl radical and NO, polluted air and smoking.

The interaction of NO with superoxide radical leads to formation of peroxynitrite ($ONOO^-$), a reaction that occurs at a threefold faster rate than that of the dismutation of superoxide by SOD (Beckman *et al.*, 1993). Therefore the formation of peroxynitrite depends on the concentration of superoxide and NO in the cell. At

physiological pH, peroxynitrite may be able to diffuse over several cell diameters to produce cell damage by oxidising lipids, proteins and DNA. Peroxynitrite can be generated by the reaction of NO• and superoxide anion Peroxynitrite is a cytotoxic species and causes tissue injury and oxidizes LDL. Peroxynitrite ($OONO^-$) can cause direct protein oxidation and DNA base oxidation.

2.3.2 The role of free radical in oxidative damage to biomolecules

The free radicals have been suggested to induce oxidative damage to biomolecules, such as some proteins, DNA, PUFA, nucleic acid, and almost any biological molecule it touches, and this damage causes aging, cancer and several diseases (Imlay and Linn, 1988; Aruoma, 1998; Aviram, 2000; Anderson, 2004; Dalle-Donne *et al.*, 2006). The degree of oxidative damage suffered by an organism, tissue or organ may be evaluated by the measurement of a number of molecules which are indexes of oxidative stress. Carbohydrates and proteins also suffer an oxidative attack. Carbonyl groups in proteins are excellent indicators of oxidative damage to these molecules (Aruoma, 1998; Aviram, 2000; Anderson, 2004, Halliwell, 2007)

2.3.2.1 Oxidative damage to protein

Proteins can be oxidatively modified in three ways: oxidative modification of specific amino acid, free radical mediated peptide cleavage, formation of protein cross linkage due to reaction with lipid oxidation product, formation of protein carbonyl and loss of sulfahydral groups (Stadman, 2001; Levine and Stadman, 2001). Protein containing aminoacids such as methionine, cystein, arginine and histidine seem to be the most vulnerable to oxidation (Freeman and Crapo, 1982). Moreover, oxidatively modified proteins may contain very reactive chemical groups that could contribute to secondary damage to other biomolecules (Halliwell, 1997). As a result of free radical exposure, many changes can occur in proteins, including amino acid modification,

fragmentation, aggregation, changes in absorption and fluorescence spectra increases in susceptibility to enzyme proteolysis, adverse effects on heat stability and alteration of signal transduction mechanisms decrease or loss of biological function (Davies, 1987; Dean, 1997). All these modifications can be used as markers of protein damage by free radicals *in vivo* and *in vitro* study.

2.3.2.2 Lipid peroxidation

Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages biological membranes and generates a number of secondary products that possess toxic action to develop diseases condition in many diseases including liver oxidative stress. Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids, i.e. those lipids containing more than two carbon-carbon double covalent bonds (Halliwell, 2007). Several experimental evidences indicate that extensive lipid peroxidation results in loss of membrane integrity, impairment of the function of membrane-transport proteins and ion channels, disruption of cellular ion homeostasis and eventual rupture leading to release of cell and organelle contents such as lysosomal hydrolytic enzymes (Jackson, 1999). Due to lipid peroxidation, a number of compounds are formed for example alkanes, malonaldehyde and isoprotanes. These compounds are used as markers in lipid peroxidation assay and have been verified in many diseases as neurodegenerative diseases, ischemic-reperfusion injury and diabetes (Lovell *et al*, 1995; Sevanian and Ursini, 2000).

2.3.3.3 Oxidative damage to DNA

DNA is crucial molecular target for free radical mediated oxidative damage (Valko *et al.*, 2004). ROS cause extensive base modification as well as single-strand breaks in both mitochondrial and genomic DNA and these are responsible for roughly

an estimated 10,000 DNA base modifications per cell per day (Ames, 1993), which have long been considered as an important underlying event in chronic inflammation leading to mutation, carcinogenesis and various degenerative diseases including Alzheimer's disease, Parkinson's disease (Imlay and Linn, 1988; Cooke *et al.*, 2003). Oxidized nucleotide as glycol, dTG and 8-hydroxy-2`-dexoyguanosine is found to be increased during oxidative damage to DNA under UV radiation or free radical mediated damage. It has been reported that mitochondrial DNA are more susceptible to oxidative damage that have role in many diseases including cancer. Among the four DNA bases, guanine has the lowest oxidation potential and is most easily oxidized. Recently, 8-hydroxy guanosine has generated considerable interest as a biomarker of oxidative stress that can be used to estimate DNA damage in humans.

2.4 Antioxidant defenses and detoxification system in human body

Organisms have evolved sophisticated antioxidant defence systems and repair systems for protection against free radicals and free radical damages at different sites (Jaeschke *et al.*, 2002; Cesaratto *et al.*, 2004). There are a number of antioxidants present in the body and derived from the diet. Based on their location in the body, they can be divided into intracellular enzymatic antioxidant and detoxification system and extracellular antioxidants in the body. The enzymic detoxification of xenobiotics has been classified mainly into Phase I and Phase II detoxification. Phases I and II involve the conversion of lipophilic, non-polar xenobiotics into more water-soluble molecules, which can then be eliminated more easily from the cell (Jones and Czaja, 2008) Phase I processes are catalyzed mainly by the cytochrome P-450 (CYP450) system. In a major detoxification reaction, conjugation to glutathione (GSH), which is catalyzed by the Glutathione S transferases (GSTs), is the major phase II reaction in many species (Neve and Ingelman-Sundberg, 2008).

2.4.1 Enzymatic antioxidant and detoxification system

Superoxide dismutase (SOD) is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) in order to maintain a low concentration of superoxide anion. It catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide (Sohal *et al.*, 1996; Fang *et al.*, 2002).

Catalase is a heme protein that catalyses the detoxification of hydrogen peroxide. Catalase provides a protective role that is similar to that of glutathione peroxidase because both are important means of removing hydrogen peroxide. This iron-containing enzyme is primary found in peroxisomes. It is predominantly concentrated in liver and it detoxifies hydrogen peroxide by catalyzing a reaction between two hydrogen peroxide molecules, resulting in the production of water and oxygen (Jackson, 1999)

Glutathione peroxidase (GPx) is a cytoplasmic and mitochondrial enzyme that is important for detoxifying peroxides in the cell (GSTs are a large group of multifunctional proteins that catalyse the conjugation of GSH to various electrophilic substrates. GSTs appear to play an important role in protecting cells against oxidative damage by: 1) binding glutathione in such a way that the sulfur is induced to ionize more completely, and 2) binding a second molecule close by so that a reaction can be facilitated. This reaction is necessary to detoxify xenobiotic materials such as toxins, drugs, and other foreign compounds (Mc Cord, 2000)

The flavoprotein, glutathione reductase, uses the reducing power for the pentose phosphate pathway (NADPH) to keep the glutathione pool in a cell in a much reduced state. Cells contain at least 100 reduced glutathione molecules for every

molecule of glutathione disulfide. The net result of this cycle is to use NADPH to reduce hydrogen peroxide to water, a process that requires two electrons. Other reductases can also catalyze reactions that reduce lipid peroxides, i.e., LOOH, instead of hydrogen peroxide (Freidovich *et al.*, 1999)

2.4.2 Non-enzymic antioxidant and detoxification agents in the body

2.4.2.1 Glutathione

One of the most interesting parameters to determine oxidative stress is the glutathione redox status. Indeed, a glutathione redox ratio (GSH-GSSG) gives us an indication of the redox state of the cells and thus indicates a global level of oxidation of the whole organism (Sies, 1999, Fang *et al.*, 2001). Glutathione carries out an important number of metabolic functions and one of the most important is protection of cells against oxidants and other xenobiotics. Glutathione is synthesised and degraded by the gamma glutamyl cycle and the liver is the major organ where this peptide is synthesised (Jaschke, 2002).

2.4.2.2 Metal-binding proteins

These proteins ensure that metals (iron and copper) are maintained in a non-reactive state and avoid formation of hydroxyl radicals. Transferrin and lactoferrin bind iron while albumin binds copper (Halliwell, 1999).

2.4.2.3 Hydrogen donating non-enzymatic compound

Those compounds, which can donate hydrogen atoms to the free radicals can scavenge free radicals and prevent lipid peroxidation. These groups of antioxidants include polyphenols, vitamin A, E, C, carotenoids, lipoic acid, glutathione etc. Flavonoids, the most important single polyphenols group are glycosides with a benzopyrene nucleus (Halliiwell, 1997, Fang *et al.*, 2002). The flavonoids including flavones, flavonols and anthocyanins are based on the common structure of carbon

skeleton. Antioxidant mechanism of polyphenolic compounds are based on hydrogen donation abilities and chelating metal ions (Bravo, 1998). Flavonoids have the most potent antioxidant activities because of the chemical structure with o-diphenolic group, a 2,3-double bond and hydroxyl group at position 3,5. Flavonoides can make complexes with metals and effective hydroxyl radical and peroxyl radical scavengers. Polyphenols possess ideal structural chemistry for the radical scavenging activity. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donor, as chain breaking function or ability to chelate metal ions. Phenolics may change the fluidity of membranes, thus could sterically hinder the diffusion of free radical and restrict peroxidation reaction (Rice-Evans, 1995).

2.4.3 *In vitro* methods for antioxidant activity evaluation

It is admitted that antioxidant capacity of plant derived natural antioxidant are contributed due to its inherent structure. On the other hand, the reactivity of potent antioxidants varies for different nature of free radicals available *in vitro* as well as *in vivo* condition (Wang *et al.*, 1998). Differences between the antioxidant potential of selected compounds can be measured using many different techniques. Because most phytochemicals are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems (Frankel and Meyer 2000; Moreno, 2002; McAnalley *et al.*, 2003; Sharma *et al.*, 2007).

ORAC (Oxygen Radical Absorbance Capacity) assay depends upon the unique property of phycoerythrin, which is used as a target of free radical. In this assay, AAPH (2, 2-azobis (2-amidino propane) dihyrochloride is used as a peroxyl radical generator. The ORAC assay combined both inhibition time and inhibition percentage of free radical action by antioxidants using an area under curve technique

for quantification. This assay has been applied for the evaluation of the total antioxidant status in animal tissues and availability status of different compounds like Vit C, Carotenoides and phenolic compounds from plants (Cao and Cutler, 1993).

TEAC (Trolox Equivalent Antioxidant Capacity) assay was first reported (Miller *et al.* 1993). This assay is based on inhibition of the absorbance of radical cation ABTS (2, 2'-azinobis (3-ethyl benzothiazoline, 6-sulphonate) by antioxidants which has characteristic wavelength at 415 nm and secondary absorbance at 660 nm and 734 nm. In this assay, Trolox is used as standard in which results are expressed in terms of Trolox Equivalent Antioxidant Capacity (TEAC) value in terms of Trolox equivalent (Re *et al.*, 1999).

FRAP (Free Radical Absorbance Parameter) assay involves Fe^{3+} as an oxidant and after reduction; it changes to Fe^{2+}. In this assay, there is no prooxidant and regarded as direct test of total antioxidant power of any compound (Benzie and Strain, 1996).

Scavenging of Superoxide ion assay involves the scavenging activity of antioxidant is measured in terms of inhibition of generation of O_2^- with the hypoxanthine-Xanthine oxidase Superoxide generating system. The generated Superoxide radical reduces NBT (Nitroblue tetrazolium) into formazone and we measure it at 560 nm. The added antioxidant mixture reduces the absorbance and inhibits the formazone production (Kakkar *et al.*, (1984).

Hydrogen peroxide scavenging activity is easily measured by using peroxide assay systems (Ruch *et al.*, 1989). The common system is horseradish Peroxidase, which uses H_2O_2 to oxidize scopolitin into a fluorescent compound.

Hydroxyl radical scavenging is often calculated by using the deoxyribose assay. A mixture of $FeCl_3$ and EDTA in the presence of ascorbate reacts to form iron

(II)-EDTA plus oxidized ascorbate, H_2O_2 then reacts with iron (II)-EDTA plus hydroxyl ion, the so called Fenton's reaction. These radicals degrade deoxyribose into a series of fragments, some or all of which on heating with thiobarbituric acid to give a pink chromozone (Halliwell *et al.*, 1987; Halliwell and Gutteridge, 2000). The scavenging activity towards hydroxyl ion of a substance added to reaction to a mixture is measured on the basis of inhibition of the degradation of deoxyribose.

Scavenging of the stable radical 2, 2` diphenyl1-picryl hydroxyl (DPPH) assay is based on the measurement of the scavenging ability of the antioxidant towards stable radical DPPH. The free radical DPPH is reduced to the corresponding hydrazine when it reacts with the hydrogen donors. We follow the colorimetric evaluation by the decrease in absorbance at 515-528 nm (Brand-Williams *et al.*, 1995).

2.5 Natural antioxidants

It is generally concluded that dietary intake of plant-derived phytochemicals from vegetables, fruit, spices, herbs and medicinal plants may contribute to shift the balance toward an adequate antioxidant status and ultimately playing crucial role in the prevention or treatment of many human diseases (Halliwell *et al.*, 1997; Lugasi *et al.*, 2003; Krishnaiah *et al.*, 2007; Pham-Huy *et al.*, 2008). It has been reported and convinced from recent decade scientific contribution that many health implications including anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant as well as free radical scavenging mechanism as part of their role in mitigating menace of such diseases (Perry *et al.*, 2000; Capasso, 2006; Proestos *et al.*, 2008).

2.5.1 Relevance of natural antioxidants in oxidative stress mediated human diseases

Due to depletion of defense system consisting of a network of enzymatic as well as non-enzymatic antioxidants in oxidative stress maladies, consuming or intake of external antioxidants as free radical scavengers may be necessary and preventive measures for such maladies (Halliwell, 1994; Halliwell 1999a; Salonen, 1999). Thus, recent scientific studies have suggested strongly the potential of natural plant products such as vitamin E, vitamin C, β-carotene, curcumin, etc. to serve as antioxidant agents against various diseases induced by free radicals mediation directly or indirectly (Salles *et al.*, 1999; Halliwell 1997).

2.5.2 Relevance of natural antioxidants as hepatoprotective agents

Since, it is established that a correlation exists between hepatotoxicity and oxidative stress. Therefore, the anti-hepatotoxic drugs and antioxidant compounds could be of great help in protection against liver abnormalities (Seef *et al.*, 2006; Lee *et al.*, 2008). The role of plant derived natural antioxidants in protection against liver oxidative damage has been evidenced by several research attempts worldwide as well as in Indian medicinal plant perspectives. To broaden the views and scope of natural antioxidants as hepatoprotective agents, several review articles have been summarized time to time (Guillermo and Moreno-Cuevas *et al.*, 2008). As, natural antioxidants available from plants constitute diverse class, there are several reports on hepatoprotective agents from plant sources having phenolic and glycosidic in nature (Luper, 1998; Xi *et al.*, 2008; Lin et al., 2008).

2.5.3 Benefit of natural plant natural antioxidants over synthetic, microbial and animal origin antioxidants

Many synthetic chemicals like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert. butyl-hydroxyquinone (TBHQ) were earlier recommended as synthetic antioxidants for use in health, food and cosmetic industry (Barlow, 1990). There are dubious concern about the safety of these synthetic compounds in relation to their metabolism and possible absorption and accumulation in human body organs and tissues. There are also reports about serious side effects including the liver toxicity and cancer development (Hayashi *et al.*, 1993; Williams *et al.*, 1999). Plants are especially susceptible to damage by active oxygen (exposed to radiation UV light) this is why plants developed numerous antioxidant defense systems those results in certain numbers of very potent antioxidants. Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases (Pieta, 2000; Laguerre *et al.*, 2007).

2.5.4 Importance of Indian medicinal plant as a source of natural antioxidants and hepatoprotective agents

In the line of worldwide importance given to plant derived natural antioxidants and hepatoprotective agents, there is wide scope to explore Indian dietary and medicinal plant doe their diverse plant flora (Dahanukar *et al.*, 2000). The geographical location, diverse climatic condition and topographical features have been considered to be a major factor for diversity of natural antioxidant classes. In Indian perspectives, these factors are quite compatible and enriching for our innumerable plant diversity. The enthusiastic results obtained from natural antioxidant therapy in several diseases have prompted several research groups in India to explore

our vast reservoir of several therapeutic antioxidant agents in Indian medicinal plant. A number of research attempts have been made to explore the natural antioxidants in the Indian medicinal plant perspectives (Scartezznini and Speroni, 2000). In due course of scientific developments, informative review articles have been summarized to give the scope of diverse nature of natural antioxidant classes present in Indian flora (Sharma, 2004). The potential application of these therapeutic agents in the cure of many human diseases including liver protection have been scientifically validated (Ali *et al.*, 2008; Samarth *et al.*, 2008).

2.5.4.1 Mechanism of action of natural antioxidants

The natural antioxidants may directly act on specific ROS and converts it into less toxic compounds. This process of scavenging is directly through enzymatic reaction (Sies, 1997; Pieta, 2000). The free radical scavenging activity of phenolic compounds may occur non-enzymatically either through donation of hydrogen atom or addition of electrons. Antioxidant may prevent by chelating to transition metals ions such as Fe and Cu. It may act as a chain breaker during the propagation of sequential production of free radical ions. Some antioxidant may act as modulator of proteins which are involved in balance of redox state inside the cell (Halliwell, 1999). Antioxidant may act as inhibitors of the enzymes which act as a source of free radical generation (Cook and Samman, 1996). Antioxidant may modify the gene expression through the ARE (Antioxidant response element) and associated transcription factors like Nrf-1 & Nrf-2 (Halliwell, 1997; Rice-Evans, 1995; Mira, 2002; Havsteen, 2002). On the basis of different mechanisms demonstrated by phenolic antioxidants against toxic free radicals, it has been suggested that these groups of natural antioxidants are highly capable of acting on different nature of reactive oxygen species *in vivo* and *in vitro* condition (Rice-Evans, 2002).

2.5.5 Classification of plant derived natural antioxidants

2.5.5.1 Introduction on phenolic compounds and their structure-activity relationship

Phenolic compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom (Pieta, 2000). Phenolics display a vast variety of structures; here only flavonoids and phenolic acids are reviewed. The structural basis for all flavonoids is the flavone nucleus (2-phenyl-benzopyrane) but, depending on the classification method, the flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar (Middleton, 1996). Important flavonoid structures divided into eleven classes are presented in Fig. 2.3 (Harbonne, 1980). The essential two groups of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids (Fig. 4), both of which are derived from non-phenolic molecules benzoid and cinnamic acid, respectively (Rice-Evans, 2000). This structure shows the tricyclic $C_6.C_3.C_6$ skeleton of Flavanoids. It is worth noting that the phenolic groups are usually found in positions 5 and 7 in ring A and in 3', 4' and 5' in ring B. The different classes of flavanoid have different oxidation states in the central ring C. The flavonols are based on the same skeleton but with a different oxidation state of the centre 'C' ring (flavone rather than flavan). They invariably occur naturally as glycosides (with D-glucose or other sugars). Hydrolysis removes the sugar to give the aglycone. Flavonoids generally consist of two benzene rings (rings A and B) linked by an oxygen-containing heterocycle (ring C) (Fig 2.3). There are six classes of flavonoids including: flavanones, flavones, flavonols (catechins and proanthocyanidins), isoflavonoids, anthocyanins, and flavans, which vary in structure (Middle). The antioxidant properties of flavonoids have been well studied and are structure-

dependent with the major contributing factor being the catechol structure at ring B (two adjacent hydroxyl groups at 3′,4′-positions) (Pieta, 2000).

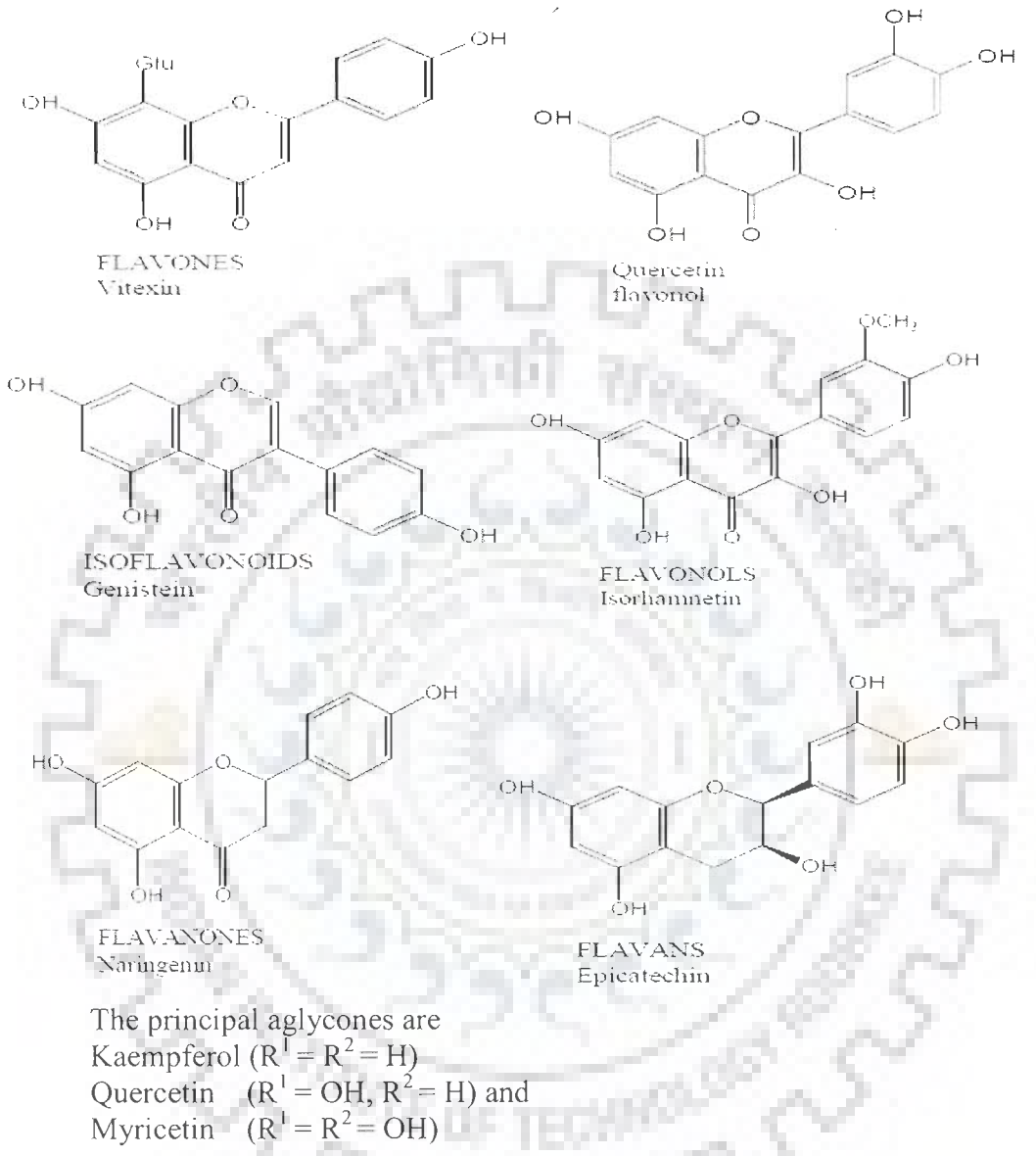

Fig 2.3 Different classes of flavonoids with their skeleton structure. Source (Harborne, 1980)

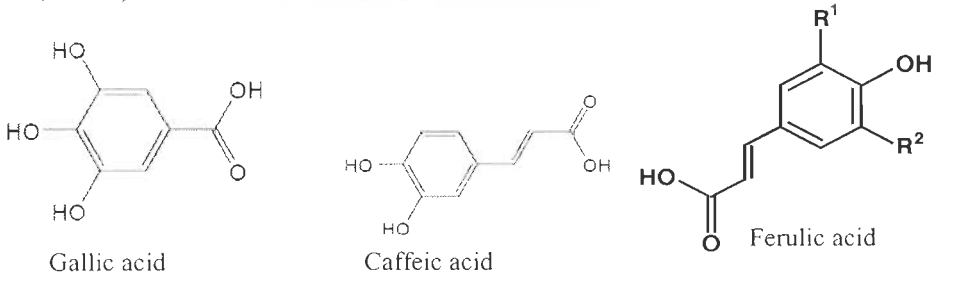

Fig 2.4 Hydroxy benzoic acid and cinnamic acid derivative phenolic acid structure. Source (Harborne, 1980)

Hydroxyl substituents on the flavonoid ring increase the antioxidant activity, while substitution by methoxy groups decreases this activity. The mechanism of hydrogen donation of quercetin indicate that hydroxy groups on the chromane-like flavonoid of quercetin do not appear to participate directly in oxidation, instead, it is the hydroxy groups of the catechol moiety, the B ring, that donate or accept hydrogen. The antioxidant capacity of a compound is based on its structural features, such as number and position of double bonds, hydroxyl-groups and modification like linkage to sugar-moieties (Rice-Evans *et al.*, 1995). Phenolic acids and their esters have antioxidative activity that is dependent on the number of hydroxyl groups in the molecule. The electron-withdrawing properties of the carboxylate group, which have a negative influence on the H-donating abilities of hydroxy benzoates, can be prevented by steric hindrance (Rice-Evans *et al.*, 1995; Cao *et al.*, 1997). The monohydroxy benzoic acids act as very weak antioxidants: owing to the electronegative potential of a single carboxyl group, only *m*-hydroxy bezoic acid has antioxidative activity. This activity increases considerably in the case of dihydroxy substituted benzoic acids, whose antioxidant response is dependent on the relative positions of the hydroxyl groups in the ring. Gallic acid (3,4,5-trihydroxy benzoic acid) is the most potent antioxidant of all hydroxybenzoic acids (Rice-Evans *et al.*, 1995; Cook and Samman, 1996). Insertion of an ethylenic group between a phenyl ring carrying a hydroxyl group and the carboxylate group, has a highly favourable effect on the reducing properties of the OH group. The total antioxidant activities of hydroxycinnamic acids are higher than those of the respective hydroxybenzoic acids.

2.5.5.2 Relevance of polyphenol as antioxidants and hepatoprotective

From the earlier work, it has been determined that the major contributing constituents of plant products form their antioxidant effect is mainly due to phenolic compounds, such as flavonoids, phenolic acids, tannins and diterpenes etc (Chung *et al.,* 1998; Pietta, 2000; Dimitrios, 2006). The polyphenols isolated from various dietry and medicinal plant sources have potential therapeutic roles in the prevention and treatment of many human diseases related to excessive oxidative stress due to their antioxidant properties (Middleton *et al.*, 1996 Pieta, 2000). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as a source of polyphenol antioxidants in reducing free radical induced oxidative tissue injury. Natural phenolic antioxidants from tea, wine, fruits, vegetables and spices, some natural antioxidant (e.g. rosemary and sage) are already exploited commercially (Mantle *et al.,* 2000). From the earlier work, it has been determined that the major contributing constituents of plant products form their antioxidant effect is mainly due to phenolic compounds, such as flavonoids, phenolic acids, tannins and diterpenes (Cook and Samman, 1996; Schinella *et al.,* 2000). Flavonoids are group of phenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Frankel, 1995). Thus, phenolic compounds from sage (*Salvia officinalis* L.) and rosemary (*Rosmarinus officinalis* L.), in thyme (*Thymus vulgars* L.) were reported as strong antioxidative compounds. Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease. (Cao, 1997; Cook and Samman, 1996). Their metal-chelating capabilities and radical scavenging properties have enabled phenolic compounds to be thought of as effective free radical

scavengers and inhibitors of lipid peroxidation (Miller, 1997; Rice-Evans and Miller, 1997; Terao and Piskula, 1997; Lien *et al.*, 1999).

2.5.6 Glycosides

2.5.6.1 Introduction about glycosides

Glycosides are a group of secondary metabolites where aglycone structure is conjugated with sugar moiety. Mainly they are classified on the basis of nature of aglycone including flavonoids glycosides, sterol glycoside and triterpenoids glycosides. Plant sterols occur also as glycosylated conjugates, in which a carbohydrate is connected with a β-glycosidic bond to the hydroxyl group in C-3 of the sterol. The most common carbohydrate in steryl glycosides is D-glucose, but also conjugation to other carbohydrates like galactose, mannose, xylose and gentiobiose has been reported. The structure of sitosterol glucosides and rutin is presented in Fig. 2.5a and b, respectively.

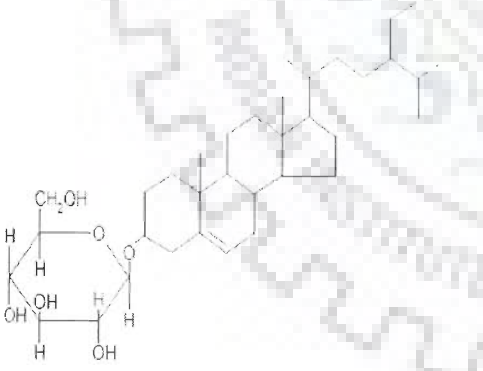

Fig 2.5a. Steryl glycosides

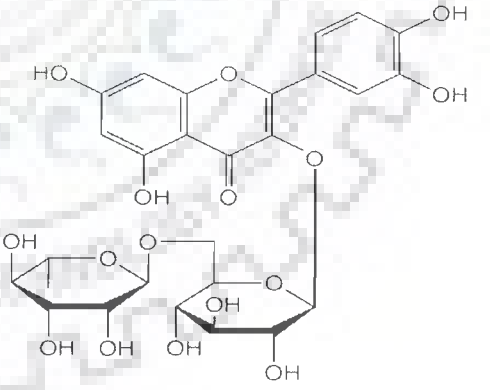

Fig 2.5b. Flavonoids glycosides

2.5.6.2 Role of glycosides as antioxidants and hepatoprotective

The glycoside nature of antioxidant and hepatoprotective agents is reported from different dietary and medicinal plants (Xi *et al.*, 2009; Lin *et al.*, 2008). The major antioxidant glycosides are from flavonoids glycoside and steryl glycoside. This group of glycosides is found in many plants and their antioxidant efficacy has been

evaluated in different *in vitro* model assay to elucidate their mechanism of action towards free radicals. The potential role of glycosidic nature of antioxidants has been explored in many human diseases including protection against liver oxidative damage (Kaur *et al.*, 2006). In Indian medicinal plant perspectives, reports are available giving scientific validation about the potential role as antioxidants (Kitts, 2000; Govindrajan *et al.*, 2005; Kang *et al.*, 2007).

2.7 Experimental plant *Euphorbia hirta*

2.7.1 About *E. hirta* and their use in traditional medicine

Euphorbia hirta L is a tree belonging to the family Euphorbiaceae; common names include Euphorbia, (Pill bearing spurge *E. pilulifera*) Asthma Weed and Catshair. This small annual herb grows, widely in most parts of India and in tropical countries, especially on roadsides and on wasteland (Kirtikar and Basu, 1963). Flowers are yellow and fragrant. Though the plant can flower through out the year, October is the main flowering period for this plant. The small green flowers constitute the inflorescence characteristic of the Euphorbias. The stem and leaves produce white or milky juice when cut (Lind and Tallantire, 1971). The leaves of *Euphorbia hirta* have been used in Indian traditional medicine such as anti-asthmatic, anti-helmetic, wound healing, antibacterial, antioxidant and diuretics, gastrointestinal disorders (diarrhea, dysentery, intestinal parasitosis etc.) and bronchial and respiratory diseases (asthma, bronchitis, hay fever etc. (Anon, 2005, Mhaskar *et al.*, 2000).

2.7.2 Phytochemistry of *Euphorbia hirta* Linn

A number of compounds have been isolated from *E. hirta* and chemically characterized (Lanhers *et al.*, 1990). These include cycloarternol, 24-methylene-cycloarternol, b-sitosterol, euphorbol hexacozonate, mb-amyrin acetate, 1-hexacosanol, ingeno-triacetate, tinyaloxin, campesterol, stigmasterol and quercitin

(Gupta and Garg, 1966). Several reports on phytochemistry of *E. hirta* showed the presence of flavonoids (Blanc and De Saqui-Sannes, 1972) and phenolic acids, saponin, and amino acids (Chen, 1991).

2.7.3 Ethnopharmacology of *Euphorbia hirta* Linn

An ethnopharmacological study of *Euphorbia hirta* L. (Euphorbiaceae) was undertaken earlier to evaluate its pharmacological properties (Lanhers, 1988). Whole plant or leaf extracts obtained from *Euphorbia hirta* is known to have a multitude of actions in the biological system. Several pharmacological properties of *Euphorbia hirta* L. are reported such as; diuretic (Johnson *et al.*, 1999), gastrointestinal motility (Hore *et al.*, 2006), sedative, anxiolytic analgesic, antipyretic, anti-inflammatory (Lanhers *et al.*, 1990; 1991) and anti-allergic reaction property (Singh *et al.*, 2006).

2.8 Experimental plant *Aegle marmelos*

2.8.1 About *Aegle marmelos* and their use in traditional medicine

The plant *Aegle marmelos* Corr. (AM) belongs to the family Rutaceae and is known as vilvam in Tamil, bael in Hindi, bilwa or sripal in Sanskrit and bael tree in English (Nadkarni, 1986). *Aegle marmelos* (Correa) L., (Rutaceae) has been widely documented in Indian traditional medicine for different medicinal purposes like pain treatment, fever, inflammation, respiratory disorders, cardiac disorders, dysentery and diarrhea. A decoction of plant leaves and fruit is used in remedies for dysentery, diarrhea, upper respiratory tract infections and heart ailments. The leaves of *Bael* are astringent, a laxative, and an expectorant and are useful in treatment of ophthalmia, deafness, inflammations, cataract, diabetes, diarrhoea, dysentery, heart palpitation, and asthmatic complications (Kirtikar and Basu, 1993). Fresh aqueous and alcoholic leaf extracts of *Aegle marmelos* were reported to have a cardio tonic effects in mammals (Haravey, 1968).

2.8.2 Reports on phytochemistry of *A. marmelos*

A number of phytochemical studies on *A. marmelos* showed the presence of coumarins, alkaloids. The chemical literature survey of *A. marmelos* showed the presence of a number of coumarins, alkaloids, lignan glucosides, triterpenoids, sterols, carbohydrates, anthraquinones and lactones (Karawya et al., 1980; Sondhi *et al.*, 2008).

2.8.3 Ethnopharmacology of *A. marmelos*.

A number of scientific attempts on the evaluation for therapeutic uses like anti-genotoxicity effects (Sondhi *et al.*, 2008), hypoglycemic, hypolipidemic, anti-hyperglycemic effects (Sharma *et al.*, 2007), anti-inflammatory, anticancer properties of leaves of *Aegle marmelos* (Arul *et al.*, 2005), anti-diarrheacal (Mazumder *et al.*, 2006) and chemoprotective activity (Shankarananth *et al.*, 2007).

Screening, Fractionation and Identification of Antioxidant Constituents

3.1 Introduction

The human body has certain antioxidant defense mechanisms to combat and reduce free radical mediated oxidative damage. In several cases of epidemiological evidence including liver disorders indicate that under excessive generation of free radicals inside body, these defense networks is not able to cope up with damaging acts of free radicals (Sies, 1997; Halliwell, 2007). The role of external supply of antioxidants has been widely considered one of promising approach in the prevention and treatment of many oxidative stress mediated human diseases including liver pathophysiological conditions (Serafini, 2000; Shen *et al.*, 2007; Shahidi, 2008). Among available source of external antioxidant agents, dietry and medicinal plant derived antioxidant agents are gaining edge over other source like synthetic, microbial and animal origin (Halliwell, 1997; Fogden and Neuberger, 2003; Krishnaih *et al.*, 2007).

In search of natural antioxidants from plant sources, several research groups have reported attempts to screen and evaluate potent source of antioxidant agent from available plant sources from many part of world (Wang *et al.*, 200; Mantle *et al.*, 2000). In Indian scenario with compatible geographical location and climatic condition, plant flora are widely diversified and endowed with several classes of phytochemicals, which has been demonstrated to deliver preventive role in many traditional as well as therapeutic medicine. In the present work, there is a need to assess different dietry and medicinal plants for their antioxidant activity on the basis

of reliable *in vitro* based assays (Moreno, 2002). While crude isolates and extracts from plants containing glycosides and phenolic compounds have been utilized in the investigation of evaluation for antioxidant activity few years ago, but developments in the isolation/purification and characterization techniques have enabled the investigation of the bioactivity of well characterized glycosides and phenolic compounds leading to the emergence of structure and bioactivity relationships (Bravo, 1998; Rice-Evans, 2000). In the present work, *E. hirta* and *A. marmelos* are selected for their strong antioxidant activity among twenty screened Indian dietry and medicinal plants. In our Indian traditional medicinal medicine, use of *E. hirta* and *A. marmelos* is widely acknowledged in physiological disorders and several scientific evidences are also reported in support of therapeutic use. A number of compounds have been isolated from *E. hirta* and chemically characterized such as cycloarternol, 24-methylene-cycloarternol, euphorbol, hexacozonate, mb-amyrin acetate, 1-hexacosanol, ingeno-triacetate, tinyaloxin, campesterol, stigmasterol and quercitin (Gupta and Garg, 1966; Lanhers *et al.*, 1987). The chemical literature survey of *A. marmelos* showed the presence of a number of coumarins, alkaloids, lignan glucosides, triterpenoids, sterols, carbohydrates, anthraquinones and lactones (Karawya *et al.*, 1980; Sondhi *et al.*, 2008). The diverse physicochemical and biological properties of glycosides and phenolic compounds have been successfully exploited in a number of therapeutic applications in many physiological disorders. The extraction and fractionation of the different plant extract yield fractions with differing chemical and biological attributes towards neutralization of free radicals. Consequently, there have been significant advances in the development of various analytical tools to ascertain the actual active fraction responsible for the desired

biological activities as in the present goal include antioxidant and hepatoprotective activity.

In the present attempts, preliminary screening for antioxidant activity was carried out among twenty Indian dietry and medicinal plants. Bioactivity guided extraction, fractionation and identification of active fraction from two selected *A. marmelos* and *E. hirta* plant was investigated.

3.2 Material and Methods

3.2.1 Chemicals and plant materials

Chemicals

All common chemicals and solvents used in the present study were of analytical grade with highest purity and purchased from SRL, S.D Fine, Himedia and MERCK India Ltd. The specific chemicals like Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid), $ABTS^{*+}$ (2, 2'-azinobis 3-ethyl benzothiazoline-6-sulfonic acid), DPPH (2, 2'-diphenyl-1-picrylhydrazyl) and BSTFA with 1% TMCS (bistrimethylsilyltrifluoroacetamide with 1% TMCS) were purchased from Sigma Chemical Co (St Louis MO, USA) and MERCK Co (Darmstadt, Germany) unless otherwise mentioned.

Plant materials

The twenty different Indian dietry and medicinal plants listed in Table 3.1 were selected for screening of their antioxidant activity and major phytochemical contents. These selected plant samples were obtained from Shantikunj, Haridwar (*Swertia chirata, Solanum nigrum, Acharas sapota, Cannavis sativa, Actinidia chinensis and Symplocos racemosa)* and local area of Roorkee, Uttarakhand, India (*Euphorbia hirta, Allium sativum, Cicer arietinum, Phaseolus vulgaris, Momordica charantia*, *Murraya koinegii*, *Beta vulgaris, Carica papaya, Ribes nigrum*, *Ananas cosmosus, Musa*

paradisiacal, Punica granatum, Aegle marmelos, Spinacea oleracea and Actinidia chinensis) and were identified by botanical expert at Shantikunj, Haridwar and in the Department of Biotechnology, IIT Roorkee, Roorkee, India. A specimen copy of these plants kept in the departmental herbarium facility.

3.2.2 Extraction, preparation and screening for antioxidant activity

The fresh plant parts from twenty different dietry and medicinal plant as given in Table 3.1 were frozen into liquid nitrogen and grinded to fine powder with the help of mortar and pestle. The aqueous and methanolic extract from 10 g of each plants were prepared by soaking in water (w/v 1:4) and methanol (w/v) for one hour. The extracts were filtered through three layered muslin cloth and filtrates were centrifuged at 10,000 x g for 45 min and clear supernatants were collected. The supernatants were stored at -80^{o}C and used for antioxidant activity evaluation and phytochemical determination.

3.2.3 Evaluation of antioxidant activity of extracts

The antioxidant activities of the aqueous and methanolic extracts of different plant used in the present were evaluated using DPPH free radical scavenging assay (Brand-Williams *et al.,* 1995) and ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996).

3.2.4 Determination of total phenolic and glycoside content

The total phenolic content of the aqueous crude extract of different plant were determined by the Folin–Ciocalteu method (Singleton *et al.,* 1999). One ml of different extract solution containing (10 mg) was mixed with 5 ml of ten fold diluted Folin–Ciocalteu reagent and 4 ml of Na_2CO_3 (0.75 g per 10 ml) was added to the mixture. The mixture was kept for incubation at 30^{0}C for one hour. After incubation, absorbance was measured at 765 nm. A calibration curve was prepared using Gallic

acid as a standard phenolic with a concentration range of (0.02–0.2 mg per ml). The linear equation y = 8.9929x - 0.0466 was obtained from Gallic acid calibration curve and was used to calculate the phenolic content in terms of Gallic acid equivalent (GAE). The total phenolic content of the samples was expressed as mg of Gallic acid equivalents (GAE) per 0.1 g extract. In the linear equation, y indicates the absorbance and x indicate the Gallic acid concentration in terms of mg per ml. For quantitative determination of total glycoside content of different extract was performed as per method described by Antan *et al.*, (1995). Briefly, 1 ml (10%) solution of different extract samples was mixed with 1 ml of 8% solution of vanillin containing 10 ml of 72% sulfuric acid. The reaction mixture was mixed thoroughly. The reaction mixture was heated at 60°C and then cooled in ice to stop the reaction. The absorption of the reaction mixture was measured at 544 nm. The standard ginsenoside was used for the preparation of calibration curve at concentrations 1 to 10 mg per ml in ethanol. The amount of total glycosides were calculated with the help of regression equation obtained from ginsenoside calibration curve (absorbance (y) = 0.08X-0.006). The total glycoside content was expressed in terms of Ginsenosides Equivalent (GEq) per g sample.

3.2.3 Extraction and fractionation of antioxidant constituents

3.2.3.1 Extraction and fractionation of glycosides enriched fraction from A. marmelos

Fresh leaves of *A. marmelos* (500 g) was frozen into liquid nitrogen and then powdered with the help of mortar and pestle. The powdered leaves sample was extracted three times with boiling water (w/v 1:3) and the extracts were pooled and filtered with three layer muslin cloth and supernatant was collected. The collected supernatant was centrifuged two times at 10,000Xg for 45 min to get ride of debris

and particulate material. The clear supernatant was lyophilized to powder and was used bioactivity guided fractionation. The crude aqueous extract lyophilized powder was redissolved in 400 ml distilled water and was further successively liquid-liquid partitioned with increasing polarity to remove fatty substances using hexane, chloroform and diethylether. The both organic and aqueous phases were evaluated for its glycoside content and antioxidant activity. This aqueous phase partitioned fraction was evaporated and lyophilized to powder which was named as crude glycoside extract from *A. marmelos* (CGAM). The 2 g of CGAM was again resuspended in 5 ml water: methanol (4:1) and fractionated on Sephadex LH-20 (Ameresham Biosciences) column (Bed volume dimension, 50 cm x 20 mm) and was eluted with methanol: water (1:1) and 5 ml fractions were collected. The each elution fractions were monitored for their glycosides content as per the method described (Antan *et al.*, 1995) and antioxidant activity using method described previously (Brand-Williams *et al.*, 1995). The elutes from 7^{th} to 10^{th} having high glycoside content and antioxidant activity were pooled and named as glycoside enriched fraction from *A. marmelos* (GAM). The schematic flow diagram for extraction and fractionation is presented in Fig. 3.1.

3.2.3.2 Extraction and fractionation of polyphenol enriched fraction from E. hirta plants.

Fresh leaves of *E. hirta* were frozen into liquid nitrogen and then powdered with the help of mortar and pestle. The fine powder was mixed 1:3 (w/v) with methanol: aqueous (1:1) and homogenized using mortar and pestle. The extraction was performed at 80°C three times for an hour each time. The pooled homogenate was filtered through three layers of muslin cloth and centrifuged at 10,000Xg for 45 min. The collected clear supernatant was further successively liquid-liquid partitioned

with increasing polarity to remove fatty substances using n-hexane, dichloromethane and chloroform. This aqueous-methanol phase partitioned fraction was evaporated and lyophilized to give a powder residue treated as crude phenolic from *E. hirta* (CPEH). The 2 g CPEH residue was again resuspended in 5 ml methanol: water (1:1), fractionated on Sephadex LH-20 (Ameresham Biosciences) column (Bed volume dimension, 50 cm x 20 mm) using eluting medium methanol: water (1:1) and elution volume was collected for 5 ml each. The elution fractions were monitored for their phenolic content with the help of spectrophotometric method (Singleton *et al.,* 1999). The collected fractions were also determined for their antioxidant activity using DPPH free radical scavenging assay. Eluates from 10^{th} to 13^{th} were then pooled and on the basis of their maximum phenolic content and antioxidant activity which is named as phenolic enriched fraction from *E. hirta* (PEH). The schematic flow diagram for extraction and fractionation of phenolic enriched fraction from *E. hirta* is depicted in Fig 3.2.

3.2.4 Qualitative and quantitative determination of antioxidant constituents in GAM and PEH

3.2.4.1 Spectrophotometric determination of glycosides in GAM and PEH constituents

For quantitative determination of glycoside content in GAM was performed as per method described (Antan *et al.,* 1995). Briefly, 1 ml (1%) solution of GAM was mixed with 1 ml of 8% solution of vanillin containing 10 ml of 72% sulfuric acid. The reaction mixture was mixed thoroughly. The reaction mixture was heated at 60 ^{o}C and then cooled in ice to stop the reaction. The absorption of the reaction mixture was measured at 544 nm. The standard ginsenoside was used for the preparation of

calibration curve at concentrations 1 to 10 mg per ml in ethanol. The total glycoside content was expressed in terms of Ginsenosides Equivalent (GEq) per g sample.

3.2.4.2 Spectrophotometric estimation of phenolic and flavonoid GAM and PEH

Total phenolic content was determined using the standard method as described by (Singleton *et al.*, 1999) and detailed method is described in earlier section. Total flavonoid content was estimated using the protocol described by Chang *et al.*, (2002) with slight modification. Briefly, 0.5 ml of 10 mg per ml concentration of GAM and PEH was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with Perkin Elmer UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing catechin solutions at concentrations 12.5 to 100 µg per ml in methanol. The total flavonoid content of GAM and PEH was expressed in terms of mg catechin equivalent per g samples.

3.2.4.3 UV visible spectroscopy of GAM and PEH antioxidant constituents.

The active fraction GAM and PEH were dissolved in methanol: water (1:1) and their UV-VISIBLE spectra was collected in the range of 200-600 nm.

3.2.4.4 Thin layer chromatography identification of active constituents in GAM and PEH

TLC method was employed for the qualitative observation of glycosides in GAM with the help of suitable developing reagents and solvent system. The GAM constituents and standard glycosides sitosteryl glucosides, lupeol and rutin were subjected to thin-layer chromatography (TLC) on silica gel plates (0.25 mm silica gels) using the solvent system methanol/distilled water (4:1). Visualization of glycosides on developed plates was done by spraying with 50% (v/v) sulphuric acid.

The sprayed chromatograph were allowed to dry for 15 min at room temperature and then heated at 105°C for 3 min in an oven until the colour developed. For qualitative analysis of PEH and standard phenolic compounds like quericitin, ferulic acid and gallic acid, we used silica gel plate using mobile phase toluene: methanol: formic acid (60:30:10) and plates were developed using 10% methanolic $FeCl_3$ spray.

3.2.5 Analysis and identification of GAM and PEH constituents using HPLC analytical tools

3.2.5.1 HPLC separation of GAM constituents

Glycosides enriched fraction (GAM) was characterized by HPLC technique. The GAM and standard glycosides sitosteryl glucosides, lupeol (triterpenoids) and rutin were first dissolved in methanol and filtered with 0.22 micron membrane filter (Millipore). The HPLC was performed using a Symmetry C-18 RP column, 4 µm, (250 mm x 4.6 mm i.d) using mobile phase acetonitrile: water (60:40, v/v) with flow rate of 1.0 ml per min and detected at 210 nm and 360 nm (Water, model 600E).

3.2.5.2 HPLC separation of PEH constituents

The PEH constitunets and standard polyphenol compounds like quercitin, ferulic acid and gallic acid were first dissolved in methanol and filtered with 0.22 micron membrane filter (Millipore). The HPLC was performed on a Symmetry C-18 RP column, 4 µm, (250 mm x 4.6 mm i.d) using mobile phase methanol: water (50:50, v/v) with flow rate of 1.0 ml per ml and detected at 280 nm, 320 and 360 nm (Water, model 600E).

3.2.6 FTIR characterization of GAM and PEH

Lyophilized powder of GAM and PEH was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra of the pellets were recorded between 4000–500 cm^{-1} on a Thermo Nicollet FTIR Spectrometer.

3.2.7 GC-MS characterization of GAM and PEH constituents

3.2.7.1 Derivatization of plant samples for GC-MS analysis

The GAM and PEH constituents were filtered with 0.25 µm membrane and solvents were evaporated with the help of rotary evaporimeter. The aqueous phase of samples was dried with the help of lyphilization. After lyphilization, samples were dried over dry nitrogen to avoid presence of moisture in the samples. Trimethylsilyl ethers (Derivatization) of the GAM and PEH were prepared by the addition of equal amounts 100 µl of BSTFA (bistrimethylsilyltrifluoroacetamide with 1% TMCS) and 50 µl of dry pyridine. Samples were allowed to react for one hour at 100°C in tubes stoppered with Teflon-lined caps.

3.2.7.2 Gas chromatography-mass spectrometry analysis of antioxidant fractions

Mass spectra were recorded on a Perkin-Elmer Clarus 560 gas-liquid chromatography-mass spectrometer having Turbomass 5.2 software. Gas-liquid chromatography columns were CP-SIL 1 (30 m length and 1.5mm Dia). The ionizing electron energy was 70 eV except as stated otherwise. Mass spectra were scanned in 3-5 seconds on the apex of the gas-liquid chromatography peak represented on the total ionization monitor. Temperatures were as follows: ion source 250°C and 270°C; carrier gas was helium. Gas-liquid chromatographic column temperature for GAM analysis was programmed at 80°C hold for one min, then up to 180°C at the rate of 8°C per min and up to 280°C at the rate of 10°C per min. The column temperature for PEH was programmed at 80°C hold for one min, then up to 180°C at the rate of 5°C per min and up to 300°C at the rate of 10°C per min. The injector temperature was maintained at 250°C and detector temperature was maintained at 280°C. The mass spectra and total ion chromatogram (TIC) analysis were performed with the help of

Turbomas software. The mass spectra search was done with the help of NIST and WILLEY data base library.

3.2.8 Direct infusion electrospray ionization-mass spectrometry (ESI-MS) characterization of antioxidant constituents from GAM and PEH fraction

Electrospray mass spectrometric analyses were performed on a Brucker LC-MS-NMR instrument equipped with a Brucker mass electrospray. Nitrogen was used as nebulizing gas at a pressure of 50 psi and a temperature of 300°C. Direct infusion conditions: the samples were analyzed by direct infusion in ESI-MS by means of a syringe pump at flow-rate of 1 min per ml in both positive and negative scan mode. Each plant extract was analyzed by direct infusion in ESI-MS at a flow-rate of 10 ml per min. The antioxidant fractions GAM and PEH were dissolved in water: methanol (50:50) and filtered with 0.25 micron membrane before injection in mass spectrometer. Mass spectra were acquired in scan mode detection and ESI-MS conditions from 100-1000 m/z.

3.2.9 Statistical analysis

All qualitative and quantitative experiments in separation and fractionation of antioxidants constituents from *A. marmelos* and *E. hirta* were carried out in triplicate and six replicates respectively. The experimental results represent the mean of three/six identical studies. Standard deviation (SD) and standard error (SE) were calculated using following formula:

$$SD = \frac{\sqrt{\sum X^2}}{N}$$

$$SE = \frac{SD}{\sqrt{N}}$$

3.3 Results and Discussion

3.3.1 Screening of twenty Indian medicinal plants for their antioxidant activity

In the present work, an attempt has been made to do systematic evaluation of antioxidant potential of twenty different plants. The list of twenty Indian dietry and medicinal plant and the respective plant parts used in the study are presented in Table 3.1. Since the antioxidant activity of plants are associated with their phytochemical constituents which differed a lot in their structure and solubility. Therefore, in the present study, both aqueous and methanol extract has been used for evaluating antioxidant potential.

The antioxidant potential of a preparation can be determined by a single assay. It has been suggested that a battery of assays based on different property and mechanism could reveal the antioxidant property of diverse class of phytochemical constituents.

Therefore in the present study, screenings for the antioxidant potential of selected plant were performed by DPPH and FRAP two different assays which work on two different principles. The results of antioxidant activity are summarized in Table 3.2. As clear from the result that there found to be great variation in antioxidant activity of the different plants in both aqueous as well as methanolic preparation. Among the twenty plant tested (*Cicer arietinum, Phaseolus vulgaris, Beta vulgaris, Ribes nigrum, Acharas sapota, Cannavis sativa* and *Actinidia chinensis*) showed poor antioxidant activity. On the other hand both the aqueous as well as methanolic extract from *E. hirta* and *A. marmelos* showed highest free radical scavenging activity (IC_{50} 0.06 and 0.05 mg per ml) and reducing power (98.21 µmol and 110.26 µmol GAE per g extract). Thus both *E. hirta* and *A. marmelos* was found to have strong antioxidant activity. The variation in the antioxidant activity has also been observed in

previous studies (Koleva, 2002; Koleckar *et al.*, 2008). These variations are found due to different phytochemical constituents among different plants. The absorbed differences in the antioxidant in the present study may be due to variations in the active constituents and difference in phytochemical constituent which is logical and expected. It has been reported from the previous study that antioxidant activity is attributed due to a number of phytochemical constituents (Benzie, 2003; Mantle *et al.*, 2000). Therefore in order to get an indication about the nature of active constituent present in the preparation, the qualitative estimation was performed and results are summarized in Table 3.3 and 3.4. It is observed that nature of phytochemical constituents varied among different plants in both aqueous as well as methanolic extracts.

Both aqueous and methanol preparation from *A. marmelos* and *E. hirta* showed high content of phenolic and glycosidic nature of compounds. Since, the phenolic and glycosides have been reported for antioxidant activity in various previous studies (Bravo, 1998; Bors, 2001; Hseu *et al.*, 2008; Yang *et al.*, 2008. It is likely that the higher antioxidant activity observed in both *E. hirta* and *A. marmelos* could be be due to their high phenolic and glycosides contents. Though phenolic and glycosides are attributed for antioxidants activity in many plants as established in earlier studies. It is not necessary that higher contents of phenolic and glycosides in *E. hirta* and *A. marmelos* are the soul contributor for their antioxidant activity. Since, some compounds though present in low quantity but could contribute greatly for the antioxidants potential of particular plants. Therefore, the observed higher antioxidants activity in *E. hirta* and *A. marmelos* is indeed due to their high phenolic and glycoside contents need to be further investigated and validated.

The aqueous and methanol extracts of the respective plant parts used in the present study were prepared as described in material and method section (3.2.2). The preliminary phytochemical analysis of aqueous and methanol extract from selected plants is presented in Table 3.2 and 3.3, respectively. The preliminary phytochemical analysis of the selected plant indicated that in most of plants mainly phenolic, flavonoids, glycosides, saponin and terpenoids were present in good amount. On the basis of this preliminary study, total phenolic and glycosides content of these selected twenty plants are summarized in Table 3.5. The total phenolic content was estimated spectrophotometrically and expressed in terms of mg GAE per 0.1 g extract. Total glycosides content was measured spectrophotometrically and expressed in terms of mg GEq per g extract. On the basis of phytochemical analysis *A. marmelos* showed highest yield of total glycosides content (12.67 mg GEq per 0.1 g extract) in aqueous extract in comparison to the methanol extract among all selected twenty plants. On the basis of phytochemical analysis, *E. hirta* showed the highest content of phenolic content (16.34 mg GAE/ 0.1 g extract) in both aqueous as well as methanol extract among the selected plants for screening study. In a number of earlier phytochemical studies, polar natures of compounds are reported from aqueous/methanolic extraction and our phytochemicals study conforms to the same observations. In the present attempt, the two *in vitro* methods selected to screen out plants have been used by other scientific groups and have recommended the reliability of these assays to validate the antioxidant activity *in vitro* condition (Arouma, 2003). In phytochemical study of several plant sources, there is assumption that most of polar extractable constituents may be found in water/methanol extraction (Harbonne, 1980). In this study, results could support the same facts as most of plants extract demonstrated high

phenolic and glycosides content in comparison with the non-polar nature of compounds.

3.3.2 Evaluation of antioxidant activity and phytochemical content from different parts of *A. marmelos* and *E. hirta*.

Since the preliminary studies showed that *E. hirta* and A. marmelos have strong antioxidants activity. The following studies where focused on these two plants. In order to select most effective plant parts. The antioxidants activity of different plant parts was investigated. The aqueous and methanolic extracts of different part of *A. marmelos* (leaf, fruit, bark) and *E. hirta* (flowers, leaves, and root) were prepared as described earlier in material method section (3.2.2). Antioxidants activity of aqueous and methanolic preparation of these parts was determined using DPPH free radical scavenging described before. The antioxidant activity of different plant parts extract is summarized in Fig 3.3. The result clearly indicated that aqueous and methanolic extracts from leaf part of *A. marmelos* demonstrated maximum free radical scavenging activity of (aqueous 75.21% and methanolic 66.76%) at the concentration 20 µg per ml in comparison to low scavenging activity of fruit and bark. The findings from the present studies support earlier traditional use of these plants for their leaf derived medicine. Similarly the aqueous and methanolic extracts of *E. hirta* leaf showed maximum antioxidants activity (aqueous 74.19% and methanolic 80.16%) compared to low scavenging activity of root and flower parts.

Total phenolic, flavonoids and glycosides content of aqueous and methanolic extracts of different parts of these two plants were also determined. The result indicated in Table 3.6. There found a very much variation in aqueous 75.21% and methanolic 66.76% in phenol, flavonoids and glycoside content of fruit, bark and leaves of *A. marmelos*. The leaves of both plants found to have high phenolic and

glycoside contents compared with other parts of plants. As suggested previously that the high antioxidants activity in leaves of *E. hirta* and *A. marmelos* may be due to their high contents of phenolic and glycosides respectively. But that need to be confirmed for further experimental analysis. Since the leaves found to be the best source for antioxidant activity as compared other parts. The leaves extracts of these two plants *A. marmelos* and *E. hirta* have been used for purification and analyzing the active constituents and evaluation of the antioxidants potential. The findings of the present study support the traditional use of leaves derived medicine from these two plants. The results also support the fact that during water and methanolic extraction, major phytochemical constituents suggested being polar in nature including phenolic, glycosidic and saponins (Pieta *et al.*, 2000).

3.3.3 Total phenol, flavonoids and glycosides content of liquid-liquid partitioned organic solvent fraction from *A. marmelos* and *E. hirta* extracts.

Since, major phytochemical constituents are polar in nature during aqueous and methanolic extraction, which include phenol and glycoside etc. and attempts was made to fractionate the active constituent by liquid- liquid portioning. The schematic flow diagram of liquid- liquid partitioning of leave aqueous extract and *E. hirta* leaves aqueous: methanolic (1:1) are shown in the Fig 3.1 and Fig 3.2 respectively. The yield of the respective stage of fractionation method is given. The liquid- liquid portioning of 20 g of *A. marmleos* extracts resulted in yield of hexane (2.4 g) , diethyl ether (1.8 g), chloroform (3.4 g), and water phase 12.1 g, respectively. Most of phytochemical constituents extracted into aqueous phase (12. 1 g, 60.12 %) in comparison to the other partitioned fractions. Total (500 g) fresh leaves of *E. hirta* yielded (25 g, 5%) aqueous-methanolic extract. After liquid-liquid partitioning of 25 g aqueous-methanolic extract as depicted in Fig. 3.2, most of phytochemical constituents

extracted into aqueous-methanolic phase (17.87 g, 68.75%). Table 3.7 show the total phenolic, flavonoid and glycosides content of different liquid-liquid partitioned fraction n-hexane, diethylether, chloroform and water from aqueous extract of *A. marmelos* leaves. Table 3.8 presents the total phenolic, flavonoid and glycosides content of different liquid-liquid partitioned fraction n-hexane, dichloromethane, chloroform and water from methanolic-aqueous extract of *E. hirta* leaves. The water phase of partitioned fraction showed highest phytochemical constituents indicating amount in decreasing order as glycoside (212.94±25.34 GEq per g extract), phenolic (mg GAEq per g extract, 107.12±11.56) and flavonoids (mg Catechin Eq per g extract, 66.47±5.52). The aqueous-methanolic phase from partitioned fraction of *E. hirta* extract demonstrated maximum amount of phenolic content (phenol (mg GAE per g extract, 315.92±32.58) followed by flavonoid (mg Catechin Eq per g extract, 242.68±21.32) and glycoside content GEq per g extract (86.76±11.78). The phytochemical yield from *A. marmelos* and *E. hirta* partitioned fractions clearly support the earlier view that the polar natures of compounds are usually extracted in water and methanolic solvents (Rice-Evans, 2000). In this study, the liquid-liquid fractionation using non-polar solvents to polar solvents demonstrated that most of major phytochemical constituents like glycosides in case of aqueous extract from *A. marmelos* and phenolic compounds from *E. hirta* extract were extracted in water/methanolic phase. In this extraction method, the findings indicate that during phase partitioning, major portion of glycosides from *A. marmelos* leaf extract could link the earlier view about nature of phytochemical present in *A. marmelos* leaf part. In case of *E. hirta*, phytochemical extraction in water/methanol phase and their probable nature of phenolic compounds could link the earlier evidences giving the idea about the presence of polar nature of constituents.

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3.3.4 Total antioxidant activity of liquid-liquid partitioned organic solvent fractions from *A. marmelos* and *E. hirta* extracts.

Free radical scavenging activity of different liquid-liquid partitioned from *A. marmelos* and *E. hirta* is presented in Fig 3.4 in terms of % DPPH free radical scavenging ability at 20 µg per ml concentration. Ferric reducing antioxidant power (FRAP) activity of liquid-liquid partitioned fraction from *A. marmelos* and *E. hirta* is summarized in Fig. 3.5 in terms of µmol GAE per g extract. Aqueous phase from *A. marmelos* extract showed maximum 84.21% free radical scavenging activity. The methanolic-aqueous phase from *E. hirta* extract demonstrated most of antioxidant activity in terms of 88.83% free radical scavenging activity. In the correlation with free radical scavenging activity, aqueous phase fraction from *A. marmelos* extract showed highest reducing power in terms of 180.21 µmol GAE/ g extract. The methanolic-aqueous phase from *E. hirta* extract exhibited most of reducing power with 212.75 µmol Trolox Eq/g extract. The results obtained clearly indicated that aqueous phase (*A. marmelos*) and methanolic-aqueous phase (*E. hirta*) contained most of free radical scavenging activity and reducing power. In earlier several liquid-liquid pertaining experiment from plants demonstrated that polar phase like aqueous and methanolic fraction contain mostly polar water soluble nature phytochemical constituents like phenolic, flavonoids and glycosides. In the line of above observations, it could be suggested that polar aqueous methanolic phase contained phenolic, flavonoid and glycosides group of compounds.

3.3.5 Bioactivity guided fraction of active constituents from *A. marmelos* and *E. hirta*

Since most of the major active constituents are retained in the aqueous fraction from *A. marmelos* and aqueous: methanol phase from *E. hirta*. These fractions also

have high phenolic and glycosides content. The phenolic and glycoside compounds are reported to be contributing factor for the antioxidant activity in many plants. Therefore, an attempt was made to purify the active constituents, which is responsible for the antioxidant activity in *A. marmelos* and *E. hirta* by bioactivity guided fractionation method. The aqueous phase obtained from *A. marmelos* and aqueous: methanol phase from *E. hirta* were fractionated on Sephadex LH-20 column chromatography as described in material and method section. The results of fractionation of *A. marmelos* and *E. hirta* are presented in Fig 3.5 and the Fig. 3.6, respectively. The antioxidant activity and glycoside content of eluted fraction were determined in *A. marmelos* (Fig 3.6). In case of *A. marmelos*, the most of antioxidant activity is retained in 7^{th}-10^{th} fraction. These fractions also accounted for the most of the glycoside content. These results clearly indicate that a shown correlation between antioxidant and glycoside content during bioactivity guided fraction of active constitunets for *A. marmelos*. Thus, active constituents in case of *A. marmelos* were found to be glycosides. On the other hand, the most of antioxidant activity from *E. hirta* was retained in the fraction 10^{th} -13^{th}, which also contained most of phenolic contents,. This clearly indicated that correlation between antioxidant activity and phenolic components. Thus, it is interpreted that antioxidant activity of *E. hirta* extract is due to their phenolic components. The glycosides and phenolic compounds have been reported to demonstrate antioxidant activity in several other plant sources (Middleton, 1996; Miliaukas, 2004; Lee *et al.,* 2008). The antioxidant activity is described in terms of percentage DPPH free radical scavenging activity with 5 µg per ml fraction. Total glycosides were expressed in terms of µg GEq per 0.1 mg fraction amount. The pooled active 7^{th}-10^{th} fraction demonstrated maximum free radical scavenging activity 53.81% and correlated maximum amount of glycosides content

expressed in terms of 72.72 µg GEq per 0.1 mg fraction. Fig. 3.7 depicts the bioactive fractionation of polyphenol enriched fraction from *E. hirta* methanol-aqueous extract on Sephadex LH-20 column chromatography. The antioxidant activity is described in terms of percentage DPPH free radical scavenging activity with 5 µg per ml fraction. Total polyphenol content is expressed in terms of µg GAE per 0.1 mg fraction amount. Results showed that pooled active fraction from 10^{th}-13^{th} indicated maximum free radical scavenging activity expressed in terms of 56.84% activity at concentration 5 µg per ml fraction. The total phenolic content found to be highest showing 82.96 µg GAE per 0.1 mg fraction. From the data, it is clear that the pooled active fraction from *A. marmelos* demonstrating highest antioxidant activity contained glycosides as major constituents. On the basis of bioactivity fractionation study from *E. hirta*, results suggest that the active fraction exhibiting highest antioxidant activity contained phenolic compounds as major phytochemical constituents. Our results are in consonance with earlier reports showing strong antioxidant activity of several plants with glycosides and phenolic compounds as major phytochemical constituents. In this study, the bioactivity guided fractionation of active constitunets was based on the idea that plant system are served with a number of classes and nature of compounds for their own metabolic purpose. To ensure the antioxidant activity of particular group of compounds, which is present in major amount, there is need to fractionate these several class of phytochemical constituents on the basis of their solubility, size and ability to interact with other phytochemicals. For the separation of crude plant extracts, conventional column chromatography methods are widely used. To partition polar components, such as phenolic or glycoside compounds, stationary phases of polyamide, cellulose, silica gel, and Sephadex LH-20 were mostly used by several phytochemical fractionation study

(Hort *et al.*, 2008; Zin *et al.*, 2008). During fractionation by above column chromatographic technique, most often aqueous alcoholic solutions (methanol/water or ethanol/water) used for their elution. In the present study, results indicated that such combination of eluting medium is most productive in terms of yield and fractionation of particular class of constituents.

3.3.6 Analysis and identification of GAM from *A. marmelos* and PEH from *E. hirta*

The active fraction (GAM) obtained from *A. marmelos* and PEH obtained from *E. hirta* from Sephadex LH-20 column chromatography were analyzed for its components using various techniques such as TLC, HPLC, FTIR, GC-MS and ESI-MS.

3.3.6.1 TLC analysis of GAM and PEH constituents

In order to identify the active components, GAM of *A. marmelos* was separated on precoated silica gel 60 F254 plate. The TLC result is shown in Fig 3.8. This figure represents the qualitative TLC observation for the presence of glycosides mainly rutin, sitosterol glucosides and lupeol in GAM fraction from *A. marmelos*. The Rf factor of standard lupeol (0.86), rutin (0.24) and sitosterol (0.86) matched with the GAM constituents TLC profile demonstrating the presence of glycosides nature of compounds. The visualizing agent 8% vanillin in sulphuric acid has been reported to be suitable for glycosides nature of compounds from earlier studies in several other plants. All the three identified components are found to be glycosidic compounds. There are several reports showing glycosides as major components with antioxidant activity from other plant sources (Xi *et al.*, 2008; Lin *et al.*, 2008). On the other hand, TLC separation of PEH constituents from *E. hirta* was performed on precoated silica gel 60 F254 plate using toluene: ethyl acetate: formic acid: methanol (30:30:9:1.25)

solvent system and results are presented in Fig 3.9. The TLC analysis resulted in their major constituents with Rf value (0.38, 0.45 and 0.71), which have been matched with the Rf value of standard phenolic compounds gallic acid (0.41), ferulic acid (0.57) and quericitin (0.68). Thus, it is clear that the antioxidant constituents of PEH from *E. hirta* is due to its phenolic components including (gallic acid, ferulic acid and quercetin).

3.3.6.2 FTIR characterization of GAM and PEH constituents

The GAM and PEH were also analyzed by FTIR. The FTIR spectra of GAM constituents of *A. marmelos* is shown in Fig 3.10a. The FTIR spectra analysis of standard glycosides like ginsenoside and triterpenoids, show the characteristic peak at 1008 cm^{-1} (glycosidic bond) and 3440 cm^{-1}. Thus, the appearance of FTIR spectra from GAM constituents suggests that glycosidic nature of compounds is present as major components. The FTIR spectra analysis has been used for identification of glycosides in earlier studies (Wolfender *et al.*, 1995). In this study, the FTIR spectra indicate the peak in the region of 1008 cm^{-1} and as per the spectral analysis of different nature of bonds and their characteristic spectra, the peaks in this region is assigned for glycosidic bonds and we could suggest that glycosides nature of compounds as major phytochemical present in active fraction from *A. marmelos*.

The FTIR spectra of PEH constituents from *E. hirta* is shown in Fig. 3.10 b. The major peak at 3445 and 1638 cm^{-1} was been indicated and suggested to account for the OH group and carbonyl group, respectively. The observed peak at 3445 cm^{-1} and 1014 cm^{-1} in PEH fractions suggest the presence of phenolic nature of compounds. In earlier reports showing FTIR characterization of phytochemical constituents, broad peak in the range of 3300-3400 cm^{-1} indicate the presence of hydroxyl goups (Rice-Evans, 2000).

3.3.6.3 UV-VISIBLE spectroscopy study on GAM and PEH constituents

The UV-VISIBLE spectra of active fraction from GAM active fraction is illustrated in Fig 3.11a and indicated the major peak at 205, 336 and 361 nm. On the basis of earlier study of glycosides constituents from plant sources, it has been reported that major absorption occurs in far UV-VISIBLE range 200-220 nm. The UV-VISIBLE spectra of standard glycosides compounds like Ginsenosides and sitosterol glucosides observed the peak in the range of 200-220 nm, which is characteristics peak of glycosides nature of compounds. The other peaks observed in the Fig 3.12a suggest the presence of flavonoid glycosides nature of compounds on the basis of standard spectra of flavonoid glycosides (rutin). The UV-VISIBLE spectra of active fraction from *E. hirta* are depicted in Fig 3.11b. The major peaks observed in the range of 250-330 nm and indicated the presence of phenolic compounds. Our results are supported by matching the standard phenolic compound spectra and spectral reports from other plant sources (Summner *et al.*, 1996; Peng *et al.*, 2003). On the basis of UV-VISIBLE spectroscopic study, we suggest the presence of glycosides nature of compound in active fraction from *A. marmelos* and phenolic compound in active fraction from *E. hirta* extract.

3.3.6.4 GC-MS characterization of GAM and PEH constituents

The GC-MS total ion chromatogram (TIC) of GAM constituents is represented in Fig 3.12a. Results indicate the major peaks at 9.98, 13.34 and 15.12 min retention time (RT). The GC-MS peaks from standard glycosides like rutin, sitosterol and lupeol was obtained and matched with the TIC of GAM fraction, results suggest that glycosides are major phytochemical constituents in the GAM fraction. On the basis of the matching results of mass ion spectra, it is clearly observed that glycoside compounds such as rutin, sitosterol glucosides and Lupeol represent the major portion

of phytochemical present in active fraction GAM from *A. marmelos*. The characteristic mass ion spectra of major peak at 13.34 min are given in Fig 3.12b with fragmentation ion at 255 and 345 m/z mass to charge ratio. The obtained mass ion spectra were matched with spectra pattern from NIST database and reported fragmentation pattern for the standard glycosides compounds like rutin, sitosterol glucosides and lupeol. Results could reveal that on the basis of mass ion spectra, glycosides nature of compounds including rutin, sitosterol glucosides and lupeol represent the major portion of phytochemicals present in active fraction from *A. marmelos*.

The GC-MS total ion chromatogram (TIC) of PEH constituents from *E. hirta* is represented in Fig 3.13a. The major peaks were observed at 4.6, 11.17, 14.07 and 16.12 min retention time (RT). These major peaks were matched with the standard phenolic compounds and results indicate that the presence of rhamnose (4.6), gallic acid (11.17), ferulic acid (14.07) and quericitin (16.12) as major constituents in PEH fraction. The mass ion spectra of major peak were obtained and spectra pattern were matched with the NIST database library and reported spectra of phenolic compounds from various plant sources (Lien *et al.*, 1999; Dimitrious, 2000). The mass ion spectra of the major peak at 16.12 min RT is presented in Fig 3.12b and analysis of fragmentation pattern with NIST library confirms the characteristic mass ion at 223, 263, 297, 312, 327 and 342 m/z which is characteristic fragmentation pattern for quericitin compound. Thus, it is clear that PEH of *E. hirta* confirmed to contain rhamnose, gallic acid, ferulic acid and quercetin as major components but quercetin is most predominant as confirmed by the mass ion analysis.

3.3.6.5 Qualitative characterization of GAM from A. marmelos and PEH E. hirta

The qualitative characterization of GAM and PEH constituents were performed using HPLC-UV technique. Fig. 3.14 A and B show the HPLC UV chromatogram at 280 nm and 320 nm of *E. hirta* active fraction (PEH). The major peak at 7.86, 9.21 and 11.3 retention time were observed at both 280 nm and 320 nm absorbance. On the basis of matched HPLC chromatogram from standard phenolic compounds like gallic acid, ferulic acid and quericitin, results of HPLC chromatogram indicate the presence of these phenolic compounds. From the previous HPLC analytical study performed on phenolic constituents analysis from varied plant sources suggest that these compounds usually indicate the peak at 280 and 320 nm (Arin *et al.*, 1995; Tsaoa and Deng, 2004; Lee *et al.*, 2007). In this study, the combination of water and methanol produced good separation and indicate the possible phenolic nature of compound as suggested from earlier phytochemical analysis using HPLC technique (Synder and Kirkland, 1975; Wang *et al.*, 1998). It has been indicated that the phenolic nature of compounds observed from the absorption maxima at 280 and 320 nm and it is possibly due to their aromatic ring structure (Decker *et al.*, 1995). On the basis of HPLC chromatogram results, it is possible to suggest that phenolic compounds mainly gallic acid, ferulic aid and quericitin are present in major portion of active fraction from *E. hirta*.

Fig 3.15 a and b. represents the HPLC UV chromatogram of GAM from *A. marmelos* at 210 nm and 330 nm, respectively. Results from HPLC chromatograph at 210 nm observed major peaks at 8.92 and 7.16 min retention time. On the other hand a major peak at 12.86 (Rutin) min was obtained in HPLC UV chromatogram at 330 nm. The analysis of HPLC chromatogram with the standard glycosides indicated the presence of sitosterol glucosides (7.16 RT), lupeol (8.92 RT) and rutin (12.86 RT) as

major phytochemical constituents in GAM from *A. marmelos*. In earlier reports of glycosides analysis with HPLC technique, glycosidic compounds indicate the absorbance in the range of 200-220 nm (Antan, 2001). The absorbed peaks at 210 nm clearly suggest the presence of glycosidic nature of compounds. The other peak 330 nm is due to rutin glycosides having quericitin as an aglycone part.

3.3.6.6 Direct infusion ESI-MS characterization of GAM from A. marmelos and PEH from E. hirta

The analysis of the GAM from *A. marmelos* and PEH from *E. hirta* was also done using ESI-MS technique. Fig. 3.16 depicts the direct infusion ESI-MS spectra of GAM fraction in negative ion mode and the scanning range was 100-1100 m/z. On the basis of fragmentation ion spectra analysis, mass ion at 144.2, 163.4, 179.3, 202.1, 301.2, 413.4, 425.7 and 609.2 m/z was observed. On the basis of comparison with the analysis of standard glycosidic compounds the results of present study indicate the presence of rutin, sitosterol glucosides and lupeol as major active components of GAM fraction.

Fig 3.17 presents the direct infusion ESI-MS spectra of PEH fraction in negative ion mode and the scanning range was 100-1000 m/z. The analysis of spectra observed the fragmentation mass ion at 144.8, 163.4, 169.4, 193.5, 301.2 and 447.8 m/z. The spectral analysis on comparison with the standard phenolic compounds, the results indicated the presence of gallic acid, ferulic acid, quericitin and rhamnose as major components in PEH fraction from *E. hirta*. ESI-MS proves very fast and versatile employing little sample preparation to yield immediate compositional information of the most polar ESI-MS ionizable compounds. These unique features of direct infusion ESI-MS have recently been applied for fingerprinting of complex mixtures.

Conclusion

The aqueous and methanolic extracts leaves of *A. marmelos* and *E. hirta* found to have strong antioxidant activity among twenty different Indian dietary and medicinal plants tested in the present study. There found to be strong correlation between antioxidant activity and their glycoside and phenolic contents suggesting that antioxidant activity may be due to glycoside and phenolic. A glycosides enriched fraction (GAM) from *A. marmelos* and phenolic enriched fraction (PEH) from *E. hirta* having strong antioxidant activity were obtained from their aqueous extract by liquid-liquid partitioning and column chromatography. On further analysis using various analytical tools showed rutin (flavonoid glycosides), β-sitosterol glucoside and lupeol (triterpenoids) as major components in GAM while quercitin, gallic acid and ferulic acid identified as major phenolic compounds.

Table 3.1 List of twenty different dietary and medicinal plant and their used parts.

S.No	Local Name	Scientific Name	Family	Plant parts used
1.	Dudhi	*Euphorbia hirta* L	Euphorbiaceae	Stem and leaf
2.	Garlic	*Allium sativum* Linn	Liliaceae	Bulb
3.	Gram	*Cicer arietinum* L.	Leguminaceae	Green seed
4.	Jangli baigan	*Solanum nigrum* L.	Solanaceae	Whole plant
5.	Chiraita	*Swertia chirata* Buch- Ham	Gentinaceae	Stem
6.	Beans	*Phaseolus vulgaris* L.	Leguminaceae	Pod
7.	Bitter gourd	*Momordica charantia* L.	Cucurbitaceae	Whole fruit
8.	Curry leaves	*Murraya koinegii* (L.) Sprengel	Rutaceae	Leaf
9.	Lodhra	*Symplocos racemosa*	Chenopodiaceae	Root
10	Beet	*Beta vulgaris* L.	Carecaceae	Whole fruits
11	Papaya	*Carica papaya* L.	Sapotaceae	Whole fruits
12.	Pine apple	*Ribes nigrum*	Bromoleaceae	Whole fruits
13	Cheeku	*Acharas sapota* L.	Musaceae	Whole fruits
14.	Pomegranate	*Ananas cosmosus* (L.) Merr	Punicaceae	Whole fruits
15.	Banana	*Musa paradisiaca* L.	Rutaceae	Leaf
16.	Pomegranate	*Punica granatum* .L.	Saxifragaceae	Whole fruits
17.	Bael	*Aegle marmelos* Correa.	Cannabiaceae	leaf
18.	Bhang	*Cannavis sativa* L.	Euphorbiaceae	leaf
19.	Spinach	*Spinacea oleracea* L.	Chenopodiaceae	Leaf
20.	kiwifruit	*Actinidia chinensis*	Actinidiaceae	Whole fruits

Table 3.2 Total antioxidant activity of aqueous and methanol extract of twenty Indian dietary and medicinal plant.

Scientific Name	Aqueous extract *		Methanol extract *	
	DPPH IC_{50} (mg/ml)	FRAP µmol GAE /g extract	DPPH IC_{50} (mg/ml)	FRAP µmol GAE Eq. /g extract
Euphorbia hirta L	0.06±0.01	98.21±8.28	0.05±0.94	110.26±0.94
Allium sativum Linn	0.23±0.04	14.805±1.2	1.54±0.07	16.59±1.2
Cicer arietinum L.	>2	3.99±0.56	>2	1.185±0.04
Solanum nigrum L.	0.478±0.0.3	39.24±3.98	0.432±0.06	31.665±4.3
Swertia chirata	0.279±0.03	45.63±6.32	0.271±0.03	44.52±6.4
Phaseolus vulgaris L.	>2	3.915±0.75	>2	3.66±0.06
Momordica charantia	0.865±0.11	28.77±4.32	0.765±0.03	31.33±3.6
Murraya koinegii (L.)	0.548±0.06	32.175±5.32	0.632±0.04	27.84±2.6
Symplocos racemosa	0.974±0.18	7.065±0.96	1.08±0.02	2.46±0.02
Beta vulgaris L.	>2	6.12±0.32	>2	1.53±0.05
Carica papaya L.	0.896±0.12	10.98±0.85	0.96±0.06	3.15±0.01
Ribes nigrum	>2	0.66±0.02	>2	0.52±0.03
Acharas sapota L.	>2	4.335±0.17	>2	3.825±0.02
Ananas cosmosus (L.)	0.754±0.06	16.59±1.2	0.697±0.03	19.92±2.2
Musa paradisiacal L.	>2	1.034±0.03	>2	4.845±0.03
Punica granatum .L.	0.284±0.02	34.65±3.7	0.267±0.02	43.17±3.7
Aegle marmelos Correa.	0.04±0.01	111.16±6.32	0.05±0.01	99.98±4.2
Cannavis sativa L.	>2±	14.22±1.4	>2	4.08±0.02
Spinacea oleracea L.	0.367±0.08	126.19±7.43	0.398±0.02	35.67±3.2
Actinidia chinensis	>2	8.685±0.86	>2	2.46±0.21

* Data presented are given as mean±SEM, n=3 Three different experiments with three replicates

Table 3.3 Preliminary phytochemical evaluation in aqueous extract of twenty different Indian dietary and medicinal plant.

Scientific Name	Phenolic test	Flavonoid test	Glycosides/saponin test	Steroid/ terpenoids test	Alkaloid test
Euphorbia hirta L.	+++	+++	+	+	+
Allium sativum	++	+	+	+	
Cicer arietinum L.	+	+	+	+	+
Solanum nigrum L.	+	++	+	+	+
Swertia chirata	+	++	+	+	+
Phaseolus vulgaris	++	+	+	++	++
Momordica charantia	+	++	++	+	+
Murraya koinegii	++	++	+	+	++
Symplocos racemosa	++	++	++	+	++
Beta vulgaris L.	+	++	+	++	+
Carica papaya L.	++	+	+	++	+
Ribes nigrum	++	++	+	+	++
Acharas sapota L.	+	++	++	+	++
Ananas cosmosus	+	++	++	+	+
Musa paradisiaca	+	+	+	++	++
Punica granatum	++	+	++	+	+
Aegle marmelos	+	++	+++	++	+
Cannavis sativa L.	+	++	+	+	++
Spinacea oleracea	+	++	+	++	+
Actinidia chinensis	++	+	++	+	++

\+ Poor ++ Average +++ Good

Table 3.4 Preliminary phytochemical evaluation in methanol extract of twenty Indian dietary and medicinal plants.

Scientific Name	Phenolic test	Flavonoid test	Glycosides/saponin test	Steroid/ terpenoids test	Alkaloid test
Euphorbia hirta	+++	+++	+	+	+
Allium sativum	++	+	+	+	
Cicer arietinum L	+	+	+	+	+
Solanum nigrum	+	++	+	+	+
Swertia chirata	+	++	+	+	+
Phaseolus vulgaris	++	+	+	++	++
Momordica charantia	+	++	++	+	+
Murraya koinegii	++	++	+	+	++
Symplocos racemosa	++	++	++	+	++
Beta vulgaris L.	+	++	+	++	+
Carica papaya L.	++	+	+	++	+
Ribes nigrum	++	++	+	+	++
Acharas sapota	+	++	++	+	++
Ananas cosmosus	+	++	++	+	+
Musa paradisiaca	+	+	+	++	++
Punica granatum	++	+	++	+	+
Aegle marmelos	+	++	+++	++	+
Cannavis sativa	+	++	+	+	++
Spinacea oleracea	+	++	+	++	+
Actinidia chinensis	++	+	++	+	++

\+ Poor ++ Average +++ Good

Table 3.5 Total phenol and glycosides spectrophotometric determination in aqueous and methanol extract of twenty Indian dietary and medicinal plants.

Scientific Name	Aqueous extract *		Methanol extract *	
	Phenolic content mg GAE / 0.1g extract	Glycoside mg GEq/0.1g extract	Phenolic content mg GAE / 0.1g extract	Glycoside mg GEq/0.1g extract
Euphorbia hirta L.	10.34±0.83	6.54±0.76	9.37±0.87	6.23±0.56
Allium sativum Linn.	3.8±0.24	1.7±0.12	4.2±0.28	1.845±0.12
Cicer arietinum L.	0.4±0.03	0.315±0.02	0.643±0.02	0.595±0.03
Solanum nigrum L.	7.34±0.76	3.26±0.46	6.92±0.49	2.945±0.18
Swertia chirata	8.45±0.94	3.71±0.39	9.45±0.76	3.825±0.43
Phaseolus vulgaris L.	1.44±0.12	1.095±0.08	1.98±0.08	1.72±0.16
Momordica charantia	3.56±0.33	2.305±0.17	4.46±0.31	2.565±0.16
Murraya koinegii (L.)	3.27±0.27	2.46±0.28	3.87±0.54	3.46±0.27
Symplocos racemosa	2.56±0.18	2.19±0.18	2.24±0.18	2.085±0.12
Beta vulgaris L.	1.45±0.15	1.685±0.14	1.84±0.16	1.3±0.08
Carica papaya L.	2.65±0.28	2.255±0.21	3.87±0.27	2.145±0.17
Ribes nigrum	1.02±0.08	1.355±0.12	1.20±0.09	0.705±0.05
Acharas sapota L.	0.82±0.05	1.33±0.09	0.92±0.06	0.64±0.03
Ananas cosmosus (L.)	1.97±0.18	1.535±0.11	2.16±0.12	1.71±0.27
Musa paradisiacal L.	0.23±0.04	0.67±0.04	0.67±0.06	2.29±0.19
Punica granatum .L.	5.89±0.38	3.18±	6.34±0.46	3.59±0.21
Aegle marmelos Correa.	12.67±0.97	4.295±0.31	11.82±0.98	4.17±0.31
Cannavis sativa L.	2.65±0.18	1.185±0.13	2.87±0.21	1.99±0.12
Spinacea oleracea L.	3.89±0.25	2.845±0.22	3.76±0.34	2.605±0.18
Actinidia chinensis	0.367±0.02	0.445±0.04	0.654±0.06	0.685±0.05

* Data presented are given as mean±SEM, n=3 Three different experiments with three replicates

Table 3.6 Total phenol, total flavonoids and total glycosides content of different plant parts extract from *A. marmelos* and *E. hirta*.

Sample	Methanolic extracts *			Aqueous extract *		
	Phenolic (mg GAE/g)	Flavonoids (mg Catechin Eq/g extract	Glycosides mg GEq/g extract	Phenolic (mg GAE/g extract)	Flavonoids (mg Catechin Eq/g extract	Glycosides mg GEq/g extract
Aegle marmelos plant parts						
Fruits	56.12±4.93	36.76±4.32	56.87±4.65	64.21±5.15	45.21±3.85	73.23±6.59
Bark	32.16±3.28	40.21±6.21	75.21±4.76	72.15±6.29	54.32±4.29	94.18±7.42
Leaves	76.43±6.38	120.43±9.27	186.49±11.84	165.32±10.39	149.31±11.21	200.17±8.5
Euphorbia hirta plant parts Methanolic extracts				Meth : Aqueous (1:1) extract		
Roots	89.46±8.31	74.21±6.38	45.32±4.46	98.23±5.29	87.25±7.54	48.56±4.19
Leaves	289.31±18.5	189.38±12.32	64.24±5.84	300.86±23.19	214.72±18.59	70.58±5.86
Flower	54.65±4.29	37.43±3.87	24.17±2.69	65.21±6.21	54.15±4.68	32.91±2.86

Table 3.7 Total phenol, flavonoids and glycosides content of different organic solvent fraction from *A. marmelos* aqueous extract.

Name of sample	Different organic solvent fraction from *A. marmelos* aqueous extract. *			
	Hexane	Diethyl ether	Chloroform	Water
Total phenol (mg GAE / g extract)	25.68±3.82	68.32±6.31	31.42±4.28	107.12±11.56
Total flavonoids (mg Catechin Eq/g extract)	18.28±2.43	14.96±1.04	8.18±0.83	66.47±5.52
Total glycosides (mg GEq/g extract)	21.39±2.97	17.58±1.12	11.48±0.96	212.94±25.34

Table 3.8 Total phenol, flavonoids and glycosides content of different organic solvent fraction from *E. hira* methanolic-aqueous extract.

Name of sample	Different organic solvent fraction from *E. hirta* aqueous: methanolic extract. *			
	Hexane	Dichloromethane	Chloroform	Aq-Meth
Total phenol (mg GAE / g extract)	43.12±3.57	31.58±3.21	18.57±1.06	315.92±32.58
Total flavonoids (mg Catechin Eq/g extract)	25.38±2.95	16.29±1.57	8.32±0.76	242.68±21.32
Total glycosides (mg GEq./g extract)	4.79±2.75	15.38±1.19	7.43±0.56	86.76±11.78

* Data presented are given as mean±SEM, n=3 Three different experiments with three replicates.

Table 3.9 Quantitative determination of constituents in (CGAM, GAM) from *A. marmelos* and (CPEH, PEH) from *E. hirta*.

Sample	Phenolic (mg GAE / g) *	Flavonoids (mg Catechin Eq/g extract) *	Glycosides (mg GEq./g extract) *
CGAM	120.86±8.39	43.68±3.85	214.38±13.6
GAM	221.75±15.37	94.17±6.85	418.48±37.45
CPEH	189.43±11.48	105.92±8.43	63.18±5.47
PEH	497.19±32.76	216.28±15.42	143.22±10.58

* Data presented are given as mean±SEM, n=3 Three different experiments with three replicates

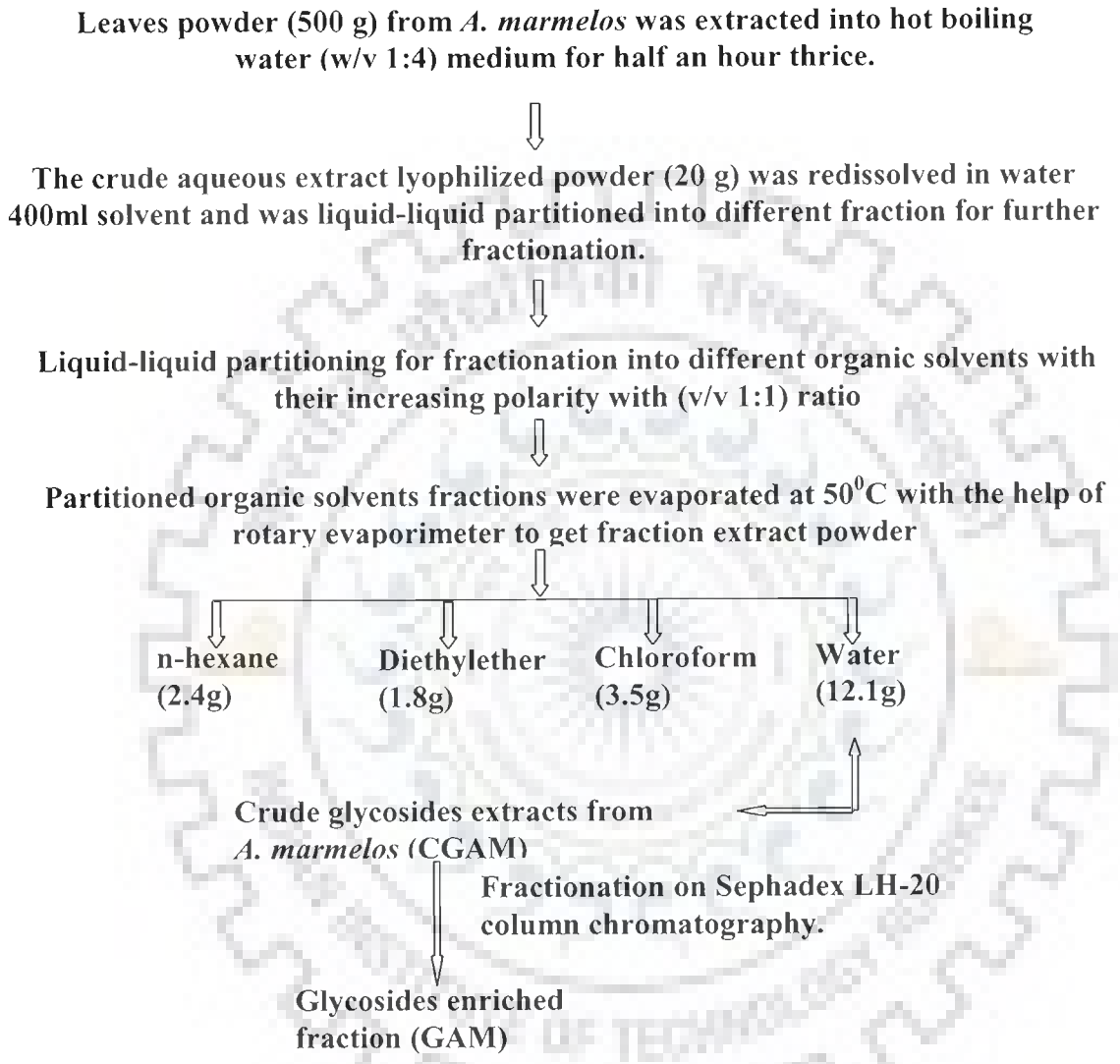

Fig. 3.1 Schematic flow diagram for bioactivity guided liquid-liquid partitioning and fractionation of glycosides enriched fraction (GAM) from *A. marmelos* leaves.

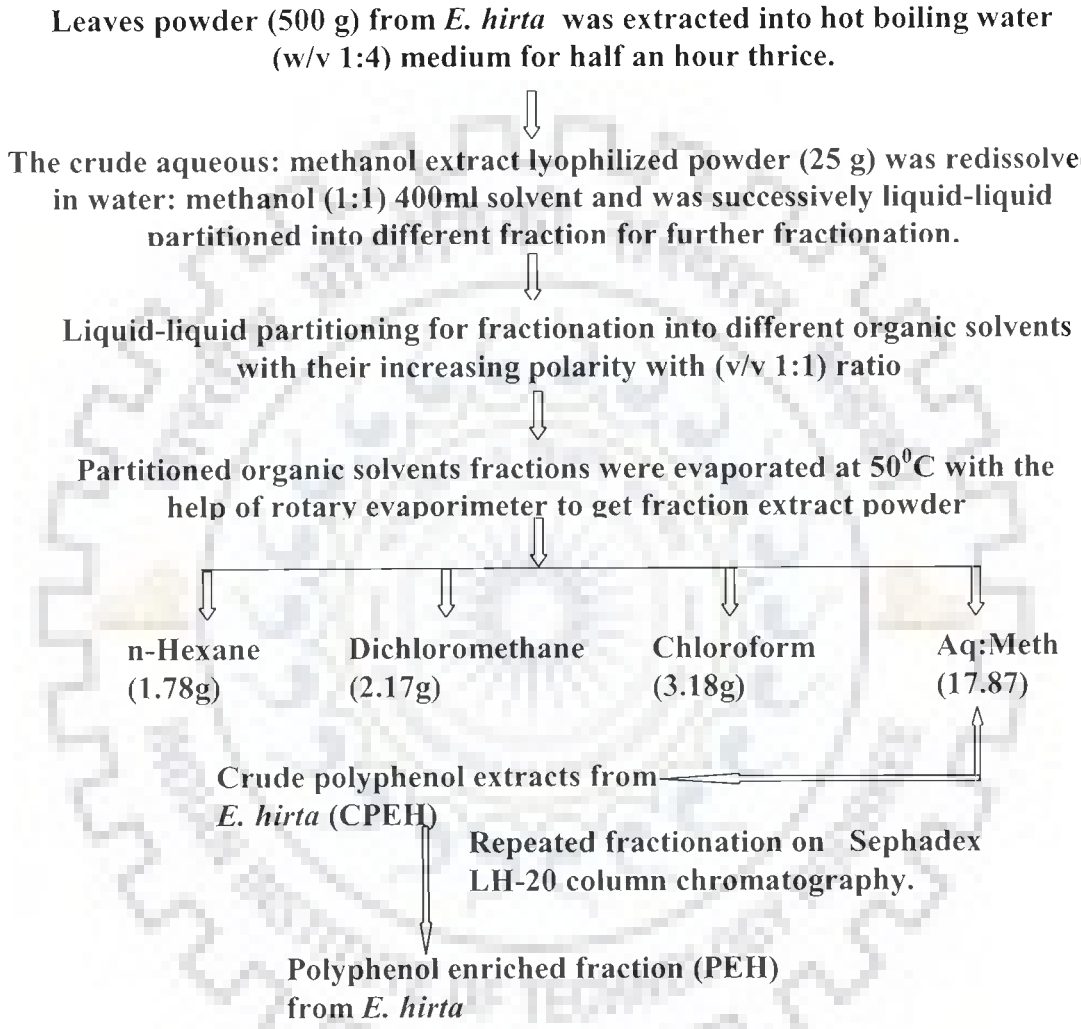

Fig. 3.2 Schematic flow diagram for bioactivity guided liquid-liquid partitioning and fractionation of polyphenol enriched fraction (PEH) from *E. hirta* leaves.

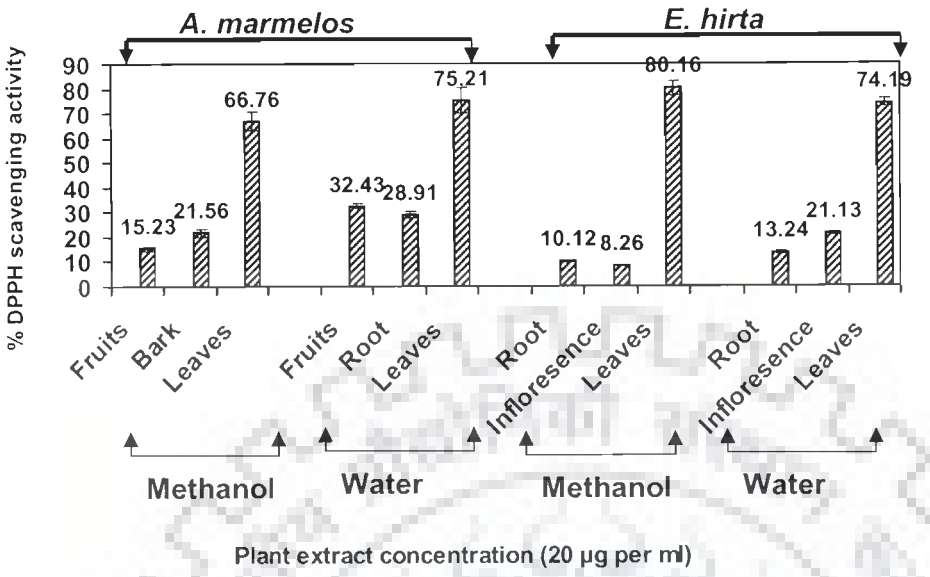

Fig 3.3 This figure depicts the DPPH free radical scavenging activity from different parts of *A. marmelos* and *E. hirta*.

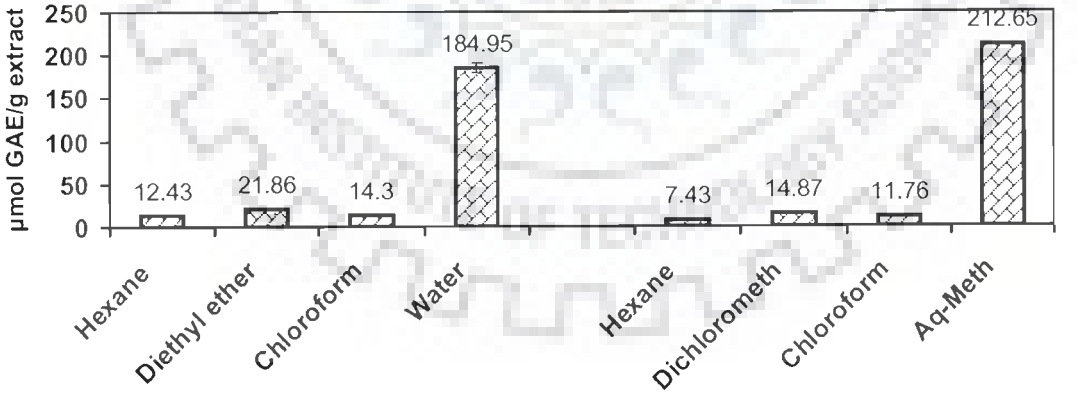

Fig. 3.4 Ferric reducing antioxidant power (FRAP) of liquid-liquid partitioned different fractions from *A. marmelos* and *E. hirta extracts*. The FRAP value is expressed in terms of µmol GAE per g extract.

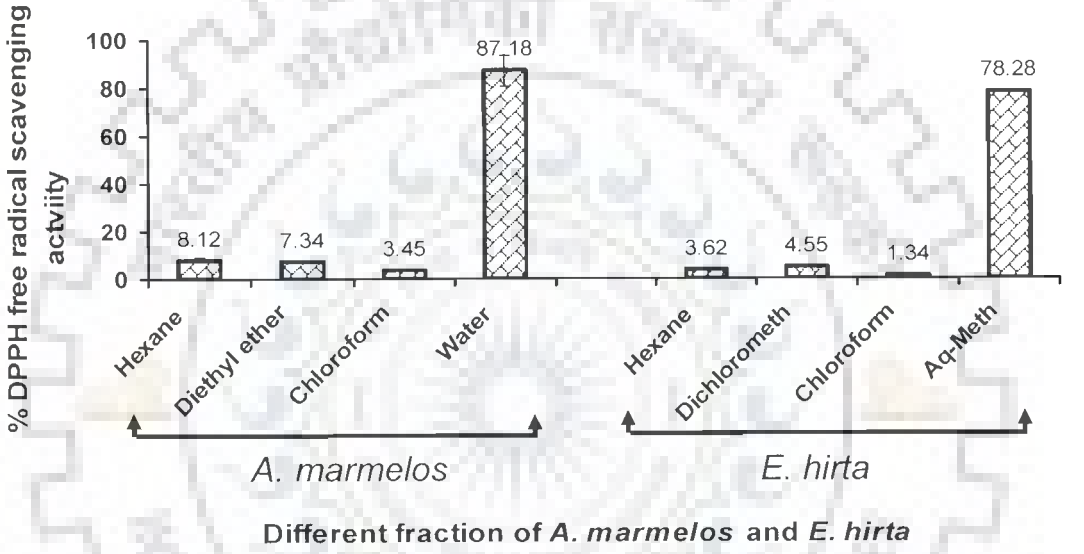

Fig. 3.5 Percentage DPPH free radical scavenging activity of liquid-liquid partitioned different fractions from *A. marmelos* and *E. hirta* extracts with final volume concentration at 20 microgram per ml.

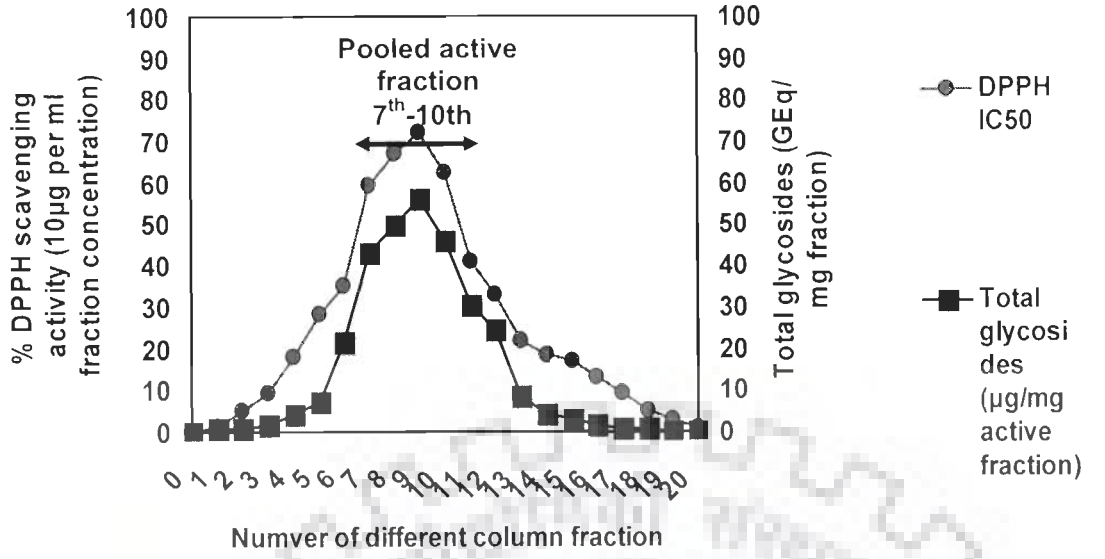

Fig. 3.6 illustrates the bioactivity guided fractionation of glycosidic enriched fraction from *A. marmelos* on Sephadex LH-20 column chromatography.

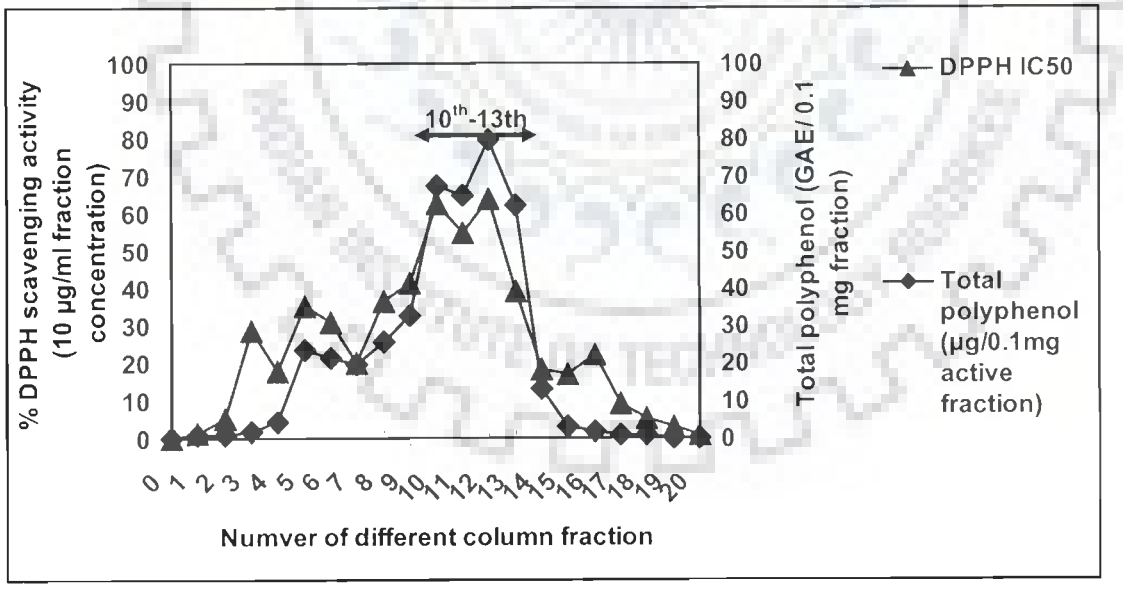

Fig. 3.7 Illustrates the bioactivity guided fractionation of polyphenol enriched fraction from *E. hirta* meth-aqueous extract on Sephadex LH-20 column chromatography.

Fig 3.8 TLC profile of GAM constituents from *A. marmelos*.

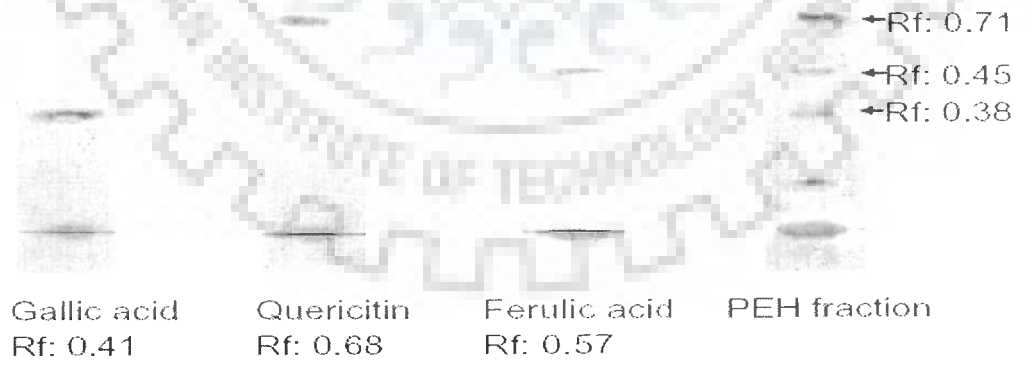

Fig 3.9 Qualitative TLC profile of PEH fraction from *E. hirta*.

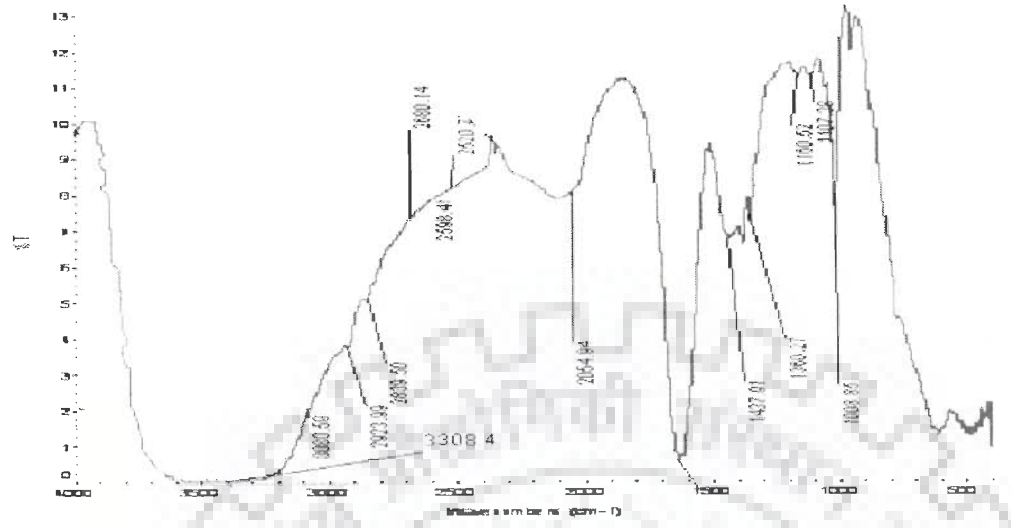

Fig 3.10a Show the FTIR spectra of GAM from *A. marmelos*

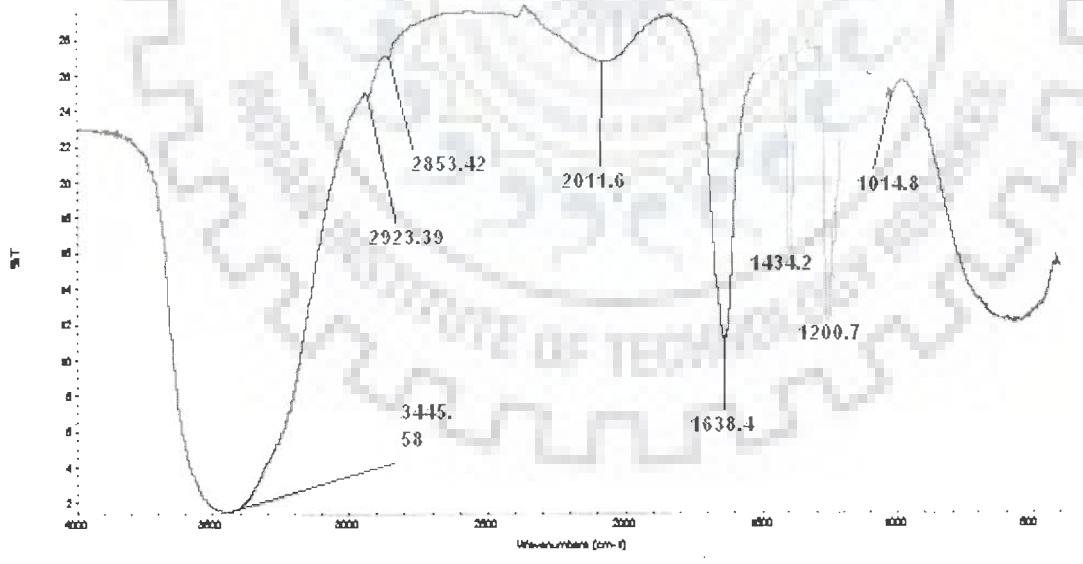

Fig 3.10b Show the FTIR spectra of PEH constituents

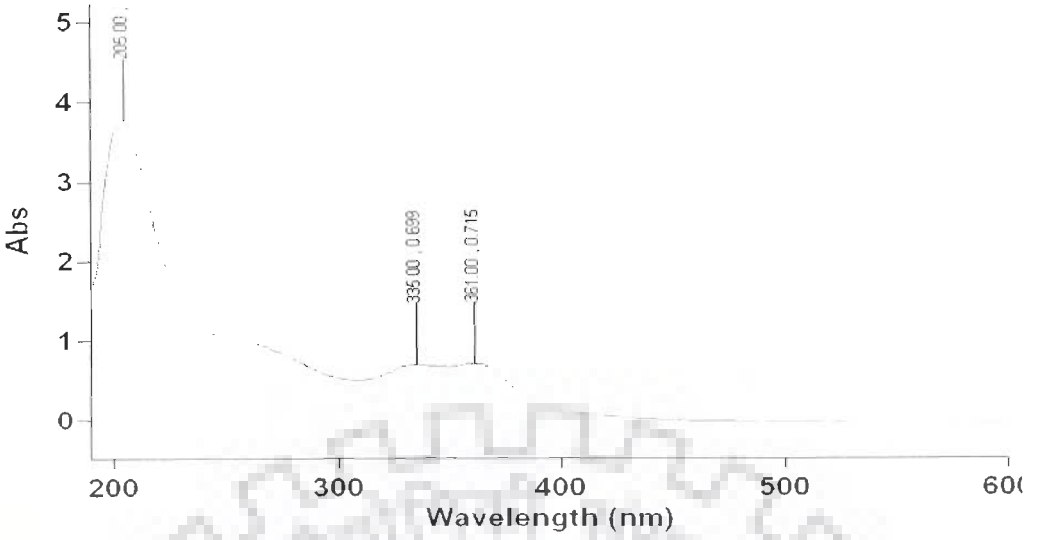

Fig 3.11a. UV-VISIBLE spectra of GAM from *A. marmelos.*

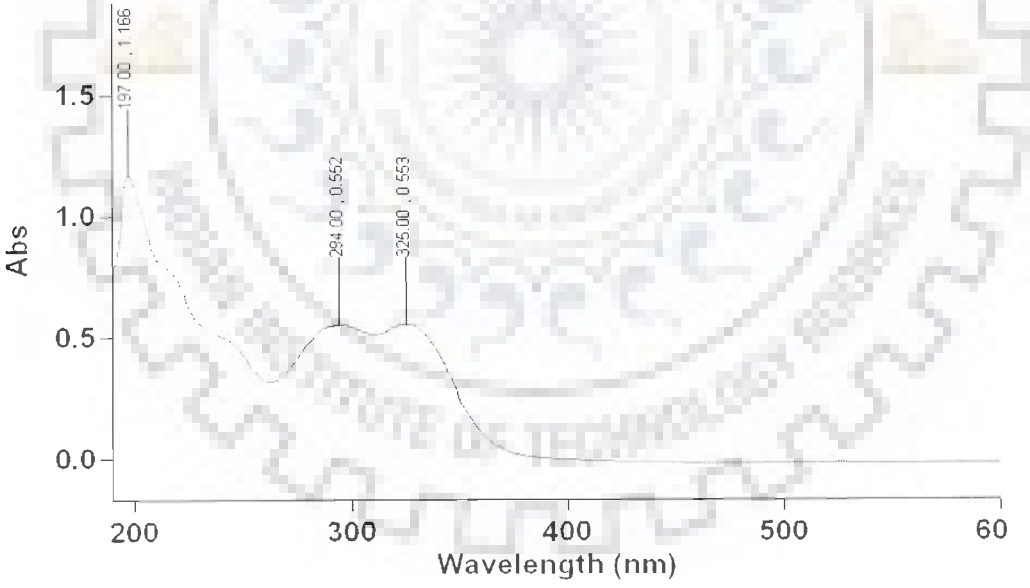

Fig 3.11b. UV-VISIBLE spectra of PEH from *E. hirta.*

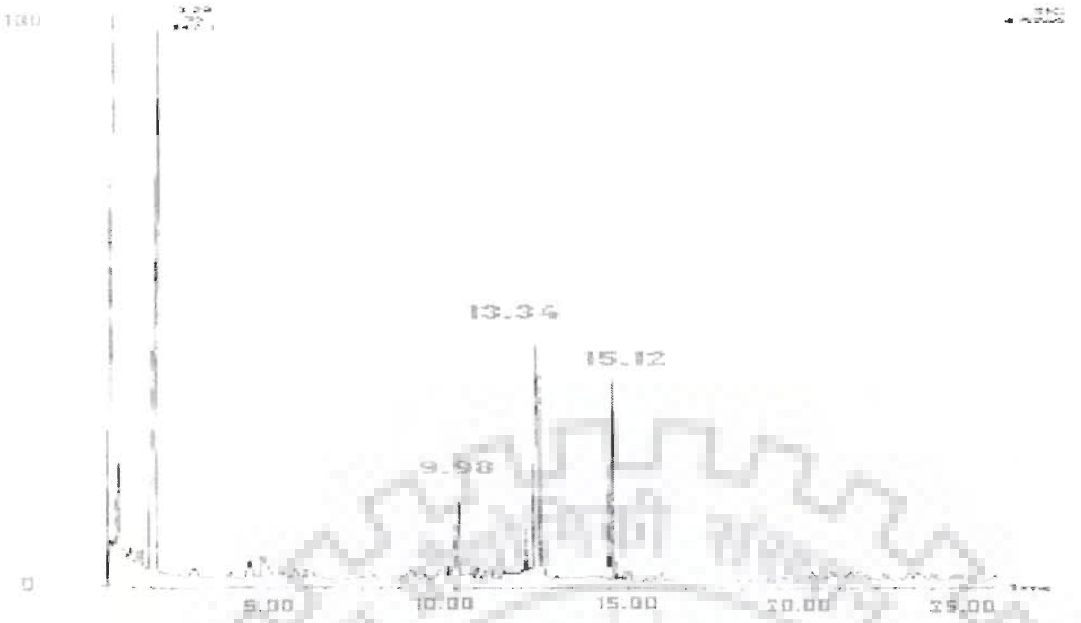

Fig 3.12a GC-MS TIC chromatogram of GAM constituents from *A. marmelos*.

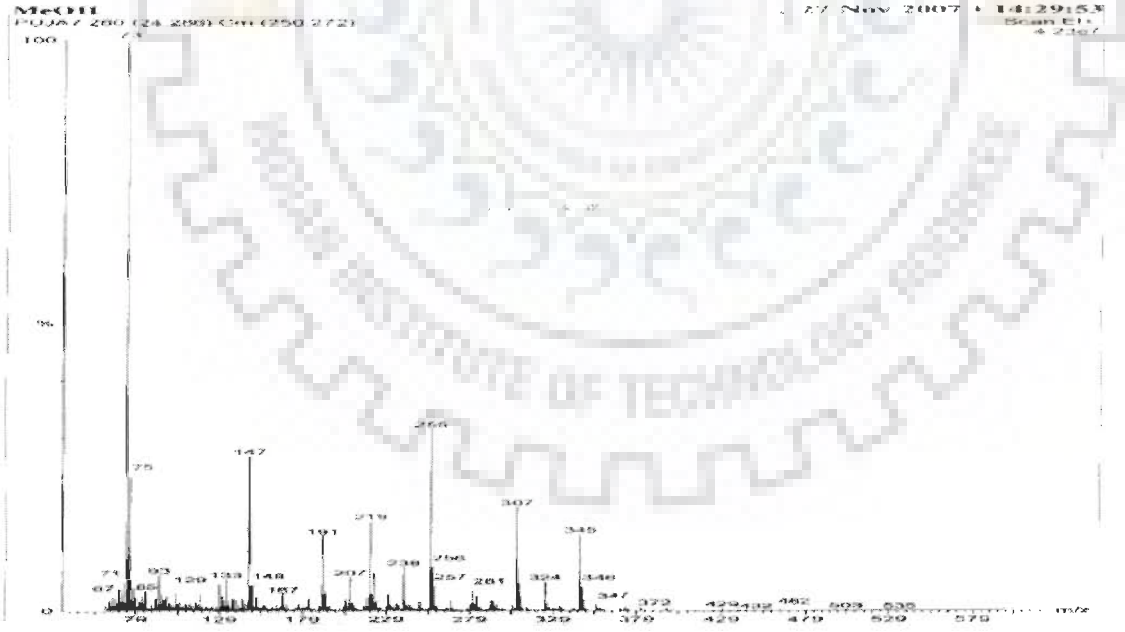

Fig 3.12b. GC-MS spectra of major peak at 13.34 min. showing mass spectra with [M-H] 255, 147 and 345 m/z. The ion peaks at 217, 207, 147 and 255m/z clearly indicate the presence of sitosterol glycoside structure.

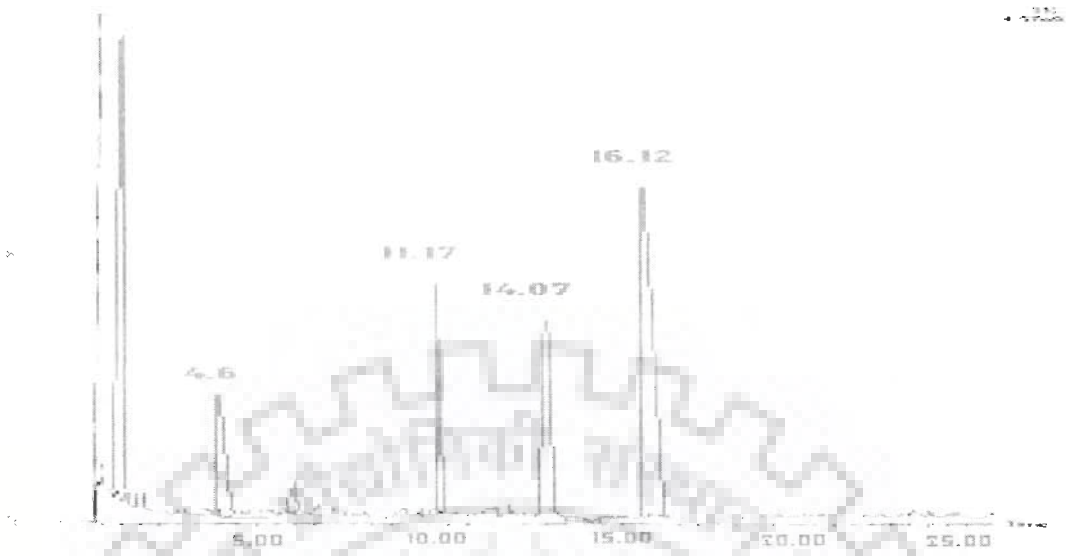

Fig. 3.13a GC-MS Total ion chromatogram of PEH constituents from with major peaks at 4.6. (Rhamnose) 11.17 (Gallic acid), 14.07 (ferulic acid) and 16.12 (Quericitin) RT min.

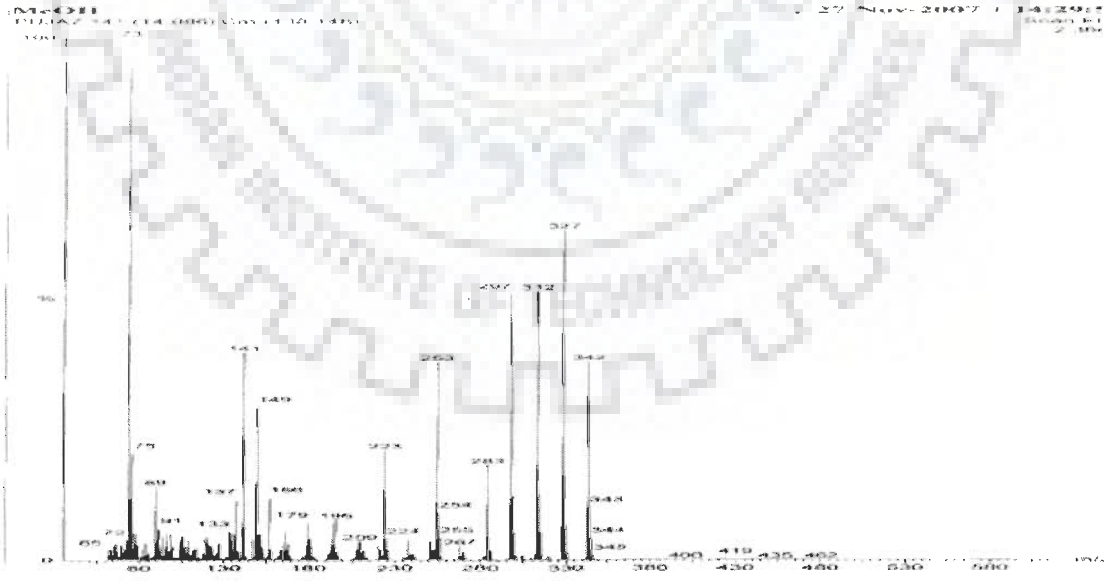

Fig 3.13b. GC-MS spectra showing major peak at 16. 12 min in TIC of PEH fraction. showing characteristic mass spectra of Quericitin at 141, 263 and 327 m/z.

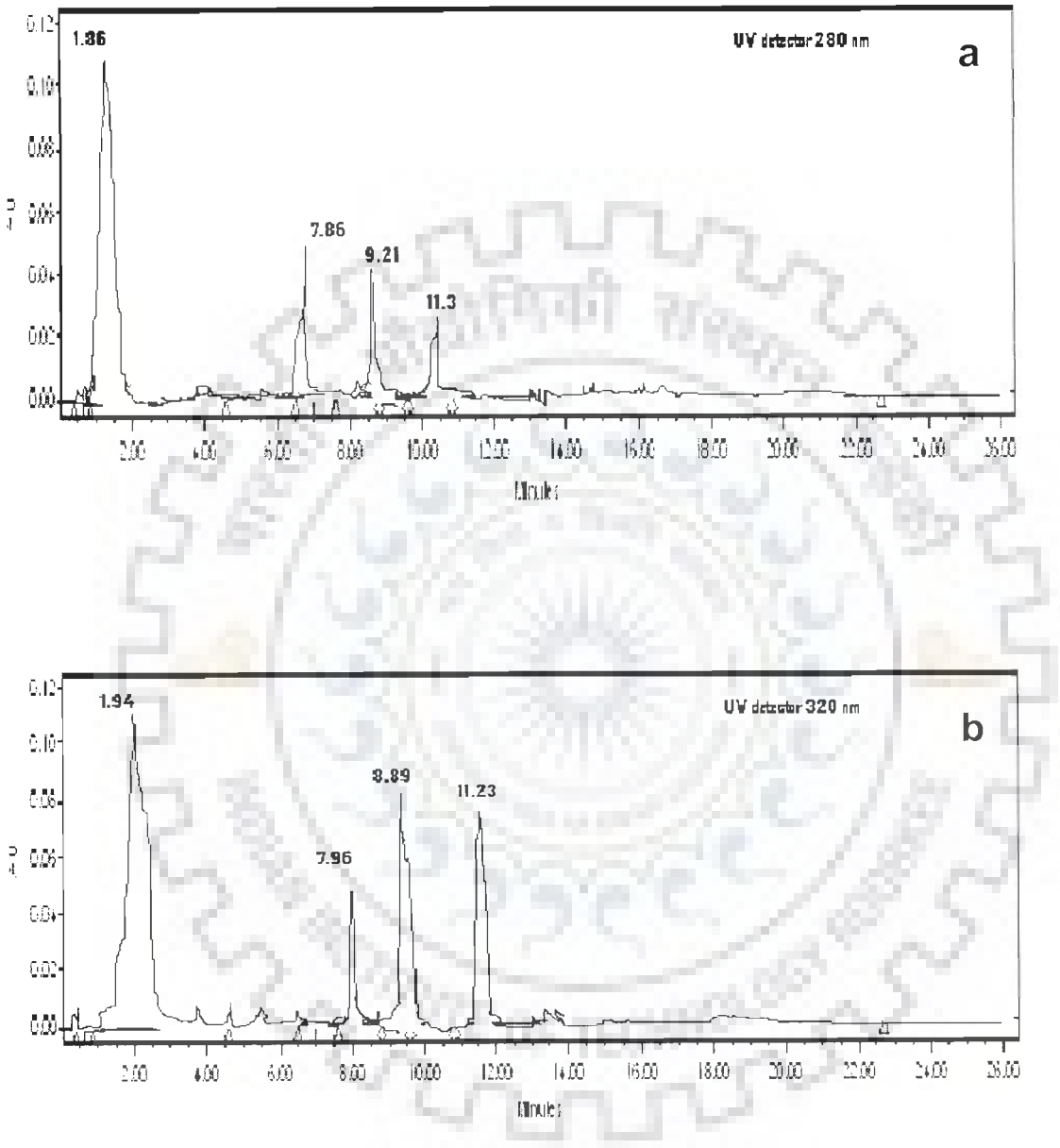

Fig. 3.14a and b Show the HPLC UV chromatogram A (280 nm) and B (320 nm) of *E. hirta* active fraction (PEH). The major peak at RT 7.86 (Gallic acid), 9.21 (ferulic acid) and 11.3 (quericitin) matched with standard compounds.

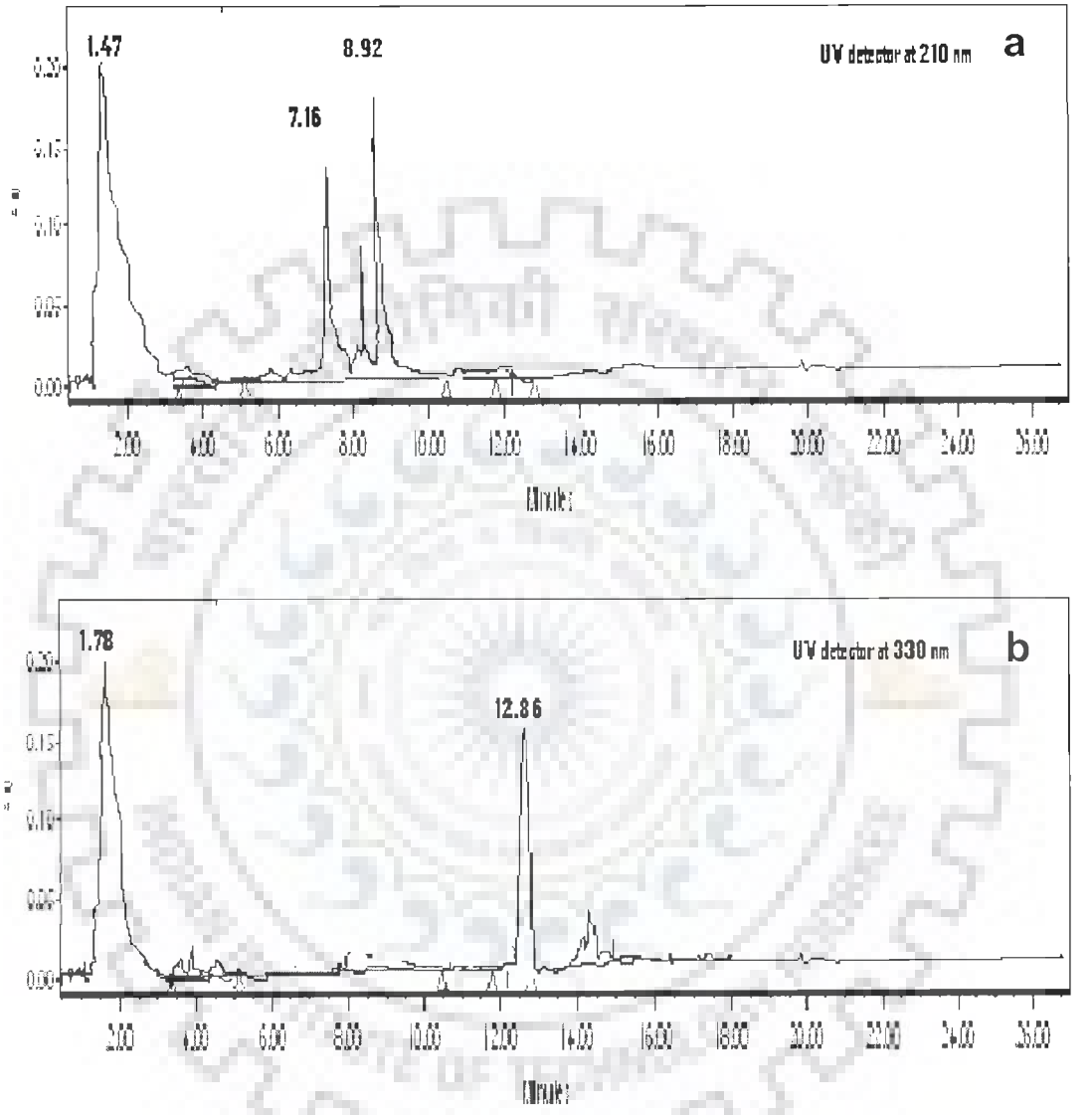

Fig 3.15a and b depicting the HPLC UV chromatogram of glycosides active fraction from *A. marmelos* (GAM) . A (210 nm) with major peak at RT 7.16 (sitosterol glucosides) and 8.92 (lupeol). Fig. 7 B depicts the HPLC UV chromatogram at (330 nm) with major peak at 12.86 (Rutin) was identified.

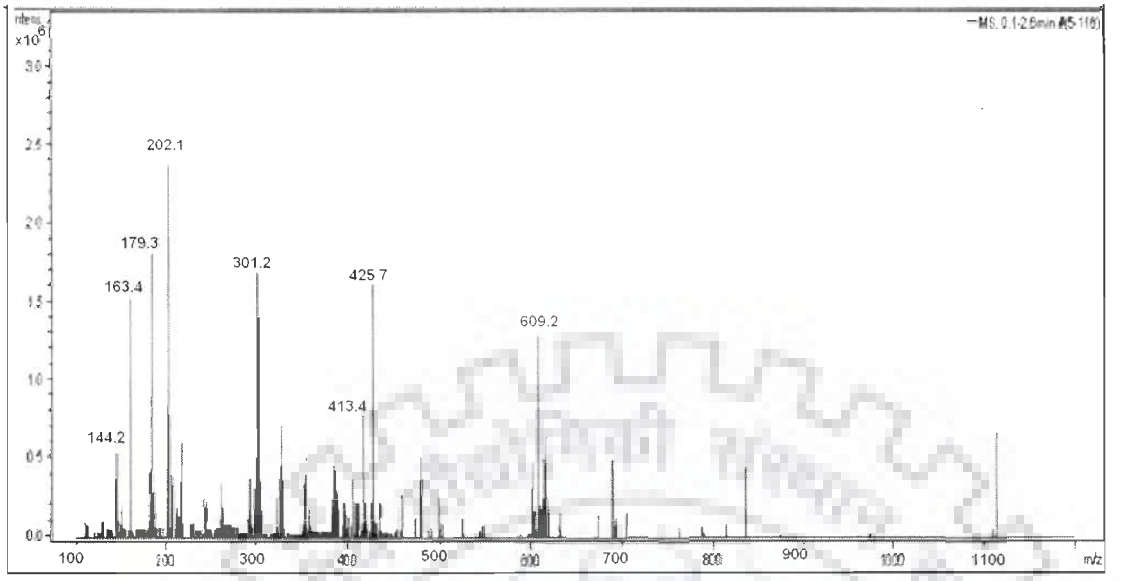

Fig. 3.16 Direct infusion ESI-MS spectra of GAM fraction in negative ion mode and the scanning range was 100-1100 m/z. [M-H].

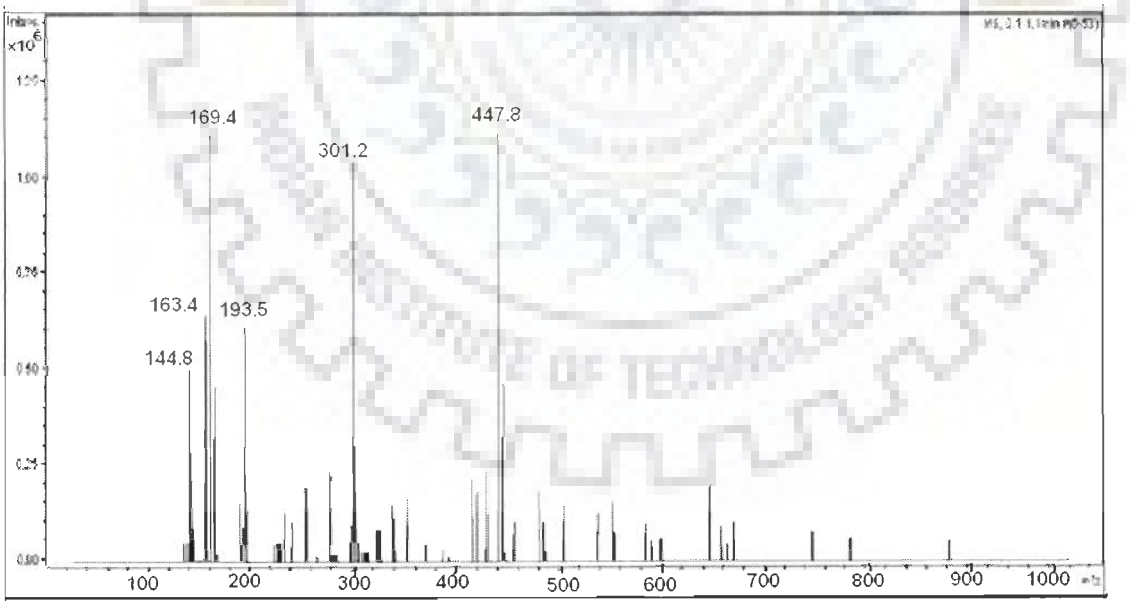

Fig. 3.17 Direct infusion ESI-MS spectra of PEH fraction in negative ion mode and the scanning range was 100-1000 m/z. [M-H].

Evaluation of Antioxidant Activity and Protective Potential of GAM and PEH against Oxidative Damage to Biomolecule

4.1 Introduction

ROS in the form of superoxide ($O_2^{\bullet-}$), Hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•) mostly attacks biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury ultimately resulting in diseases (Valko *et al.*, 2007; Halliwell *et al.*, 2007). Although the organism possesses defense mechanism to arrest the damaging properties of ROS, however, continuous exposure to chemicals and oxidative stress may lead to an increase in the amount of free radicals in the body beyond control, and cause irreversible oxidative damage (Arouma, 1998; Jackson, 1999; David *et al.*, 2000; Valko *et al.*, 2004). The extent of damage caused to particular biological molecules depends on the type of ROS species and its concentrations. The generation of free radicals and their several classes showing different reactivity and attributes towards oxidative damage to biomolecules and cellular functions is widely studied (Andersen, 2004; Loft, 2008). Further, it has been widely conceived that free radical mediated oxidative damage to biomolecules including DNA, protein and lipid is one of the noticeable pathways leading to oxidative induced cell death and diseases development (Sies, 1997; Valko *et al.*, 2007). There are several reports emphasizing the generation of several marker oxidation products during oxidative damage to biomolecules (Dalle-Donne *et al.*, 2006). It has been reported that some agents are very prompt to inflict damage to biomolecules like hydroxyl radicals, transition metals like iron and copper, hydrogen

peroxide, peroxyl radicals and trichloromethyl radical (Moller and Loft, 2006). Both part of DNA including sugar as well as nucleotide bases are crucial molecular target for free radical mediated oxidative damage. Free radicals cause extensive base modification as well as single-strand breaks in DNA (Marnett, 2000). Oxidative modification of protein include free radical mediated peptide cleavage, formation of protein cross linkage due to reaction with lipid oxidation product, formation of protein carbonyl, loss of sulfahydral groups and amino acid modification (Stadman, 2001; Levine and Stadman, 2001). All these modifications can be used as markers of protein damage by free radicals *in vivo* and *in vitro* study. A number of marker products like malonaldehyde and isoprotanes are formed during lipid peroxidation and are used as markers in assessment of lipid peroxidation level (Lovell *et al*, 1995). Therefore, there is need for sensitive and reliable tools to monitor the biomarkers produced to due oxidative damages of biomolecules. In the recent decades, there has been development of several analytical methods for the measurement of such marker *in vitro* as well as *in vivo* experiments, so that we could evaluate the level of protection by interest antioxidant agents against oxidative damage to biomolecules (Halliwell *et al.*, 2007).

Role of antioxidants in protecting our body against damage caused by ROS is well established (Halversen *et al.*, 2002; Dragland *et al.*, 2003; Pham-Huy *et al.*, 2008; Ali *et al.*, 2008). Therefore, antioxidants have great relevance as prophylactic and therapeutic agents in diseases in which oxidants are implicated (Halliwell, 1999; Frei, 2004, Shen *et al.*, 2007). In this context the role of plant derived natural antioxidants have been appreciated and encouraged to prevent such implications (Benzie, 2006). As it is known fact that plant derived natural antioxidants belongs to several classes of phytochemicals mainly phenolic, glycosides, terpenoids and

saponins etc, which are reported to show different extent of antioxidant activity and mechanism towards free radicals (Rice-Evans, 2000; Arouma, 2002; Koleckar *et al.*, 2008). On the basis of varied nature of free radical *in vivo* and *in vitro* conditions, the protective mechanism against such free radicals is suggested to be different for structurally varied natural antioxidant agents (Halliwell, 1996; Sanchej-Moreno, 2002; Shahidi, 2008).

Due to diversified nature of antioxidant and their different mechanisms towards free radicals, the evaluation of antioxidant properties is not an easy task. Many methods can be used to determine their activity and during determination substrates, conditions, analytical methods and concentration can affect the estimated activity (Nyska and Kohen, 2002; Benzie, 2003). In this direction, *in vitro* systems are easier, faster and more cost-effective compared to traditional bioassays in *vivo*. Therefore, test of the direct antioxidant activity *in vitro* is useful, because if a substance that is poorly effective *in vitro* will not be better *in vivo* (Frei, 1999; Aruoma, 2003). It can also be evaluated about some possibility of damaging effects. However, the true antioxidant potential of a preparation or pure compounds could not be rely solely on a single *in vitro* assay. Therefore, it is suggested and logical that the antioxidant efficacy of a particular preparation or compound need to be evaluated using a battery of *in vitro* antioxidant assay involving different mechanisms including hydrogen donation ability, chain breaking property, metal chelation and modulators of innate antioxidant defense system (Sanchej-Merino, 2002).

Keeping the above logic and facts in mind, the aim of the present study was to assess the antioxidant efficacies of glycoside enriched fraction from *A. marmelos* (GAM) and phenolic enriched fraction from *E. hirta* (PEH) described previously in (Chapter 3). Attempts have been made to evaluate the antioxidant activity of fraction

GAM and PEH using a battery of *in vitro* assays. Their protective potential was also investigated against oxidative damage to DNA, protein and lipid employing spectrophotometric, electrophoresis and immunoblot techniques.

4.2 Material and Method

4.2.1 Material

All common chemicals and solvents used in the present study were of analytical grade with highest purity and purchased from SRL, S.D Fine, Himedia and MERCK India Ltd. Calf thymus DNA and pUC18 plasmid were purchased from Bangalore Genei Pvt Ltd. India. Primary anti-DNP antibody and secondary antibody goat anti-rabit IgG were purchased from Sigma Chemical Co (St Louis MO, USA) unless otherwise mentioned.

4.2.2 Evaluation of *in vitro* antioxidant activity of antioxidant constituents from *A. marmelos* (CGAM & GAM) fraction and *E. hirta* (CPEH & PEH)

The *in vitro* antioxidant potential of (CGAM, GAM) from *A. marmelos* and (CPEH, PEH) from *E. hirta* was evaluated using a battery of *in vitro* based assay including free radical scavenging, hydroxyl radical, superoxide radical, hydrogen peroxide and metal chelating assay and are described below separately.

4.2.2.1 Free radical scavenging activity of (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The free radical $ABTS^{*+}$ (2, 2'-azinobis 3-ethyl benzothiazoline-6-sulfonic acid) scavenging assay was performed according to standard protocol by Re *et al.*, (1999) with slight modifications. The free radical 2,2'-azinobis 3-ethyl benzothiazoline-6-sulfonic acid ($ABTS^{*+}$) was produced reacting ABTS solution (7 mM) with (2.45 mM) potassium persulfate and mixture was allowed to stand in dark for 12-16 hours before use. For aqueous extract ABTS was diluted with PBS (7.4 pH)

to an absorbance of 0.700±0.002 at 734 nm and for methanolic extract diluted with ethanol and Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid) was used as standard for calibration curve and activity was expressed in terms of mM TEAC (Trolox Equivalent Antioxidant Capacity) value equivalent to 1 mg per ml concentration of CGAM, GAM, CPEH and PEH.

Free radical scavenging activity was also performed by DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay as described by Brand-Williams *et al.*, (1995). In this assay, 2 ml of $6x10^{-5}$ M methanol solution of DPPH was mixed with 10 µl of (CGAM, GA, CPEH and PEH) with concentration range (0, 5, 10, 20, 30, 40 and 50 mg per ml). The decrease in absorbance was recorded at 517 nm and inhibitory concentration value (IC_{50}) was calculated and results are presented in terms of IC_{50} µg per ml. The standard BHA was used as positive control against DPPH free radical scavenging assay.

4.2.2.2 Ferric Reducing Antioxidant Power (FRAP assay) determination of (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The ferric reducing antioxidant power (FRAP) is more precise interpretation of antioxidant potential of a sample in both aqueous and organic solvents medium. The FRAP assay was performed as per procedure described by Benzie and Strain (1996) with slight modification. The FRAP reagent was prepared by mixing acetate buffer (pH 3.6), 10 m mol TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 m mol HCl and 20 mmol ferric chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was warmed to 37^0C for one hour in water bath before use. The varied concentration (0.2, 0.4, 0.6. 0.8, 1.0 mg per ml) of antioxidant samples were added to 3.0 ml of the FRAP reagent. The absorbance of the reaction mixture was taken at 593 nm after 6 min. The standard gallic acid solution with concentration

range (100 µM-2000 µM) was used for calibration curve preparation. The FRAP value of antioxidant samples was expressed in terms of µmol GAE per g extract.

4.2.2.3 Hydroxyl radical scavenging potential of (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The hydroxyl radicals were generated on the basis of Fenton reaction as per the method of Halliwell *et al.*, (1987) with slight modification. In brief, the reaction mixture included, 10 mM KH_2PO_4-KOH, pH 7.4, 100 µM Fe $(SO_4)_2$ $(NH_4)_2$-EDTA, 1.42 mM H_2O_2, 1 mM ascorbic acid and 2.8 mM deoxyribose in a final volume of 1.0 ml. The same hydroxyl radical generation system was allowed to run in the presence or absence of half an hour preincubation with varied concentration of CGAM and CPEH (100, 150, 200 and 250 µg per ml) and for GAM and PEH (25, 50, 75 and 100 µg per ml) in final concentration. Along with antioxidant constituents, Trolox was used as positive standard. The same deoxyribose degradation experiment was carried out without the addition of ascorbic acid as reducing agent, to assess the prooxidant activity of the tested antioxidants for a given concentration. The extent of deoxyribose degradation due to hydroxyl radical was estimated directly from the aqueous phase by the TBA method with slight modification (Okhawa *et al.*, 1979).

4.2.2.4 Superoxide radical scavenging potential determination of (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The super oxide radical scavenging activity was determined using the method described by Kakkar *et al.*, (1984) with slight modification. Solutions containing 156 µM NBT dissolved in 50 mM phosphate buffer, pH 7.4, 468 µM NADH and varied concentration (50, 100, 150, 200, 250 and 300 µg per ml) of antioxidant samples were mixed and the reaction started by adding 100 µL of 60 µM PMS solution. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was

measured against control samples (without NADH). All tests were performed in triplicate. The percentage of scavenging activity (%) was calculated as follows: Scavenging activity = [1-(absorbance of sample at 560 nm)/ absorbance of control at 560 nm)] x 100. Trolox was used as positive control in this assay.

4.2.2.5 Metal chelating potential of (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The metal chelating effect of CGAM, GAM, CPEH and PEH was determined by the ferrous ion chelating assay as per the modified method of Dinis *et al.,* (1994). In brief, the reaction mixture contained varied concentration (20, 40, 60, 80, 100 and 120 µg per ml) of CGAM, CPEH, GAM and PEH sample, 40 µl of 2 mM $FeCl_2$ and 80 µl of 5 mM ferrozine to activate the reaction mixture. After vortex, the reaction mixture was incubated at room temperature for 10 min, and its chelating activity was measured at 562 nm. EDTA and BHA were used as positive standard. The metal chelating effect was calculated by using the following equation:

$$(\%)\ \text{Chelating effects} = \left(1 - \frac{\text{Absorbance of sample at 562 nm}}{\text{Absorbance of control at 562 nm}}\right) X100$$

4.2.2.6 Hydrogen peroxide scavenging activity determination of (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The hydrogen peroxide scavenging abilities of CGAM, CPEH, GAM and PEH and PEH were determined according to the method of Ruch *et al.,* (1989) with minor modification. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). The varied concentration (10, 20, 40, 80, 200 µg per ml) of antioxidant samples in 3.4 ml phosphate buffer (pH 7.4) was added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was

containing the phosphate buffer without H_2O_2. The percentage H_2O_2 scavenging ability of samples and positive standard BHA, glutathione and Trolox was calculated according to formula given below.

%Scavenged $H_2O_2 = (A_0 - A_1) / A_0 X100$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples.

4.2.3 Protective potential of GAM and PEH constituents against oxidative damage to DNA.

Different analytical methods were carried out to evaluate the protective potential of GAM and PEH constituent against oxidative damage to DNA. Among different methods available to determine protective role against DNA damage, these methods including spectrophotometric MDA determination, ethidium bromide intercalating and agarose gel electrophoresis techniques are widely used.

1989).

4.2.3.1 Spectrophotometric determination of calf thymus DNA oxidation using TBARS method

Spectrophotometric study to evaluate protective potential against oxidative damage to Ct. DNA was studied using the oxidation reaction mixture containing Ct. DNA (1 mg per ml) and metal catalyzed oxidation (MCO) system (Fenton reaction) [200 μM $FeSO_4$ and 500 μM H_2O_2] in final concentration. The oxidation reaction was carried in the presence or absence of varied concentration of GAM and PEH (25, 50, 100, 150 and 200 μg per ml) and positive standard querecetin (75 μg per ml). After the oxidation reaction was over, the TBARS production was determined employing TBA-MDA estimation by (Okhawa *et al.*, 1979) with slight modification.

4.2.3.2 Ethidium bromide intercalating assay for assessment of protection against oxidative damage to DNA

The ethidium bromide (EB) binding assay is considered to work on the formation of a fluorescent complex between double-stranded DNA and EB and was used performed as described (Stoewe and Prutz, 1987). In brief, reaction mixture contained 20 mM phosphate buffer (pH 7.0), 1 mg per mL calf thymus DNA and metal catalyzed oxidation (MCO) system (Fenton reaction) [200 µM $FeSO_4$ and 500 µM H_2O_2]. The oxidation reaction was carried in the presence or absence of varied concentration of GAM and PEH (25, 50, 100, 150 and 200 µg per ml) and positive standard querecetin (75 µg per ml). The reactions were carried out at 37°C for 10 min and terminated by the addition of EDTA to a final concentration of 10 mM. After incubation, 4 µl of 1 mM EB solution was added and fluorescence spectra and intensity was recorded with a spectrofluorometer (VARIAN) with excitation at 510 nm and emission at 590 nm. The reduction in fluorescence was used to measure the extent of DNA damage.

4.2.3.3 Electrophoretic study on oxidative damage to Calf thymus DNA induced by MCO system.

The reaction mixture contained 4 µl of calf thymus DNA (0.2 mg per ml), oxidation system MCO [$FeSO_4$ (2 µl, 1 mM) and H_2O_2 (4 µl, 1 mM)]. The oxidation reaction was carried out in the presence or absence of varied concentration of GAM and PEH (10, 20, 40 and 60 µg per ml) and positive standard querecetin (50 µg per ml). Incubation was allowed for 15 min at 37^0C. After incubation, the reaction was stopped with 100 µM EDTA (final volume) and electrophoresed onto 1% agarose gel as per the standard protocol Sambrook *et al.*, (1989).

4.2.3.4 Supercoiled to Nicked Circular-Conversion (SNCC Assay) using pUC18 plasmid

The SNCC reaction mixture contained DCC (double closed circular) pUC18 plasmid (0.5 µg), oxidation system MCO [$FeSO_4$ (2 µl, 1 mM) and H_2O_2 (4 µl, 1 mM). The reaction mixture was incubated at 37°C for half an hour. The oxidation reaction was carried in the presence or absence of varied concentration of antioxidant sample (10, 20, 40 and 60 µg per ml) and positive standard Querecetin (50 µg per ml). After the reaction was over, the plasmid samples were run on 1% agarose gel as per the standard protocol. After staining with ethidium bromide, the plasmid bands were visualized, photographed and analyzed with the Gel Documentation system (Bio-Rad, Hercules, CA, USA). The level of plasmid nicking was expressed as % DNA in relaxed form of plasmid (Form II).

4.2.4 Protective potential of GAM and PEH against oxidative damage to protein

The protective potential of GAM and PEH was evaluated using spectrophotometric, FTIR, SDS-PAGE electrophoresis and immunoblot techniques.

4.2.4.1 Oxidative damage reaction methods to BSA

Oxidation reaction mixture contained oxidation system MCO (Halliwell *et al.*, 1987) [ascorbic acid (0.8 mM)+EDTA (0.4 mM)+$(NH_4)_2Fe(SO_4)_2$, (0.4 mM)+H_2O_2 (0.5 mM)] in 50 mM phosphate buffer, pH 7.4 and the BSA (1 mg per ml). The assay was performed in the presence or absence of varied concentration (25-200 µg per ml) of GAM and PEH. In the control tube (without generation of $OH^{\bullet}$), the H_2O_2 was replaced by water. After 30 min of incubation at room temperature, 250 µl of 20% trichloroacetic acid was added and the mixture was centrifuged at 1000Xg 30 min per 4°C. The supernatant was discarded and the pellet was resuspended in 500 µl of 0.1 M NaOH.

4.2.4.2 DNPH based spectrophotometric determination of protein carbonyl against BSA oxidation

The total carbonyl content was measured after reaction with DNPH (2, 4–dinitrophenylhydrazine) in 2N HCl. The procedure was adopted from Levine *et al.*, (1995) with some modification. The oxidized BSA pellets obtained above resuspended in 500 µl of 50 mM phosphate buffer, pH 7.4 was derivatized with 500 µl of dinitrophenyl hydrazine (DNPH) dissolved in (2.5 mol per l HCl). Both tubes were incubated for 45 min at room temperature. In order to precipitate proteins, 500 µl of (20% w/w) trichloroacetic acid was added and centrifuged for 10 min at 1000×*g*. The sediment was washed three times with 500 µl of ethanol: ethyl acetate (1:1). After the final rinse, the samples and blank were centrifuged for 10 min at 2000×*g* and the sediment was dissolved in 500 µl of 6 M guanidine in 20 mM KH_2PO_4, with final pH 2.3. The absorbance of BSA-DNP conjugates were recorded at 375 nm. The final data were expressed as nmol of protein carbonyl per mg of proteins (ε_{375} = 22,000 $mol^{-1} \cdot cm^{-1}$) for the DNPH derivates).

4.2.4.3 Determination of protein carbonyl amount using FTIR technique in BSA oxidation

The same BSA oxidation reaction mixture was analyzed for their protein content using protein carbonyl peak formation in the secondary structures of freeze-dried oxidized BSA samples with an FT-IR system (Thermo Nicollet). FTIR Spectra of the solids were obtained from pressed disks containing approximately 1 mg protein and 250 mg potassium bromide (256 scans). The spectra were scanned from 4000-500 cm^{-1}.

4.2.4.4 Analysis of oxidative damage to BSA and their inhibition by GAM and PEH using SDS-PAGE technique

To determine protein damage by reaction mixture containing oxidized BSA was stopped and precipitated with 20% TCA and pellets were subjected to SDS-PAGE. Forty micrograms of BSA (from resuspended pellet) were mixed 1:1 with loading buffer (10% glycerol, 2% SDS, 25 mM Tris–HCl (pH 6.8), 5% mercaptoethanol, and 0.1% bromophenol blue) and heated at 100°C for 1 min. The protein sample was loaded in a 12% polyacrylamide gel and electrophoresed at 100 V. After running, gels were stained with 0.2% Coomassie brilliant blue for 1 hour, distained and visualized in Bio-Rad gel documentation system for densitometric analysis using Quantity one software. The area of each band was obtained and compared with respect to control band (BSA without antioxidant constituents and H_2O_2) and 0% scavenging band (BSA only with H_2O_2 for oxidation).

4.2.4.5 Study on oxidatively damaged BSA using immunoblot technique

The same oxidatively damaged BSA Samples was separated on 12% SDS-PAGE according to standard Laemmli (1970) and determination of protein carbonyl was done with the help of western blot technique using anti-DNP primary antibody. The gels were then equilibrated in electrotransfer buffer (25 mM Tris, 191 mM Glycine, 15% methanol) and electroblotted to nitrocellulose membranes as per standard protocol Towbin *et al.*, (1979). Following the electroblotting procedure, the nitrocellulose membrane was derivatized in a solution of 2, 4-dinitrophenylhydrazine (0.5 mM) in 2N HCl for exactly 5 min and then membrane was washed three times in 2N HCl (5 min each). The DNPH derivatized membrane was immunochemically stained with a rabbit polyclonal anti-DNP primary antibody (1:2000 dilution) and

HRP conjugated goat anti- rabbit IgG (1:5,000 dilution) for 1 hour. The blots were visualized using DAB/H_2O_2 chromogenic substrates as per standard method.

4.2.5 Lipid peroxidation in rat liver microsomes and their inhibition by GAM and PEH constituents

Lipid peroxidation in rat liver microsomes was induced by iron/ascorbate and CCl_4/NADPH reaction system (Mc Gowan *et al.*, 1983). In brief, rat liver microsomes protein (2.0 mg), iron/ascorbate [($FeSO_4.7H_2O$, 200 μM) and ascorbate (200 μM)] and CCl_4/NADPH system in the absence or presence of GAM and PEH constituents (25–200 μg per ml) in 50 mM phosphate buffer, pH 7.8 to a final volume of 2 ml, were incubated at 30°C for 90 min. Reference tubes and reagent blank were also run simultaneously. After incubation, all the sets were assayed for lipid peroxide content by their reaction with thiobarbituric acid (Okhawa *et al.*, 1979). The lipid peroxide content in microsomes was expressed in nmol MDA per mg protein.

4.2.6 Statistical analysis

All *in vitro* based antioxidant evaluation studies were carried out in triplicate and six replicates respectively, experimental results represents the mean of three/six identical studies. Standard deviation (SD) and standard error (SE) were calculated using following formula:

$$SD = \frac{\sqrt{\sum X^2}}{N}$$

$$SE = \frac{SD}{\sqrt{N}}$$

4.3 Results and Discussion

The previous chapter described and discussed about the antioxidant potential of *A. marmelos* and *E hirta*. The active fractions from these plants were purified by liquid-liquid partitioning and column chromatography. The active fractions were named as glycosides enriched fraction from *A. marmelos* (GAM) and phenolic enriched fraction from *E. hirta* (PEH), respectively. The major constituents of the fraction were also identified as described previously in Chapter 3. The antioxidant potential of a number of plant derived antioxidants, such as α-tocopherol (Vitamin E), ascorbic acid (Vitamin C), β-carotene, glycoside, flavonoid and phenolic acids have been evaluated and reported to have beneficial effects in protecting against oxidative stress related diseases (Finkel and Holbrook, 2000; Brash and Harve, 2002; Cooke *et al.*, 2003, Hseu *et al.*, 2008; Yang *et al.*, 2008). Although, the medicinal potential and traditional uses of *A. marmelos* and *E. hirta* have been well documented but their antioxidant potential have not been reported so for. Therefore, antioxidant efficacy of the glycosides enriched fraction (GAM) of *A. marmelos* and phenolic enriched fraction of *E. hirta* (PEH) have been evaluated in the present study using various established and commonly used *in vitro* assay methods. There are several reports showing evidences of protective potential of plant derived natural antioxidants against oxidative damage to DNA, protein and lipid. In the present study, the protective potential of GAM and PEH was evaluated oxidative damage to biomolecules using suitable *in vitro* model techniques.

4.3.1 *In vitro* antioxidant potential evaluation of CGAM & GAM) from *A. marmelos* and CPEH & PEH) from *E. hirta*

In vitro antioxidant activity of GAM and PEH were evaluated using free radical scavenging and metal chelating power assay.

4.3.1.1 Free radical scavenging activity evaluation using DPPH and ABTS assay

The free radical scavenging activity of (CGAM, GAM) from *A. marmelos* and (CPEH, PEH) from *E. hirta* were studied using DPPH and ABTS assays and results are illustrated in Table 4.1. The calibration curve for standard antioxidants BHA against DPPH free radical and TEAC standard for ABTS are presented in Fig. 4.1.A & B. The free radical scavenging activity against DPPH free radical is expressed in terms of IC_{50} concentration (μg per ml). The data obtained showed the free radical scavenging activity with IC_{50} value in decreasing orders of CGAM (10.43 ±0.76) < CPEH (9.46 ±0.87) < GAM (3.89 ±0.21) BHA< (3.21±0.18)< PEH (2.87 ±0.46). The result clearly indicates that fractionated antioxidant constituents GAM and PEH possessed strong three fold free radical scavenging activity against DPPH free radical in comparison to their crude preparations (CGAM and CPEH). The inhibitory concentration values for both GAM and PEH constituents were found to be statistically equivalent with standard BHA compound.

The other free radical scavenging method the ABTS assay showed result similar to those obtained by the DPPH assay. The free radical scavenging activity against ABTS free radical is expressed in terms of mM Trolox equivalent to 1 mg per ml concentration of antioxidant. The TEAC values for antioxidant constituents were found in order CGAM (0.89±0.1) < CPEH (0.89±0.01) <GAM (2.84±0.18) <PEH (3.07±0.19) <BHA (3.85±0.25). The finding indicated that fractionated GAM and PEH constituents demonstrated strong antioxidant activity in terms of mM Trolox concentration to scavenge the ABTS free radical cation.

Both the GAM and PEH constituents showed discernible antioxidant activity in both ABTS and DPPH free radical scavenging assay, which primarily evaluates proton radical-scavenging ability. DPPH is one of the compounds that possess a

proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi *et al.*, 1998). Further it is well accepted that the DPPH free radical-scavenging by antioxidants is due to their hydrogen-donating ability (Chen and Ho, 1995). In the present study, the GAM and PEH showed a concentration dependent scavenging of DPPH radical and ABTS, which is attributable to its hydrogen-donating ability. Since the active constituents of GAM (rutin, sitosterol and glucosides) and PEH (gallic acid, ferulic acid and quercetin) are identified and belonged glycosides and phenolic class, thus the antioxidant activities of these preparation are due to the hydrogen donating ability of their glycosides and phenolics constituents. This is well agreement with earlier reports where antioxidants activities of plants are shown due to their phenolics and glycosides constituents (Ara *et al.*, 2005; Gulcin, 2006; Koleckar *et al.*, 2008). It has been suggested that antioxidative activity of phenolic and glycosides are related to their conjugated rings and hydroxyl groups (Middleton *et al.*, 1996, Lien *et al.*, 1999; Frankel and Meyer, 2000), so it is not surprising that GAM and PEH constituents demonstrated strong free radical scavenging activity. Plant phenolics and flavonoids, are one of the most diverse and widespread groups of natural compounds have been extensively studied for antioxidant activity (Amarowicz, 2000; Bors, 2001; Lee *et al.*, 2008; Vuckics *et al.*, 2008). These compounds possess a wide spectrum of chemical and biological activities including radical-scavenging properties. A strong relationship between total phenolic content and antioxidant activity in fruits, vegetables and grain products has been reported (Sanchej-Merino *et al.*, 2000; Dorman, 2003; Halliwell, 2006; Fiorentino, 2007).

4.3.1.2 Measurement of total reducing power of CGAM & GAM from A. marmelos and CPEH & PEH from E. hirta

Ferric reducing antioxidant power of four antioxidant constituents CGAM & GAM from *A. marmelos* and CPEH & PEH from *E. hirta* was determined by FRAP assay and results are depicted in Fig 4.2a. The FRAP value is expressed in terms of µmol GAE per g of extracts, which is calculated with the help of gallic acid calibration curve depicted in Fig 4.2b. The results shows analysis suggest that both fractionated GAM and PEH possessed three to four fold increased reducing power with respect to their crude extracts. Result of FRAP assay indicated that one gram of GAM and PEH constituent contains 1.25±0.10 and 1.42±0.11 mmol gallic acid equivalent (GAE) reducing power. From these results, it could be concluded that both GAM and PEH have strong antioxidant potential in terms of reducing power. The results were comparable with those the known potential plants reducing power such as *Gingko biloba* (0.62 mmol), *Panax ginseng* (0.61 mmol) and *Geranium pratensis* (2.22 mmol) GAE per g extract (Miliauskas *et al.*, 2004).

FRAP assay measures the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) in the presence of antioxidants. Because the ferric-to-ferrous iron reduction occurs rapidly with all reductants with half reaction reduction potentials above that of $Fe^{3+/}Fe^{2+}$, the values in the FRAP assay will express the corresponding concentration of electron-donating antioxidants. In this method, FRAP was used to determine the total reducing power of the samples for several reasons. The FRAP assay is the only assay that directly measures antioxidants or reductants in a sample. The other assays are more indirect because they measure the inhibition of reactive species (free radicals) generated in the reaction mixture and these results also depend strongly on the type of reactive species used. The FRAP assay, in contrast, uses antioxidants as

reductants in a redox-linked colorimetric reaction. Furthermore, the other assays, but not the FRAP assay, use a lag phase type of measurement. This has been difficult to standardize in previous experiments and has generated varying results among different laboratories. In the FRAP assay, pretreatment is not required, stoichiometric factors are constant and linearity is maintained over a wide range. Due to the above reasons the FRAP assay suggested to be more reliable and effective (Benzie and Strain, 1996).

Different studies have indicated that there is a direct correlation between antioxidant activity and reducing power of certain plant extracts (Duh *et al.*, 1997; Duh, 1998). The reducing properties are generally associated with the presence of reductants (Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductants are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Thus the reducing power of antioxidant candidate is sufficient interpretation for validating its *in vitro* antioxidant activity (Benzie and Strain, 1996; Duh and Yen, 1999). Our data on the reducing power of GAM and PEH suggest that it is likely to contribute significantly towards the observed antioxidant effect. Besides, it has been observed from the earlier studies that constituents possessing marked free radical scavenging activity also found to have strong reducing power potential (Arouma, 2002). Our data are in well agreement with earlier observation and the both GAM and PEH showed strong free radical scavenging activity and high reducing power as well. The obtained data are sufficient to prove that these antioxidant constituents from *A. marmelos* and *E. hirta* exhibited strong free radical scavenging activity and correlated reducing power activity. The activity is attributed to their glycosides (rutin, sitosterol glucosides and lupeol) for *A. marmelos*

and phenolics (gallic acid, ferulic acid and quercitin) in case of *E. hirta* constituents. This observation is in agreement to earlier studies where the phenolic and glycosides constituents from sources like *Panax ginseng* and Licorice could serve as a good source of reducing power and antioxidant activity (Middleton, 2005). In number of plants the high reducing power observed are linked to their phenolics (Fraser, 1999; Mantle *et al.,* 2000), flavonoids (Cook and Samman, 1996; Kakohenen *et al.,* 1999; Amarowicz *et al.,* 2000) and glycosidic nature of compounds (Kitts, 2000; Hsue *et al.,* 2008).

4.3.1.3 Hydroxyl radical scavenging potential and prooxidant activity of CGAM&GAM from A. marmelos and CPEH&PEH from E. hirta

The effect of (CGAM, GAM) from *A. marmelos* and (CPEH, PEH) from *E. hirta* on oxidative damage on deoxyribose induced by Fe^{3+}/H_2O_2 was measured by the thiobarbituric acid method. Hydroxyl radical generated in the reaction mixture, attack sugar deoxyribose and degrade it into a series of fragments, some or all of which react on heating with thiobarbituric acid at low pH to give a pink chromogen. Thus the scavenging activity towards hydroxyl radical of a substrate added to the reaction mixture is measured on the basis of the inhibition of the degradation of deoxyribose (Halliwell, 1990; Aruoma *et al,* 1993). The generation of Hydroxyl in the Fenton-reaction is due to the presence of iron ions. Some compounds inhibit color formation in the deoxyribose assay, not by reacting with hydroxyl radical but by the chelating iron ions and preventing hydroxyl radical formation. In order to check the reaction mechanism is the direct radical scavenging and not by inhibition of the Fenton reaction by chelation, the assay systems contain EDTA. This system has been used in various earlier studies to evaluate the antioxidant activity of different phenolics fraction from different plants (Ghiselli *et al.,* 1998; Tazawa *et al,* 1999; Koleckar *et*

al., 2008). The hydroxyl radical scavenging activity results of (CGAM, GAM) and (CPEH, PEH) are shown in Table 4.2a and 4.2b, respectively. The hydroxyl radical scavenging potential is expressed in terms of inhibitory concentration values determined from concentration dependent decrease in absorbance at 532 nm. The lower values of IC_{50} (µg per ml) indicates higher efficiency to scavenge hydroxyl radicals. The inhibitory concentration values in decreasing order for antioxidant samples were CGAM (156.54±8.32) < CPEH (150.38±7.32) < GAM (47.83±2.18) < PEH (38.18±3.29) < glutathione (32.34±3.78). The obtained values for antioxidant constituents exhibited that GAM and PEH demonstrated three to four fold higher efficiency to scavenge hydroxyl radicals of their crude preparations CGAM & CPEH, respectively. This was equivalent or even better than the glutathione a well known antioxidant. When we compared GAM and PEH constituents' ability to control these radicals with established plant sources, it could reveal that these constituents are at par with plant extracts like *G. biloba* and Pycnogenol (Packer *et al.*, 1997) or even much better.

The hydroxyl radical is widely studied toxic free radicals due to their inflicting role in several diseases (Younes *et al.*, 2005; Mc Cord *et al.*, 2007) The protective role of antioxidants against these deadly free radicals have been established (Benzie, 2006, Brown and Rice-Evans, 2006). In the present results, the efficacy of GAM and PEH constituents to scavenge these radicals could serve the purpose to reduce the oxidative damage on biomolecules and ultimately protective role against physiological disorders.

4.3.1.4 Superoxide radical scavenging activity (CGAM, GAM) from A. marmelos and (CPEH, PEH) from E. hirta

The superoxide radical scavenging potential of CGAM, CPEH, GAM, PEH and Trolox is shown in Fig 4.3. The superoxide radical scavenging potential is presented in terms of IC_{50} values and results are shown in decreasing order CGAM (343.23), CPEH (321.43), GAM (231.87), PEH (189.75) and Trolox (69.47) µg per ml. The superoxide radical is considered to be one of typical free radicals which is involved in the generation of other deadly free radicals and indirectly help in the oxidative damage to biomolecules (Fridowich, 2001). The ability to scavenge superoxide radicals may be of one of the approach adopted by antioxidant constituents towards free radicals. In the present study, as superoxide radicals are generated for reduction of nitro blue tetrazolium (NBT) in the presence of PMS-NBT system, which is one of the standard methods. Although it is a relatively weak oxidant, it may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate peroxidation of lipids (Halliwell, 2006). Removal of superoxide in a concentration dependent manner by antioxidant constituents from *A. marmelos* and *E. hirta* may be attributed to the direct reaction of its constituents with these radicals or inhibition of the enzymes.

4.3.1.5 Metal chelating potential of CGAM&GAM from A. marmelos and CPEH&PEH from E. hirta

The results are presented in Fig 4.4 depicts the ferrous ion metal chelating potential of (CGAM and GAM from *A. marmelos*) and (CPEH and PEH from *E. hirta*) using spectrophotometric determination of ferrozine-metal complex absorbance at 512 nm and their metal chelating efficiency was converted to percentage metal chelating potential. Two reference metal chelators EDTA and BHA were used in this

experiment to compare the metal chelating efficiency of GAM and PEH constituents. As shown in Fig 4.4, the formation of the Fe^{2+}-ferrozine complex could not be completed and reduction in the absorbance was observed in the presence of CGAM, GAM, CPEH and PEH constituents along with reference standard, indicating that the GAM and PEH have metal chelating activity and capture ferrous ion. From the analysis of this concentration dependent metal chelation graph, we calculated the metal chelating efficiency in terms of effective concentration EC_{50}. The metal scavenging effect decreased in the order EDTA (EC_{50} 34.56)> PEH (EC_{50} 44.29) > GAM (EC_{50} 59.87)>CPEH (EC_{50} 80.18)>CGAM (EC_{50} 98.34). The findings from above experiment can be correlated with promising role of GAM and PEH constituents in the *in vivo* and *in vitro* protection against oxidative damage reaction mediated by iron and ascorbate. Chelating agents may inhibit lipid oxidation by stabilizing transition metals. Ferrozine can quantitatively form complexes with Fe^{2+}, however, in the presence of other chelating agents; the complex formation is disrupted, resulting to decrease in the red color. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (Dinis *et al.*, 1994; Rice-Evans, 2000). It was reported that the chelating agents that form σ bond with a metal are effective as secondary antioxidants, because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Duh *et al.*, 1999). Metal chelating capacity is important since it reduces the concentration of the catalyzing transition metal in lipid peroxidation via the Fenton reaction (Dinis *et al.*, 1994).

Generally, it is assumed that the ability of polyphenols and glycosidic antioxidant to chelate metals is associated for their antioxidant activity (Decker,

2001). The metal chelating potential of GAM and PEH are suggested due to their glycosides and phenolics components and as observed in various other studies (van Acker *et al.*, 1998; Kang *et al.*, 2005). Thus both the hydroxyl radical scavenging and metal chelating activity suggest that GAM and PEH could play an important role as antioxidants in protection against oxidative damage to molecules and oxidative stress related diseases.

4.3.1.6 Hydrogen peroxide scavenging activity of CGAM&GAM from A. marmelos and CPEH&PEH from E. hirta

The antioxidant constituents CGAM&GAM from *A. marmelos* and CPEH&PEH from *E. hirta* showed hydrogen peroxide scavenging activity and result are presented in Fig 4.5. The potential to scavenge hydrogen peroxide is expressed in terms of their IC_{50} (µg per ml). This value corresponds to the 50% reduction in hydrogen peroxide absorbance at 340 nm in the presence of different antioxidant constituents. The reference compound Trolox, BHA and Querecetin were used a standard hydrogen peroxide scavenger antioxidants. As, it is clear that among several toxic free radicals, hydrogen peroxide is also one of crucial toxic agents that need to be controlled *in vitro* as well as *in vivo* condition to avoid damage effects on biomolecules (Valko *et al.*, 2004). The antioxidant reported from several plant sources have been reported to be a good scavenger for hydrogen peroxide *in vitro* as well as *in vivo* condition (Wang *et al.*, 2000; Tuba and Gulcin, 2008). The ability of GAM and PEH antioxidants to scavenge hydrogen peroxide reported in the present work related to their phenolics and glycosides and was found to be equivalent to some other reports of antioxidants exhibiting the equivalent efficiency in terms of inhibitory concentration (Wang *et al.*, 1998).

4.3.2 Protective effects of GAM and PEH constituents against oxidative damage to DNA

Free radical mediated oxidative damage to biomolecules including DNA, protein and lipid is one of the noticeable pathways leading to oxidative induced cell death and diseases development (Imlay and Linn, 1988; Anderson, 2004). There are several reports emphasizing the protective role of antioxidants from plants sources against oxidative damage to biomolecules (Salles *et al.*, 1999; Russo *et al.*, 2001). In the present study the protective effect of GAM and PEH fraction against free radicals damage of DNA, protein and lipids have been evaluated.

4.3.2.1 Spectrophotometric evaluation of protective potential of GAM and PEH constituents against oxidative damage to DNA

One of the most reactive radical species that induce lesions in DNA is the hydroxyl radical (OH•). This species cause cell injury when they are generated in excess or the cellular antioxidant defense is impaired. When hydroxyl radical is generated adjacent to DNA, it attacks both the deoxyribose sugar and the purine and pyrimidine bases resulting into intermediates radicals, which are the immediate precursors for DNA base damage (Marnett, 2000). In living systems many of the hydroxyl radicals are generated from the transition ion dependent breakdown of hydrogen peroxide (Lean *et al.*, 1999).

The protective effect of GAM and PEH on oxidative damage induced by Fe^{2+}/H_2O_2 (Fenton-reaction) on Ct. DNA was measured by the TBA method. The MDA formation by hydroxyl radical action during DNA oxidation was determined in the absence or presence of varied concentration of GAM and PEH (25-200 µg per ml). The results are depicted in fig 4.6a and 4.6b. A stronger concentration-dependent

inhibition of DNA oxidation was observed. Nearly 34.53 % reduction was observed in MDA content at 75 µg per ml concentration which was comparable to standard glutathione (57.47% reduction in MDA content at 75 µg per ml). Earlier, numerous workers (Halliwell, 1987; Duh, 1998) have employed this system to assess the biological activity of various plant derived antioxidants in protection against biomolecules.

Oxidative DNA damage is generally regarded as carcinogenic and actively participates in many pathological processes, including cancer and aging (Halliwell and Gutteridge, 2000). Generally, it is assumed that the hydroxyl radical scavenging and metals chelating ability of antioxidants are account for their protective activity against oxidative damage to biomolecules (Halliwell, 1996). Since GAM and PEH found to have strong free radicals scavenging activity against (hydroxyl, super oxide and H_2O_2) and metal chelating ability as described and discussed above. It is suggested that GAM and PEH protection against oxidative damage to DNA could be synergistic action of their constituents both by scavenging free radicals as well as chelating the metals. It is further suggested that the protective action is due to the glycosides in GAM and phenolic components from PEH constituents. The antioxidant potential of flavonoid, phenolic and glycosides from several plants in chelation with Fe ions and their importance in protection against oxidative damage to DNA has been reported (Van Acker *et al.*, 1999; Hsieh *et al.*, 2005; Samarath *et al.*, 2008).

4.3.2.2 Fluorescent study on protective effects against oxidative damage to DNA

The protective effect of GAM and PEH against oxidative damage to DNA was also studied by checking EtBr DNA intercalating ability. The ethidium bromide intercalating fluorescent spectra showing protective effects of GAM and PEH constituents against MCO induced oxidation are depicted in Fig 4.7a and 4.7b. It has

been suggested that MCO induced oxidation leads to reduction in ethidium bromide intercalated fluorescent intensity. The results obtained from fluorescent spectra indicated that the reduction in peak intensity was significantly reversed to normal in presence of GAM and PEH. This could be possible that in the presence of GAM and PEH, there is less damage to DNA induced due to free radicals scavenging and inhibition of their generation by iron chelating. As suggested in the earlier section, the strong free radical scavenging and metal chelating activity of GAM and PEH constituents could be one of attributes to protect DNA from oxidative damage and more EtBr intercalation. There are several of antioxidant constituents demonstrated their protective effects against oxidative damage to DNA on the basis of same facts that they are capable of neutralizing free radicals (Yoo *et al.*, 2002; Serafini, 2006).

4.3.2.3 Agarose gel electrophoresis study on evaluation of protective effects against oxidative damage to DNA

Reactive oxygen species (ROS)-induced oxidative DNA damage producing a variety of modifications at DNA level including base and sugar lesions, strand breaks, DNA-protein cross-link and base-free sites (Halliwell, 1996; Valko *et al.*, 2004). In addition to deoxyribose, free radicals also damage DNA (Halliwell and Gutteridge, 2000). To demonstrate the protective effects against oxidative damage to DNA, the DNA was exposed to metal catalyzed Fenton reaction in absence or presence of various amounts of GAM and PEH. The gels photograph showing protective effects of GAM and PEH against oxidative damages to DNA are presented in Fig. 4.8a (GAM) and Fig. 4.9a (PEH). Their corresponding band intensity is presented in Fig. 4.8b and 4.9b. From the gel photograph, it was clearly visible that the presence of GAM and PEH caused reduction in the oxidation to DNA and the inhibitory concentration found to be 54.84 (GAM) and 47.65 (PEH) µg per ml, which is

statistically equivalent to the protective ability of standard glutathione showing 72.43% inhibtion at 50 µg per ml concentration.

The protective effects of both the GAM and PEH were also confirmed by the oxidation induced strand breakage or nick in SDCC pUC18 plasmid when exposed to Fenton reaction. The SDCC plasmid was exposed to oxidation in absence and presence of GAM and PEH. The agarose gel photographs showing the protective effect of GAM and PEH constituents towards Fenton reaction induced DNA damage are shown in Fig 4.10a and 4.11a, respectively. It was observed that the control pUC18 plasmid contains mostly supercoiled (form I) DNA and only a small amount of the relaxed form (form II) (lane 1). On the other hand in the presence of MCO agent supercoiled form was converted into the relaxed plasmid (form II, lane 2). The damage of plasmid DNA results in a cleavage of one of the phosphodiester chains of the supercoiled DNA and produces a relaxed open-circular form. Further cleavage near the first fracture results in linear double-stranded DNA molecules The Densitometric band intensity analysis relaxed form plasmid form (II) is presented in Fig. 4.10b and 4.11b.

The IC_{50} values were calculated from the band intensity data and results revealed that 65.74 and 73 µg per ml were sufficient to protect up to 50% oxidative damage to DNA. In our studies, GAM and PEH constituents produced the dose dependent DNA protection against the oxidative DNA damage induced by oxidant system including metal ions, hydrogen peroxide and peroxyl radicals (Salles *et al.*, 1999). This confirms that both GAM and PEH constituents exert a site specific protection, which could be suggested to its ability to quench such reactive oxygen species involved in oxidation to DNA. On the basis of above methods to evaluate protective potential against oxidative damage, it is suggested that both GAM and PEH

constituents exhibited remarkable protection against DNA oxidation. It could be proposed that these constituents may play important role in the prevention of several human diseases. The implication of DNA damage has been recognized in a large number of human diseases (Halliwell, 2007) and importance of antioxidants preparations from various plants have been reported to show protective effect against oxidative damage to DNA (Sultana *et al.*, 1995; Desmarchelier *et al.*, 1997; Toyakumi and Sagripanti, 2000; Russo *et al.*, 2000; Moller and Loft, 2006).

4.3.3 Protective effect of GAM and PEH constituent against oxidative damage protein

Exposure of proteins to free radicals particularly $OH^{\cdot}$ and $O_2^{\cdot-}$ or both leads to gross structural modifications. The oxidatively modified proteins may undergo spontaneous protein fragmentation and cross-linking or exhibit a substantial increase in proteolysis (Davies *et al.*, 1987; Stadtman, 2001). The principles of protein modification by ROS are well established as well as the characterized reaction products of protein interactions with $OH^{\cdot}$ and $O_2^{\cdot-}$ (Stadtman, 2001). The oxidative attack of the polypeptide backbone is usually initiated by hydroxyl radical. This has been experimentally determined by generating this radical using the radiolysis of water or from a metal-catalyzed cleavage of H_2O_2 (Dean *et al.*, 1997). This eventually leads to the formation of alkyl, alkoxyl and alkylperoxyl radical intermediates, which set the stage for cleavage of the peptide bond via several means. A wide variety of reactions between ROS and amino acid chains occur and all amino acids in proteins are susceptible to modification by OH. or by OH. plus $O_2^{\cdot-}$, however, tryptophan, histidine and cysteine are the most vulnerable (Davies *et al.*, 1987; Stadtman, 2001). In addition to fragmentation, the oxidation of lysine, arginine, proline and threonine residues may also yield carbonyl derivatives. The presence of carbonyl groups has

therefore been used as a maker of ROS-mediated protein oxidation (. The mechanism of metal catalyzed oxidation of proteins and there physiological consequences have been reviewed (Stantdman, 1991; Dean *et al.,* 1997).

The potential antioxidants available from plant sources are thought to involve in protection against oxidative damage to protein *in vitro* and *in vivo* condition (Halliwell, 2007). There are several mechanisms proposed to induce protein oxidative damage, but most crucial is metal catalyzed oxidation system involvement in protein damage (Stadtman, 2001). In aim of the present study was to test whether GAM and PEH can counteract oxidative damage of proteins using different methodologies available to study protein oxidative damage including DNPH based protein carbonyl determination and fragmentation study (Mayo *et al.,* 2003).

4.3.3.1 Spectrophotometric study to evaluate protective against oxidation to protein

The protection action of GAM and PEH against metal catalyzed oxidative damage to BSA was analyzed with the help of DNPH based spectrophotometric technique. The both GAM and PEH showed a dose dependent inhibition of oxidative damage of BSA. The results are presented in Fig 4.12. The IC_{50} values were 130.64 (GAM) and 127.4 (PEH) µg per ml, respectively, as formation of protein carbonyl during oxidation to BSA was reduced to 50% at these concentrations. The standard glutathione one of the strong antioxidant showed IC_{50} value 120.32 µg per ml as which up to 56.64% inhibition was observed. The efficiency of GAM and PEH to protect BSA oxidative damage was equivalent to glutathione in terms of their inhibitory concentration. Thus, the both found to have strong antioxidant potential against oxidative damage to proteins.

4.3.3.2 FTIR based study to determine protective effects against protein oxidation

The protective effect of GAM and PEH against oxidative damage to BSA was also evaluated by measuring the carbonyl index using FTIR. The results of the FTIR spectra are shown in Fig 4.13 (GAM) and 4.14 (PEH), depicting the extent of protein carbonyl formation due to BSA oxidation in the presence of MCO oxidizing agents. The amount of protein carbonyl formed during BSA oxidation in the presence or absence of antioxidant constituents is given in Table.3 and 4. The results indicated that at 143.64 (GAM) and 138 (PEH) µg per ml, BSA oxidation was protected up to 50 percent and the values were almost equivalent to that of IC_{50} (136.73 µg per ml) value of standard glutathione. Thus, it also confirmed strong antioxidant potential of GAM and PEH and their protective role against oxidative damage to proteins.

4.3.3.3 SDS-PAGE fragmentation study to evaluate protective effects of GAM and PEH against oxidative damage to protein

The protective effect of GAM and PEH against metal catalyzed oxidative damage to BSA was also tested using SDS-PAGE method. The gel of the BSA oxidation reactions in the absence or presence of various concentrations of GAM and PEH was visualized on gel documentation system and their results are presented in Fig 4.15a (GAM) and 4.16a (PEH). A concentration dependent inhibition of BSA fragmentation was observed in comparison to the control. The densitometry analysis of BSA band was done using quantity one software and percentage band intensity was calculated and given in Fig. 4.15b (GAM) and 4.16b (PEH). The use of SDS-PAGE to evaluate the protective potential of antioxidants against protein oxidation is reported in the previous studies (Mayo *et al.*, 2003;). The results from the present study indicated that at 143.52 (GAM) and 136 (PEH) µg per ml, there observed to be a clear reduction in BSA damage. The antioxidant potential of the GAM and PEH observed

in the present study found to be better than the antioxidant extract from plants like *S. officinalis* which has inhibitory effects against protein oxidation up to 50% at the 150.65 µg per ml concentration. A procynidin rich extract of *Pinus pinaster* bark showed inhibitory effects on MCO catalyzed oxidation to BSA and our results were found to be equivalent or even better in terms of their inhibitory concentration at 143.43 µg per ml.

4.3.3.4 Immunoblot study to determine the protein carbonyl during protein oxidation with or without antioxidant constituents

The protective effect of GAM and PEH against oxidative damage of BSA was further tested by immunoblotting using Anti-DNP antibody which is a more sensitive method. The immunblot photographs showing the inhibition of carbonyl formation due to BSA oxidation in presence of GAM and PEH are represented in Fig. 4.17a (GAM) and 4.18a (PEH). The band intensity of their corresponding band is given in 4.17b and 4.18b. The data analysis supported our earlier results performed using spectrophotometric and FTIR technique that at the concentration 137.64 and 130.4 µg per ml oxidation to protein could be inhibited up to 50%.

Carbonyl groups are considered a general assay of oxidative protein damage (Dean *et al.*, 1997) and the presence of these products is increased in several tissues with aging (Fagan *et al.*, 1999). Multiple redox reactions cause the introduction of carbonyl groups into proteins, with their number being influenced by metal cations, especially Fe^{2+} and Cu^{2+} (Stadman, 2001; Levine and Stadman, 2001). Carbonyl groups represent an early stage of protein oxidation (Levine and Stadman, 2001). Carbonyl levels by themselves, although suggested to be a good index of protein damage, do not increase indefinitely, as protein degradation or other processes limit it. It has been reported in some of the studies where the carbonyl content rapidly reached

a plateau and the levels did not correlate with the increasing structural damage seen in the protein (Mayo *et al.*, 2003). Still the carbonyl index is a good marker for evaluating the oxidative damage to proteins and reduction in its level by presence of antioxidant shows it potential of protection. It is clear from the spectrophotmetric analysis, FTIR, SDS-PAGE and immunoblot experiments that both the GAM and PEH found to have strong protection against oxidative damage to proteins, as evidenced from the carbonyl index reduction. The protection efficiency was equivalent to the one of the known strong antioxidant glutathione and was found to be better than activity of the antioxidant reported from the other plants.

4.3.4 Study on lipid peroxidation inhibtion potential of GAM and PEH

In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acid in the cell membranes) generates a number of degradation products, such as malondialdehyde (MDA) and is found to be an important cause of cell membrane destruction and cell damage (Jackson, 1999). MDA, one of the major products of lipid peroxidation, has been extensively studied and measured as an index of lipid peroxidation and as a marker of oxidative stress. In the present study, we measured the potential of GAM and PEH to inhibit lipid peroxidation in rat liver homogenate, induced by two different oxidation systems.

The lipid peroxidation inhibition potential of GAM from *A. marmelos* against ascorbate/Fe^{2+} and CCl_4/NADPH oxidation system induced lipid peroxidation in rat liver microsome is presented in Table 4.5. Inhibition of lipid peroxidation was determined by MDA level and there found to be a concentration depend inhibition of lipid peroxidation. The IC_{50} values of GAM for two different assayed systems found to be 138.82±6.29 (ascorbate/Fe^{2+}) and 150.22±86 (CCl_4/NADPH), respectively and were of almost comparable to those of GSH (132.7±11.8, ascorbate/Fe^{2+}) and GSH

(126.14±8.38, CCl_4/NADPH) µg per ml, respectively. Protective potential of PEH against ascorbate/Fe^{2+} and CCl_4/NADPH induced lipid peroxidation in rat liver microsome is presented in Table 4.6. The increasing concentration of PEH strongly inhibited the extent of lipid peroxidation. The IC_{50} for PEH found to be 116.92±9.73 (ascorbate/Fe^{2+}) and 105.55±8.37 (CCl_4/NADPH) system and was almost similar to the GSH (106.74±7.47) and GSH (96.74±6.3) µg per ml, respectively. The results revealed that with increase in PEH concentration, lipid peroxidation was reduced convincingly and equivalent with the standard lipid peroxidation inhibtion agent glutathione.

Lipid peroxidation is initiated through one-electron redox reactions in which an initiating radical species combine with an unsaturated lipid and in turn with oxygen, to form a lipid peroxyl radical. These lipid peroxyl radicals are short lived and react with metals to produce a variety of products which are themselves reactive (aldeydes etc.) and start chain reaction and the end product being MDA and HNE (Sevanian and Ursini, 2000). It is important to note that peroxidation reactions to proceed, there is an absolute requirement for transition metals. It has also been postulated on the basis of earlier studies that the first electron is provided by cytochromep-450 to reduce complex iron with subsequent addition of O_2, forming a perferryl radical. Reaction of perferryl radical with unsaturated lipids to produce lipids radicals and subsequent reaction with O_2 to form lipid peroxyl radicals represent the well known initiating steps for lipid peroxidation (Morehouse and Aust, 1988).

Our results showed that protective effect GAM and PEH against lipid peroxidation could be attributed due to their efficient metal chelating ability and free radical scavenging activity. It is further suggested that this was due to the glycosides

and phenolic constituents. Similar observation has been obtained in some earlier studies (Rice-Evans, 2000). The polyphenolic and glycosides constituents identified from several plant sources like Rosemary and pine have been found to be good antioxidants against lipid peroxidation in rat liver microsomal system (Laguerre *et al.*, 2007).

Conclusion

The antioxidant potential of GAM and PEH was evaluated using various assay methods. The both GAM and PEH have strong antioxidant potentials and it was due to their free radical scavenging and metal chelating abilities. The antioxidant efficacy was almost similar to the well known antioxidant agents like glutathione and BHA and found to better than the antioxidant reported from other plants. The both GAM and PEH showed strong protective effects against oxidative damage to DNA, proteins and lipids. The protective activity was linked to both their metal chelating as well as radicals scavenging abilities. It was further suggested that the strong antioxidant activity of GAM and PEH due their glycosides and phenolics constituents.

Table 4.1 Free radical scavenging activity of CGAM, GAM, CPEH and PEH constituents using ABTS and DPPH free radical scavenging assay.

Name of Sample	DPPH assay * (IC_{50} µg per ml)	ABTS assay * (mM Trolox)
BHA	3.21±0.18	3.85 ±0.25
CGAM	10.43 ±0.76	0.89 ±0.01
GAM	3.89 ±0.21	2.84 ±0.18
CPEH	9.46 ±0.87	0.94 ±0.02
PEH	2.87 ±0.46	3.07 ±0.19

* Data presented are given as mean±SEM, n=3 Three different experiments with three replicates.

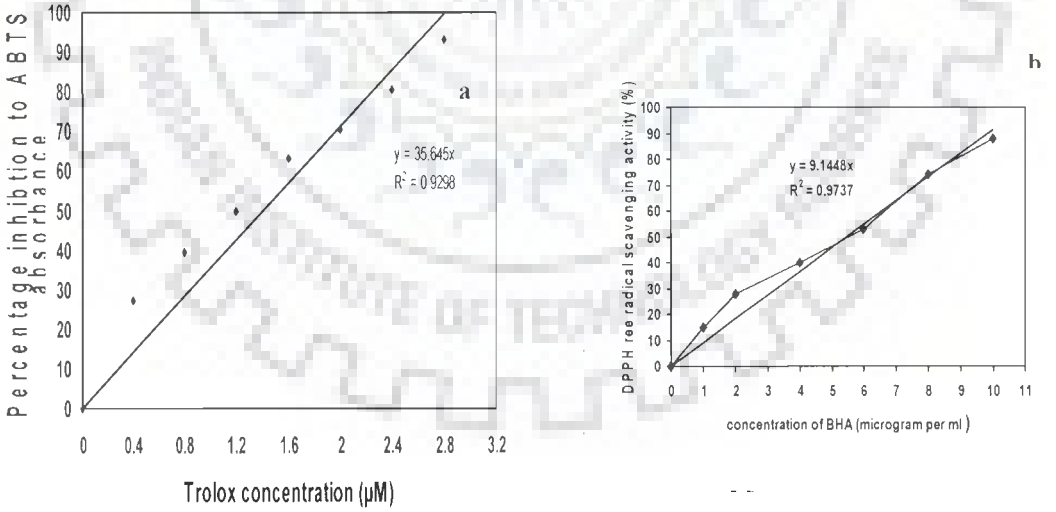

Fig. 4.1a depicts the TEAC calibration curve for ABTS assay. Fig. 4.1b shows calibration curve of BHA against DPPH free radical scavenging assay.

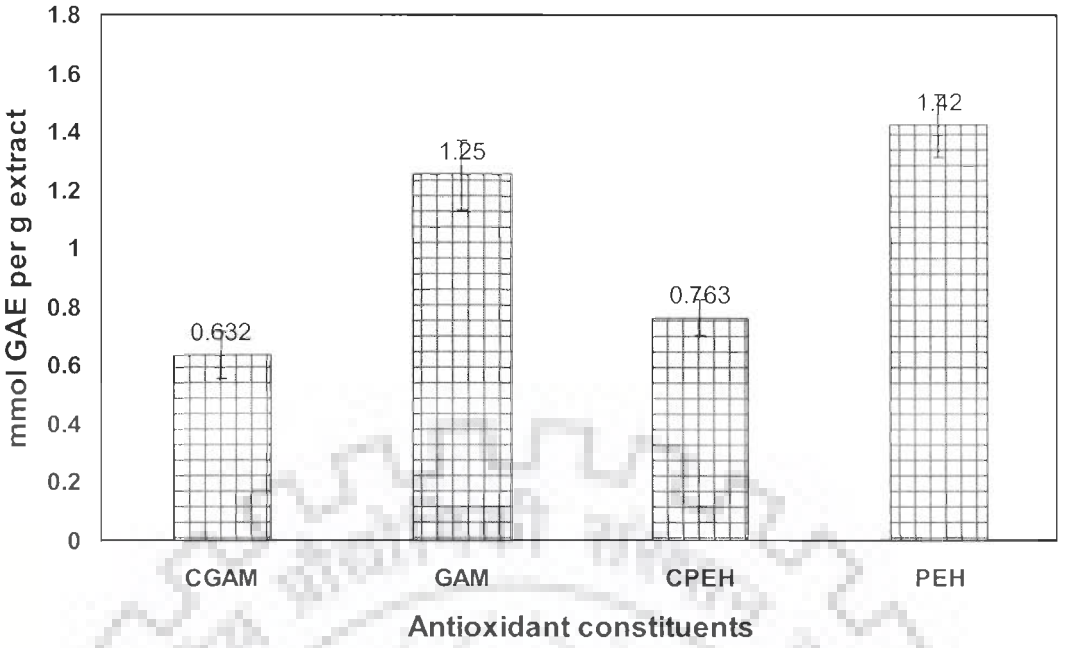

Fig 4.2a. depicts the ferric reducing antioxidant power of antioxidant constituents from *A. marmelos* and *E. hirta*.

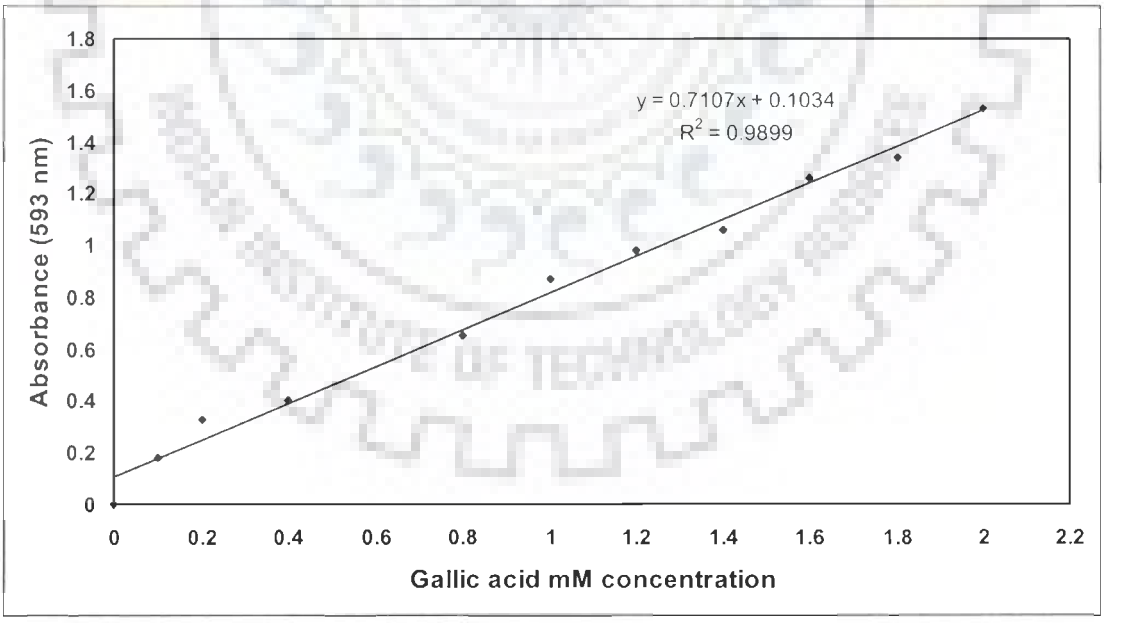

Fig 4.2 b. shows the calibration curve for standard gallic acid for FRAP.

Table 4.2a Hydroxyl radical scavenging activity of GAM constituent

Sample (µg per ml)	Absorbance (532 nm) With ascorbic acid		Absorbance (532 nm) Without ascorbic acid
Control (only Deoxyribose)	0.114		0.121
Oxidizing mixture only	0.876		0.365
CGAM 100	0.63	(IC_{50} 156.54±8.32µg per ml)	0.372
CGAM 150	0.54		0.384
CGAM 200	0.46		0.393
CGAM 250	0.39		0.403
GAM 25	0.61	(IC_{50} 47.83±2.18µg per ml)	0.378
GAM 50	0.48	(IC_{50} glutathione 32.34±3.78)	0.387
GAM 75	0.43		0.396
GAM 100	0.32		0.408

* Data presented are given as mean±SEM, n=3 Three different experiments with three replicates.

Table 4.2b Hydroxyl radical scavenging activity of PEH constituent

Sample (µg per ml)	Absorbance (532 nm) With ascorbic acid		Absorbance (532 nm) Without ascorbic acid
Control (only Deoxyribose)	0.118		0.129
Reaction mixture only	0.883		0.376
CPEH 100	0.58	(IC_{50} 150.98.38±7.32 µg per ml)	0.382
CPEH 150	0.47		0.396
CPEH 200	0.42		0.422
CPEH 250	0.36		0.426
PEH 25	0.54	(IC_{50} 38.18±3.29 µg per ml)	0.379
PEH 50	0.42	(IC_{50} glutathione 32.34±3.78)	0.393
PEH 75	0.35		0.382
PEH 100	0.26		0.418

*Data presented are given as mean±SEM, n=3 Three different experiments with three replicates.

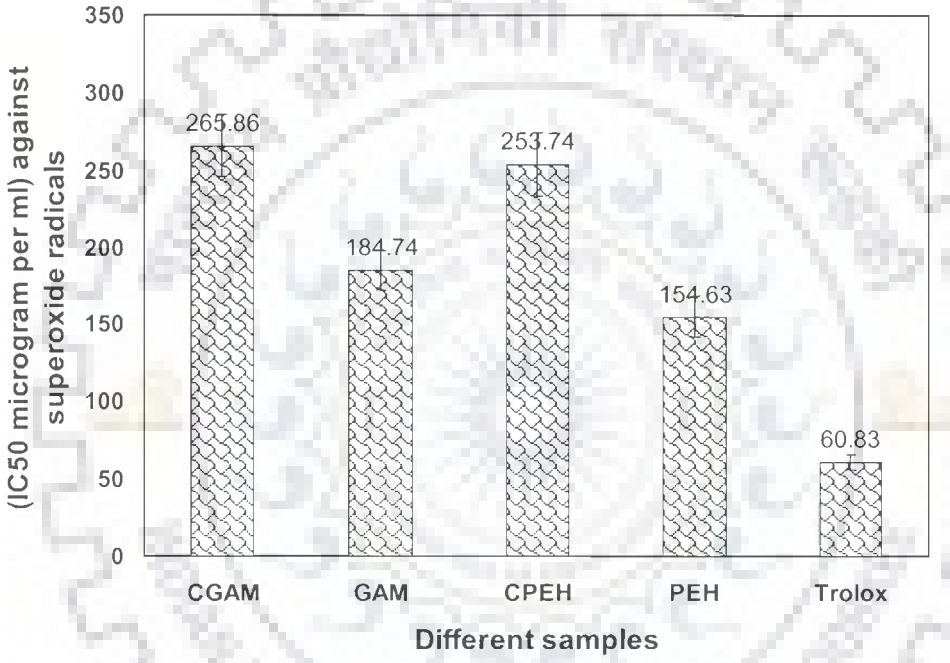

Fig 4.3. Superoxide radical scavenging potential of CGAM, GAM, CPEH and PEH constituents.

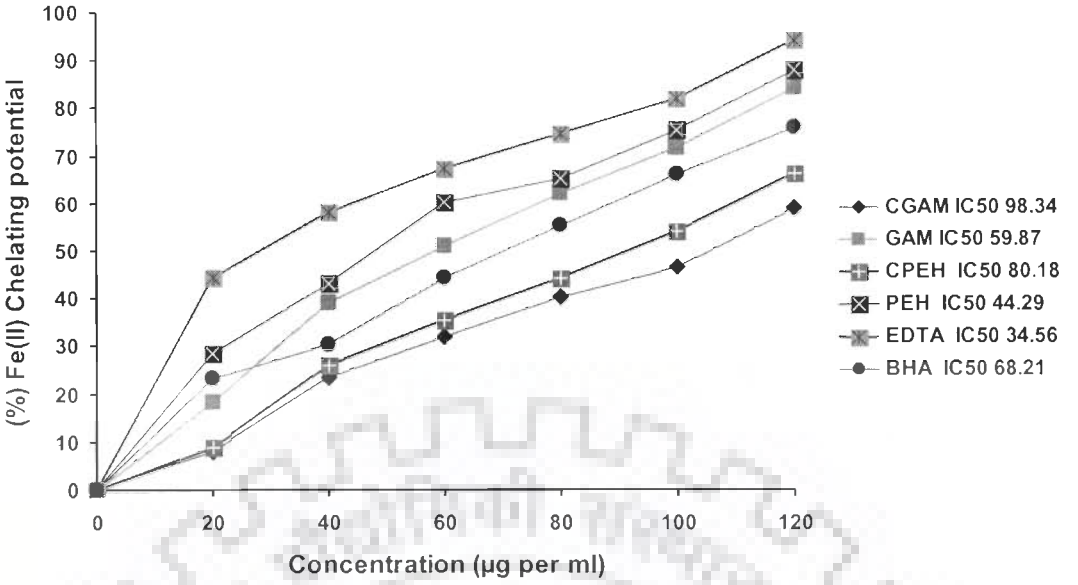

Fig. 4.4 depicts the ferrous ion metal chelating potential of (CGAM and GAM from *A. marmelos*) and (CPEH and PEH from *E. hirta*).

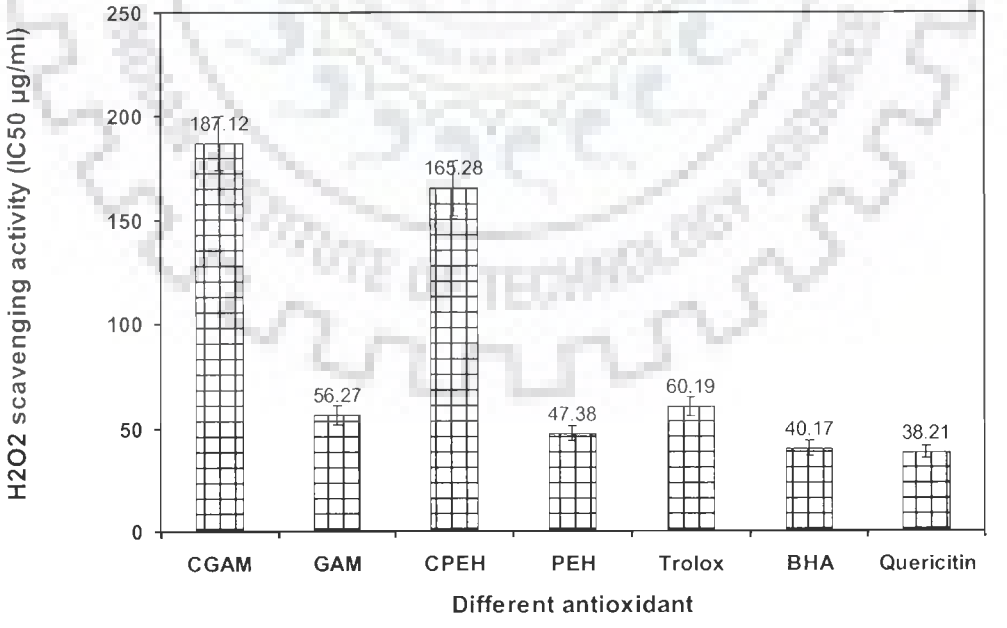

Fig 4.5 Shows the hydrogen peroxide (H_2O_2) scavenging activity of (CGAM, GAM from *A. marmelos*) and (CPEH, PEH from *E. hirta*) constituents and expressed in terms of (IC_{50} µg per ml).

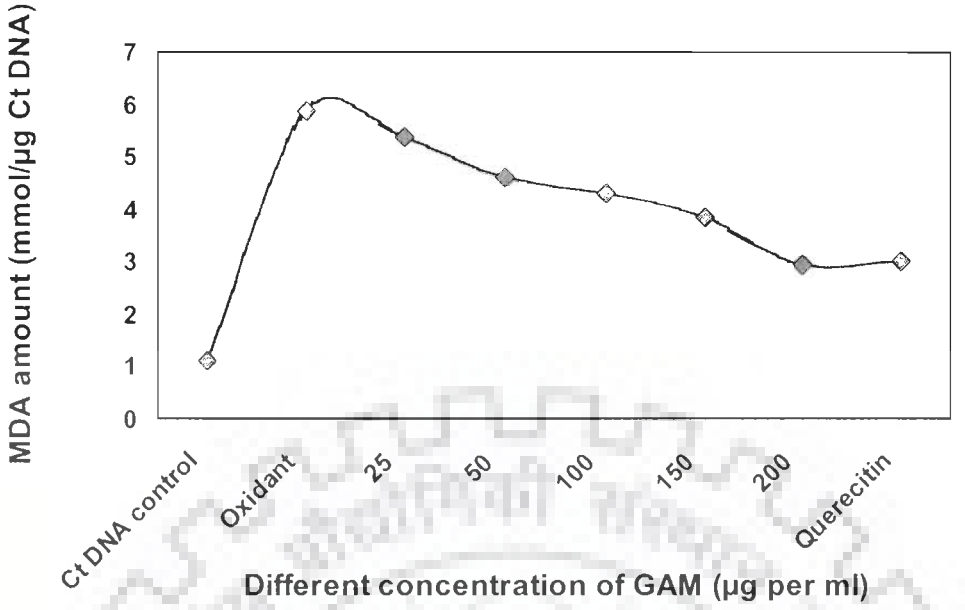

Fig 4.6a. Show the Spectrophotometric determination of MDA generation in Ct DNA induced by MCO in the presence of GAM and positive standard quercetin.

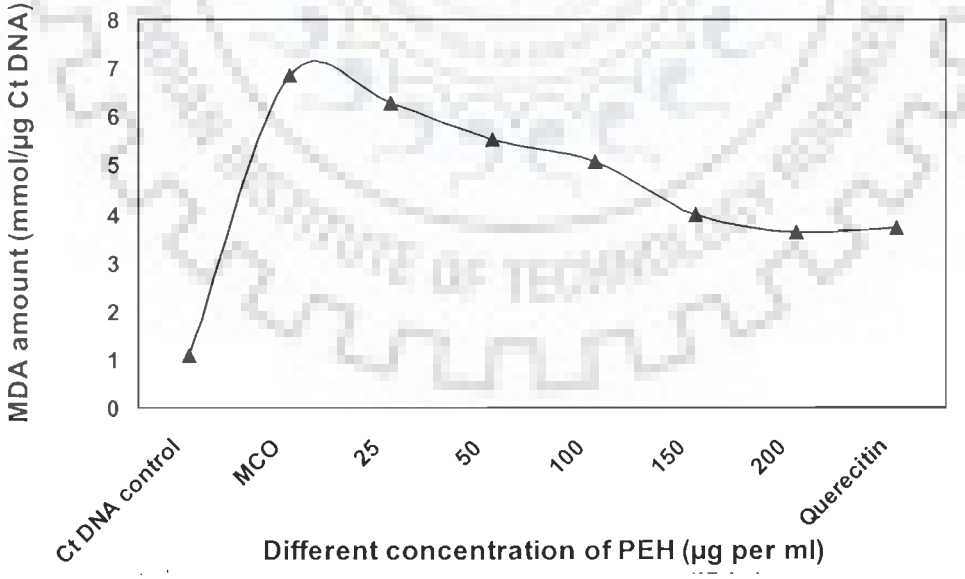

Fig 4.6 b. Show the Spectrophotometric determination of MDA generation in Ct DNA induced by MCO in the presence of PEH and positive standard querecitin.

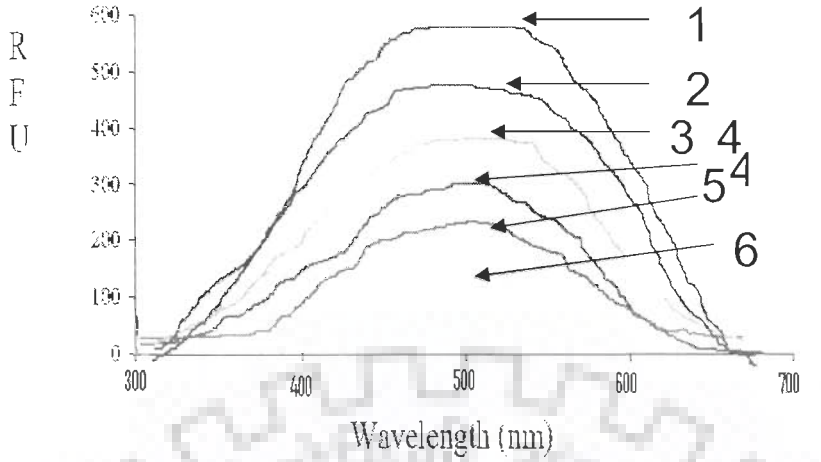

Fig 4.7a Show the ethidium bromide intercalated calf thymus DNA fluorescent spectra with excitation at 518 nm and scanned from 300-700 nm. In the figure, number denotes as 1-Control Ct DNA+EtBr, 2-(CtDNA+RM+GAM 100, 3-RM+GAM 80, 4-RM+GAM 40, 5-RM+glutathione 75 µg per ml and 6-RM (Fenton reaction+ Ct DNA) only.

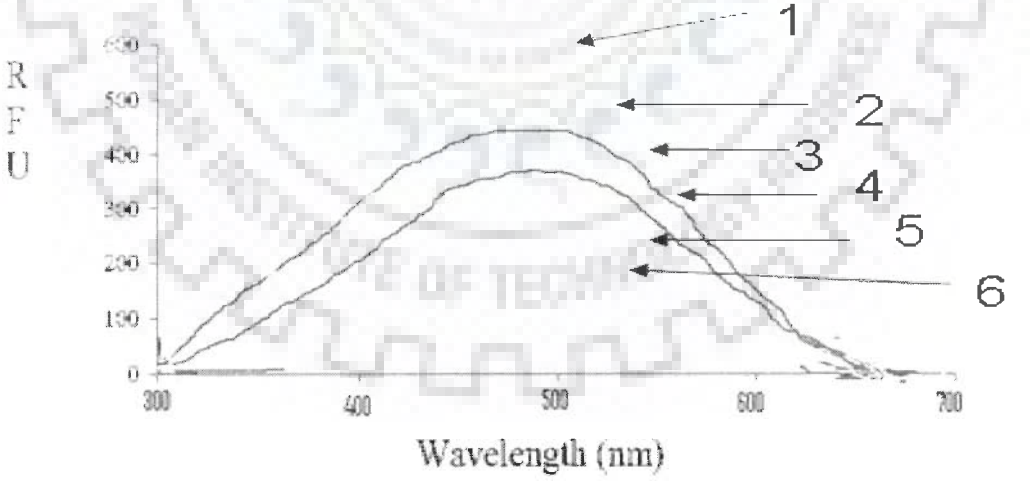

Fig 4.7b Show the ethidium bromide intercalated calf thymus DNA fluorescent spectra with excitation at 518 nm and Scanned from 300-700 nm. In the figure, number denotes as 1-Control Ct DNA+EtBr, 2-(Ct DNA +RM+PEH 100, 3- Ct DNA +RM+PEH 80, 4- Ct DNA+RM+PEH 40, 5- Ct DNA +RM+quericitin 75 µg per ml and 6-RM (Ct DNA +RM) only.

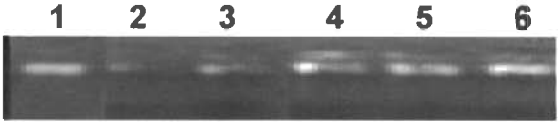

Fig. 4.8a Ethidium bromide stained agarose gel photograph showing calf thymus DNA (Ct DNA) damage by Metal catalyzed Fenton system (Fe^{2+} (1mM, 2µl) +H_2O_2 (1mM, 4µl) with or without GAM and standard quercetin. In brief, Lane 1 (Ct DNA only (0.5 µg), Lane 2 Reaction mixture (Ct DNA+MCO), Lane 3-6(Reaction mixture+10, 20, 40 and 60 µg per ml of GAM, Lane 7 (Reaction mixture+quercetin. 50 µg per ml).

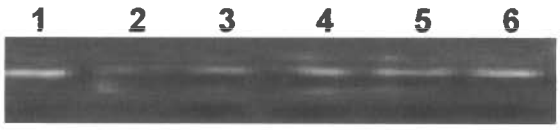

Fig. 4.9a Ethidium bromide stained agarose gel photograph showing calf thymus DNA (Ct DNA) damage by Metal catalyzed Fenton system (Fe^{2+} (1mM, 2µl) +H_2O_2 (1mM, 4µl) with or without PEH and standard quercetin. In brief, Lane 1 (Ct DNA only (0.5 µg), Lane 2 Reaction mixture (Ct DNA+MCO), Lane 3-6(Reaction mixture+10, 20, 40 and 60 µg per ml of PEH, Lane 7 (Reaction mixture+quercetin. 50 µg per ml).

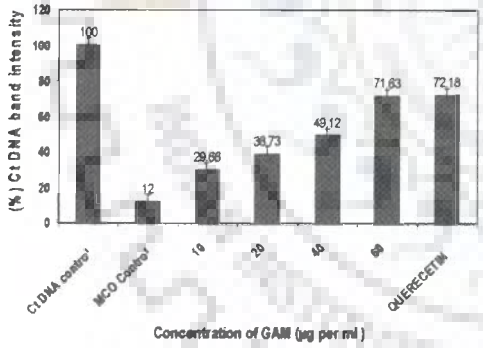

Fig 4.8b Densitometric analysis of loss in calf thymus DNA band intensity in the presence of MCO and varied concentration of GAM fractions.

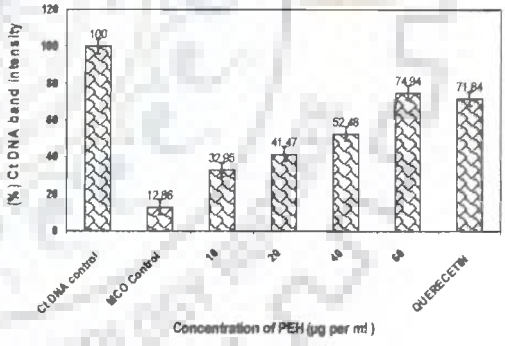

Fig 4.9b Densitometric analysis of loss in calf thymus DNA band intensity in the presence of MCO and varied concentration of PEH fractions.

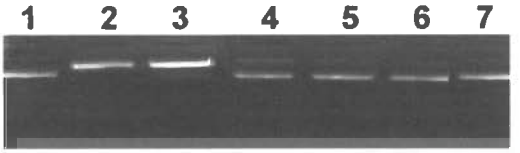

Fig. 4.10a Showing nicking of pUC18 induced by Metal catalyzed Fenton system (Fe^{2+} (1mM, 2µl) +H_2O_2 (1mM, 4µl). In brief, Lane 1 (plasmid only (0.5 µg), Lane 2 Reaction mixture (plasmid+MCO), Lane 3-6(Reaction mixture+10, 20, 40 and 60 µg per ml of GAM, Lane 7 (Reaction mixture+quercetin. 50 µg per ml).

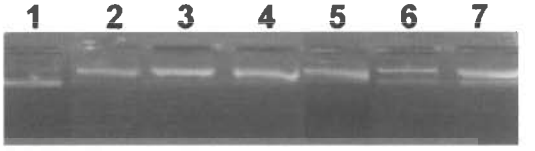

Fig. 4.10a Showing nicking of pUC18 induced by Metal catalyzed Fenton system (Fe^{2+} (1mM, 2µl) +H_2O_2 (1mM, 4µl). In brief, Lane 1 (plasmid only (0.5 µg), Lane 2 Reaction mixture (plasmid+MCO), Lane 3-6(Reaction mixture+10, 20, 40 and 60 µg per ml of PEH, Lane 7 (Reaction mixture+quercetin. 50 µg per ml).

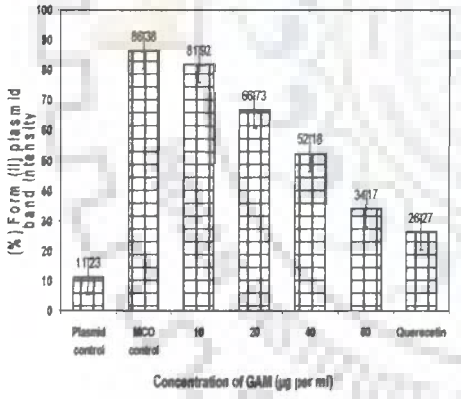

Fig 4.10b. Densitometric analysis of nicking of pUC18 plasmid in the presence of MCO and varied concentration of GAM fractions.

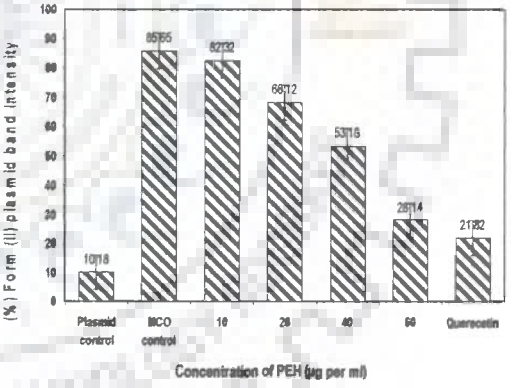

Fig 4.11b. Densitometric analysis of nicking of pUC18 plasmid in the presence of MCO and varied concentration of PEH fractions.

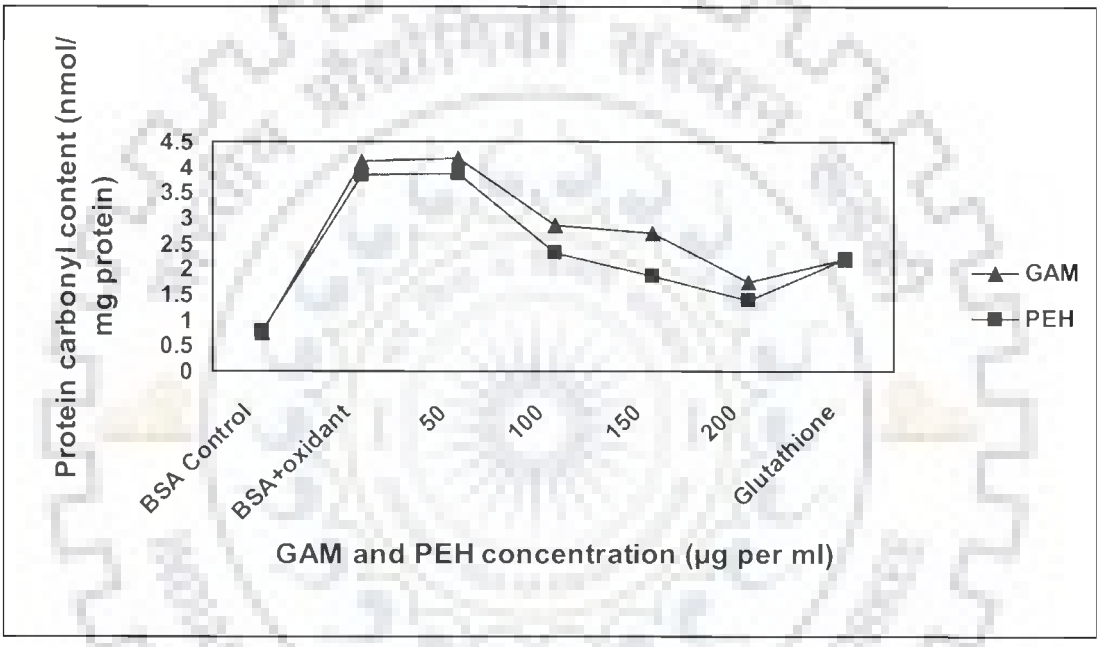

Fig 4.12 DNPH spectrophotometric determination of protein carbonyl in BSA oxidation induced due to MCO oxidant along the presence of PEH and GAM.

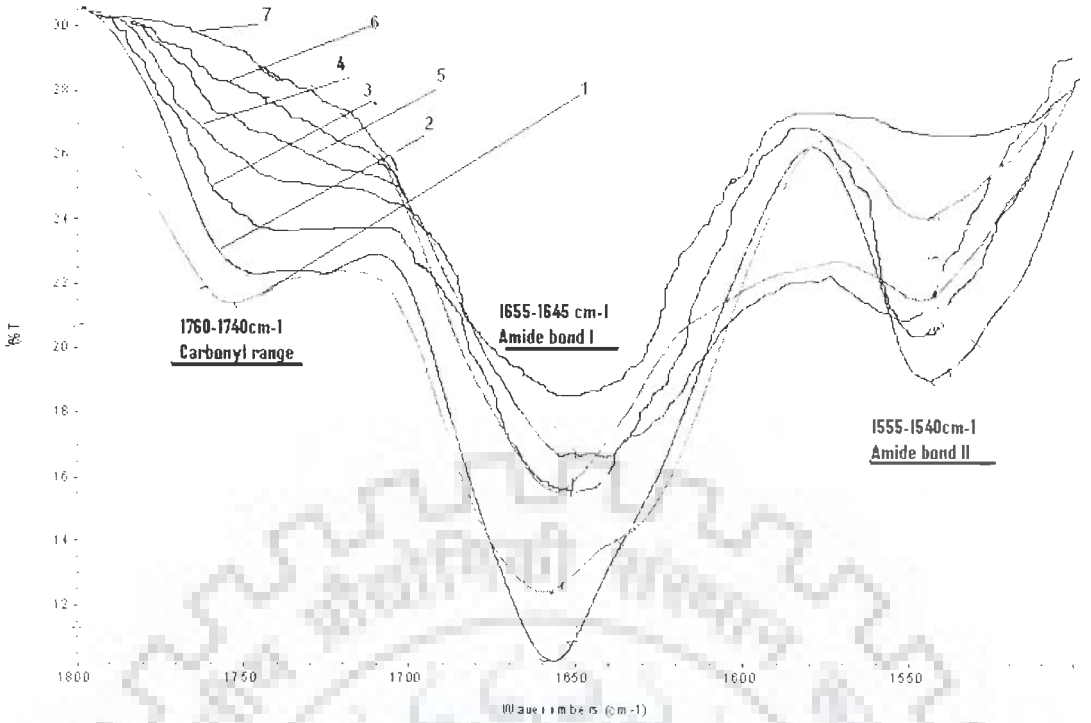

Fig 4.13 Shows the FTIR spectra of oxidized BSA sample with or without varied concentration of GAM constituents. The spectra are recorded in KBr pellets and spectra are presented from 1800-1500 cm^{-1} range.

Table 4.3. Show the arbitrary peak intensity of carbonyl group between ranges 1780-1730 cm^{-1} wavelength corresponding to spectra of oxidized BSA sample given in Fig 4.13.

FTIR graph name	Arbitrary peak area intensity
BSA Control (7)	5.12
BSA+MCO (1)	96.28
BSA+MCO+GAM100 µg per ml (2)	74.29
BSA+MCO+GAM 200 µg per ml (3)	63.18
BSA+MCO+GAM 300 µg per ml (4)	54.26
BSA+MCO+GAM 400 µg per ml (5)	38.31
BSA+MCO+Glutathione+200 µg per ml (6)	48.92

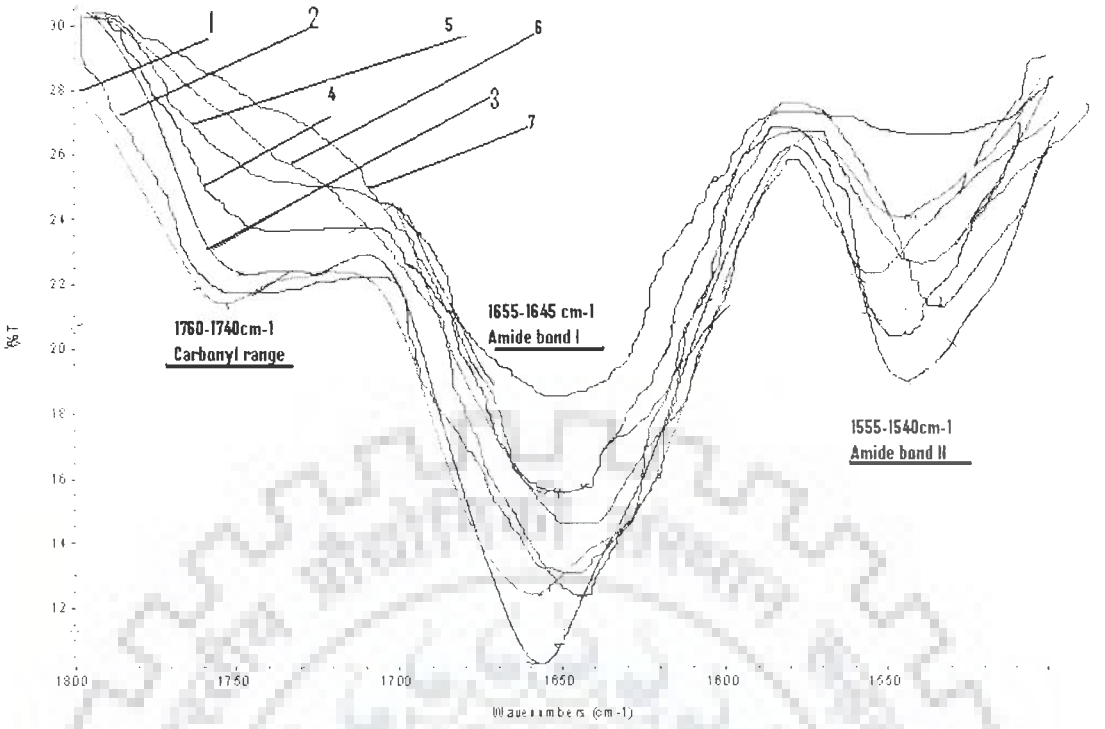

Fig 4. 14 Shows the FTIR spectra of oxidized BSA sample with or without varied concentration of PEH constituents. The spectra are recorded in KBr pellets and spectra are presented from 1800-1500 cm^{-1} range.

Table 4.4 Show the arbitrary peak intensity of carbonyl group between ranges 1780-1730 cm-1 wavelength corresponding to spectra of oxidized BSA sample given in Fig 4.14.

FTIR graph name	Arbitrary peak area intensity
BSA Control (7)	4.92
BSA+MCO (1)	94.61
BSA+MCO+PEH100 µg/ml (2)	69.83
BSA+MCO+PEH 200µg/ml (3)	56.29
BSA+MCO+PEH 300µg/ml (4)	51.01
BSA+MCO+PEH 400µg/ml (5)	37.26
BSA+MCO+Glutathione+200µg/ml (6)	46.19

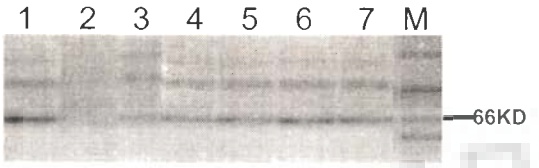

Fig 4.15a shows the SDS-PAGE results from BSA oxidation due to MCO system along with GAM and glutathione. Lane 1. Contol BSA, Lane 2. BSA+MCO Lane 3-6BSA+MCO+GAM (50, 100, 150 and 200 µg per ml). 7. BSA+MCO+ glutathione (200 µg per ml) 8.Molecular Marker.

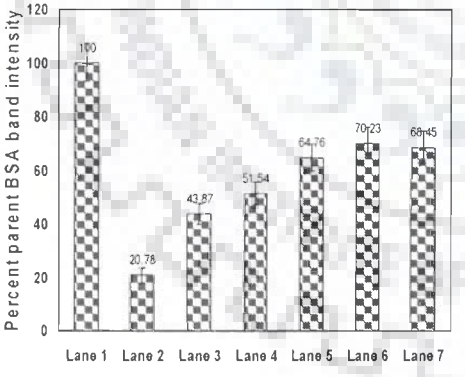

Fig 4.15b Densitometric BSA band intensity analysis of control and oxidized BSA samples with or without GAM and glutathione compounds.

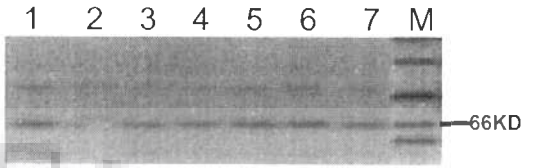

Fig 4.16a shows the SDS-PAGE results from BSA oxidation due to MCO system along with PEH and glutathione. Lane 1. Control BSA 2.BSA+MCO Lane 3-6 BSA+MCO+PEH (50, 100, 150 and 200 µg per ml). 7. BSA+MCO+glutathione (200 µg per ml) 8.Molecular Marker.

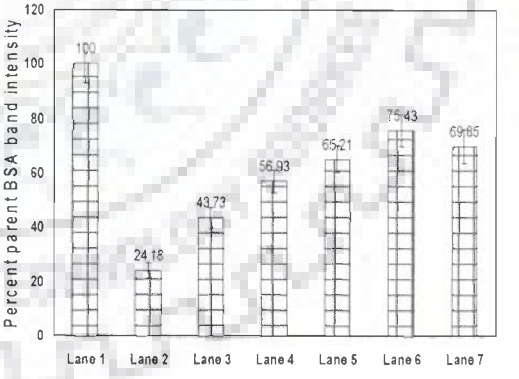

Fig 4.16b Densitometric BSA band intensity analysis of control and oxidized BSA samples with or wihtout PEH and glutathione compounds.

Fig 4.17a Immunoblot photograph of oxidized BSA sample using anti-DNP antibody. In brief, Lane 1(BSA only), Lane 2 lane 2 (BSA+MCO, Lane 3-6 (Reaction mixture GAM 50, 100, 150 and 200 µg per ml). Lane 7 Reaction mixture+glutathione 200 µg per ml.

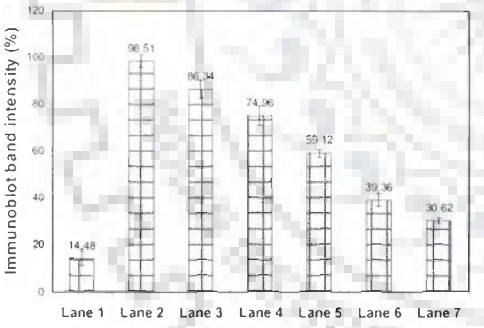

Fig 4.17b Densitometric immunoblot band intensity analysis of control and oxidized BSA samples in the presence or absence GAM and glutathione compounds.

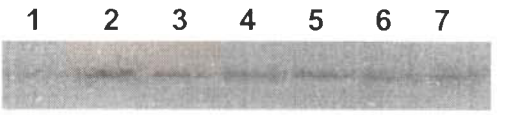

Fig 4.18a Immunoblot photograph of oxidized BSA sample using anti-DNP antibody. In brief, Lane 1(BSA only), Lane 2 lane 2 (BSA+MCO, Lane 3-6 (Reaction mixture PEH 50, 100, 150 and 200 µg per ml). Lane 7 Reaction mixture+glutathione 200 µg per ml.

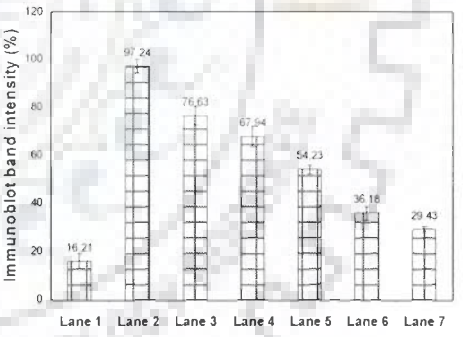

Fig 4.18b Densitometric immunoblot band intensity analysis of control and oxidized BSA samples in the presence or absence PEH and glutathione compounds.

Table 4.5 Lipid peroxidation inhibition potential of GAM constituents from *A. marmelos* against ascorbate/Fe^{2+} and CCl_4/NADPH oxidation system induced lipid peroxidation in rat liver microsome.

Sample	Lipid peroxidation system in rat liver microsome					
	Ascorbate/Fe^{2+}			CCl_4/NADPH		
	MDA * (nmol/mg protein	I% a	IC_{50}	MDA * (nmol/mg protein	I% a	IC_{50}
Control (Lipid peroxidation)	14.12±2.87	-	138.82±6.29 (µg/ml) GSH IC_{50}= 126.14±8.38	5.32±1.21	-	150.22±86 (µg/ml) GSH IC_{50}= 132.7±11.8
GAM (25 µg/ml)	10.45±3.41 **	25.99		4.69±0.91 **	11.84	
GAM (50 µg/ml)	9.43±1.98 **	33.21		4.12±0.06 **	22.55	
GAM (100 µg/ml)	8.36±0.78 **	40.79		3.42 ±0.04 **	35.71	
GAM (150µg/ml)	7.01±1.21 **	50.35		2.38± 0.07 **	55.26	
GAM (200µg/ml)	5.86±0.56 **	58.49		2.13±0.04 **	59.96	

* The values are expressed as mean ± SD (n=4). TBARS were expressed by nmol of MDA produced in the presence of varied concentration of GAM constituents.
a Percentage inhibition (I%) due to the action of extracts was calculated after deducing the basal level of Peroxidation. The unit of IC_{50} is µg per ml. ** denote the significant difference from control at $p>0.05$ value.

Table 4.6 Lipid peroxidation inhibition potential of PEH constituents from *E. hirta* against ascorbate/Fe^{2+} and CCl_4/NADPH oxidation system induced lipid peroxidation in rat liver microsome.

Sample	Lipid peroxidation system in rat liver microsome					
	Ascorbate/Fe^{2+}			CCl_4/NADPH		
	MDA * (nmol/mg protein	I% [a]	IC_{50}	MDA * (nmol/mg protein	I% [a]	IC_{50}
Control (Lipid peroxidation)	14.92±2.28	-		5.52±0.98	-	
PEH (25 µg/ml)	9.43±2.24 **	36.79	116.92±9.73 (µg/ml)	4.10±0.87 **	25.72	105.55±8.37 (µg/ml)
PEH (50 µg/ml)	8.25±1.98 **	44.70		3.40±0.12 **	38.40	
PEH (100 µg/ml)	7.65±0.91 **	48.72	GSH IC_{50}= 106.74±7.47	2.94 ±0.48 **	46.73	GSH IC_{50} =96.74±6.3)
PEH (150 µg/ml)	6.21±1.21 **	58.37		1.57± 0.67 **	71.55	
PEH(200 µg/ml)	4.97±0.56	66.68		1.21±0.12 **	78.07	

- The values are expressed as mean ± SD (n=4). TBARS were expressed by nmol of MDA produced in the presence of varied concentration of PEH constituents.

[a] Percentage inhibition (I%) due to the action of extracts was calculated after deducing the basal level of Peroxidation. The unit of IC_{50} is µg per ml. ** denote the significant difference from control at $p>0.05$ value.

In vivo Hepatoprotective Effect of GAM and PEH against CCl_4 Induced Rat Liver Oxidative Damage

5.1 Introduction

The liver is subjected to toxic injury more often than any other organ, as it is our most important detoxifying organ. When the liver is not functioning optimally, the body begins to store toxins in the tissues leading to altered physiological functions (Adams and Linder, 2007). Hepatotoxicity is a chemical driven liver damage and is connected with severe impairment of cell protection mechanisms (Lee and Senior, 2005; Jones and Czaza, 2008). Under normal condition, hepatic anaerobic metabolism involves a steady state production of prooxidants such as reactive oxygen species (ROS) which are balanced by a similar rate of their consumption by antioxidants. Imbalance in the pro-oxidant/antioxidant equilibrium in favor of pro-oxidant constitutes the oxidative stress phenomenon a condition that may induce a number of pathophysiological events in the liver (Shewita *et al.,* 2007; Halliwell, 2007; Manibusan *et al.*, 2007).

Hepatotoxicity by oxidative stress is achieved through a direct attack of ROS on essential biomolecules such as nucleic acid, lipid and protein with loss of their biological function and cell viability (Watkin and Seef, 2006; Tanikawa, 2006). Once hepatocellular function is impaired, accumulation of bile acids causes additional stress and cytotoxicity. Oxidative stress condition has been suggested to be implicated in the initiation of various liver pathological processes, such as fibrogenesis, cirrhosis and steatosis (Parola and Robino, 2000; Cesaratto *et al,* 2004; Nourissat *et al.,* 2008). Liver histology in CCl_4 induced oxidative stress is characterized by fatty

degeneration, steatosis, inflammation, mixed neutrophilic and mononuclear infiltration and necrosis (Jaeschke, 2002; Lutz *et al.,* 2003). On the basis of above observations, liver protection has been a subject of intense interest for biomedical importance because the liver plays a critical role in all aspects of metabolism and overall health (Schuppan *et al.,* 1999; Seef *et al.,* 2001). There is a need to provide the protection against toxicity and various liver disorders.

Since, it is established that a correlation exists between hepatotoxicity and oxidative stress. Therefore, the anti-hepatotoxic drugs and antioxidant compounds could be of great help in protection against liver abnormalities. Application of synthetic antioxidants such as propyl gallate (PG), Butylated hydroxytoluene (BHT) and tertiary butylhydroxyquinone (TBHQ) and other hepatoprotective drugs can assist in coping with oxidative stress. But some of the physical properties of these synthetic antioxidants such as their high volatility and instability at elevated temperatures, toxicity and higher manufacturing cost limit their use (Branen, 1975; Barlow, 1990). Thus, there is need for the search of more potent safer natural antioxidants. Plants provide a formidable source of natural products (with pharmacological activity) since they accumulate these antioxidants chemicals as secondary metabolites through evolution as natural means surviving in a hostile environment. A number of scientific attempts have demonstrated the protective role of natural antioxidants from plant sources in protection against oxidative stress induced liver damage (Medino and Moreno-Otero, 2005; Stickel and Schuppan, 2007; Singh *et al.,* 2008; Park *et al.,* 2008). In the present research work, the active antioxidant fraction GAM and PEH were evaluated for their protective effects in CCl_4 induced oxidative damage to rat liver and their possible mechanism by studying the biochemical, histological and molecular changes.

5.2 Material and Method

5.2.1 Material

All common chemicals and solvents used in the present study were of analytical grade with highest purity and purchased from SRL, S.D Fine, Himedia and MERCK India Ltd. All the biological kits used for biochemical assay were purchased from Span Diagnostics Pvt. Ltd., India. Primary anti-DNP antibody, secondary antibody goat anti-rabit IgG and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co (St Louis MO, USA) unless otherwise mentioned. The primary anti-CYP2E1 polyclonal antibody was received as a kind gift from Prof. Inglenman Sundberg (Karolinska Institute, Sweden).

5.2.2 *In vivo* material, animal husbandry and experimental design

Sixty two adult male Wister rats weighing 200–250 g obtained from Indian Vertinary Research Institute, Izatnagar, and Bareily, India were used in this study. All animals were kept under the same laboratory conditions of temperature (25 ± 2°C) and lighting (12:12 h light: dark cycle) and were given free access to standard animal feed (Ashirwad Industries, Chandigrah, India) and tap water. All rats were allowed to acclimatize for a week prior to experimentation. All experimental procedures involving animals were approved by the animal ethical committee of Department of Biotechnology, IIT Roorkee, and Roorkee, India.

The animals were randomly divided into nine groups with six rats in (Group 1, Group 2, Group 7, Group 8, Group 9) and eight rats in (Group 3, Group 4, Group 5 and Group 6). Group 1 served as controls and received an injection of vehicle (olive oil) alone; Group 2 was injected intraperitoneally (i.p.) with CCl_4 dissolved in an equal volume of olive oil (1:1) at a dose of (1ml per kg.bw per week) for four week, which is well documented to induce hepatotoxicity. The first dose of CCl_4 was given

in Group 3, 4, 5, 6 and 7 after one week of pretreatment with hepatoprotective agent GAM PEH and silymarin. The GAM, PEH and silymarin were dissolved in physiological saline and then administered to Group 3 (GAM 50 mg per kg. bw, p.o), Group 4 (GAM 100 mg per kg.bw, p.o), Group 5 (PEH 50 mg per kg.bw, p.o+CCl_4), Group 6 (PEH 100 mg per kg. bw, p.o), Group 7 (Silymarin 50 mg per kg. bw, p.o), Group 8 (GAM 200 mg per kg.bw, p.o) and Group 9 (PEH 200 mg per kg.bw, p.o). The CCl_4 doses as mentioned for Group 2 (CCl_4 treated control) were given to all treatment groups except Group 1 (normal control), Group 8 and 9. The design and treatment performed *in vivo* experiment is summarized in Table 1.

5.2.3. Total body weight, relative liver weight and lipid profile of rat liver tissue under CCl_4 toxicity

The changes in total body weight, liver weight and relative liver weight were determined gravimetrically in different treatment groups. Samples of liver tissues were homogenized in a chloroform-methanol mixture (1:1) for cholesterol determination and in a chloroform-methanol mixture (2:1) for triacylglycerol determination. Total lipids of liver were extracted by the procedure of Sperry (1954) and determined gravimetrically. Total triglyceride Abell *et al.*, (1952) and total cholesterol Jover (1963) in liver tissue were determined using assay kit manufactured by Span Diagnostics Pvt. Ltd., India. The total lipid, triglyceride and cholesterol content were expressed in terms of mg per g fresh liver tissue wt.

5.2.4 Effects of GAM and PEH pretreatment on oxidative stress marker and antioxidant status level in blood and liver tissue.

To evaluate the extent of liver damage under CCl_4 toxicity and their protection by GAM and PEH pretreatment, different biochemical parameters were examined

including serum oxidative markers level, antioxidant enzyme level and ROS production.

5.2.4.1 Preparation of blood serum and plasma sample

At the end of experiment, each rat was anaesthetized in a chloroform saturated chamber. The thoracic region was opened to expose the heart. Blood was obtained by heart puncture by means of a 5 ml hypodermic syringe collected in ice-cold 5 ml sample tubes for serum preparation and for plasma preparation in heparinized 5 ml sample tubes. The blood samples were centrifuged at 5000 rpm for 5 min. The collected blood serum and plasma were collected and stored at −20°C

5.2.4.2 Preparation of rat liver homogenate

Livers were quickly removed, weighed and perfused with ice-cold 0.15 M KCl and small pieces of liver were frozen in liquid nitrogen and stored at −80°C until biochemical analysis. To prepare rat liver homogenate, 2 g of liver tissue samples were homogenized with 8 ml (w/v 1:4) of 10 mM Tris–HCl (pH 7.4) containing 0.15M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 0.01 mM phenylmethoxysulfonyl fluoride in a Potter–Elvehjem homogenizer. The rat liver homogenized samples were centrifuged at 10,000Xg for 10 min. The supernatant as rat liver homogenate was collected and stored at −80°C for further analysis.

5.2.4.3 Assessment of oxidative stress marker enzyme level in blood serum of experimental rats

Biological kits used for ALT (Alanine aminotranferease) and AST (Aspartate aminotransferase), ALP (Alkaline phosphatase), LDH (Lactate dehydrogenase) and bilirubin estimation in serum were manufactured by Span Diagnostics Pvt. Ltd., India.

5.2.4.4 Evaluation of antioxidant status in blood plasma and rat liver tissue

The rat liver homogenate and blood plasma from different treatment group were evaluated for their superoxide dismutase (SOD) (Paoletti *et al.*, 1986), catalase (CAT) (Aebi, 1984) and reduced glutathione content (GSH) (Ellman, 1959) as levels of endogenous antioxidants system. The total antioxidant status of plasma and rat liver tissue in different treatment group was determined using FRAP (Benzie and Strain, 1996) assay. Total protein content in blood plasma and liver tissue was estimated as per standard protocol Bradford (1976). For the catalase enzyme assay, the liver homogenates and blood plasma containing 5 µg total protein were mixed separately with 700 µl of 5 mM hydrogen peroxide and incubated at 37°C. The disappearance of peroxide was observed at 240 nm for 15 min. One unit of catalase activity is that which reduces 1 µmol of hydrogen peroxide per minute. The catalase activity expressed in terms of U per mg protein of blood plasma and liver tissue.

The principle of measuring SOD is based on the oxidation of NADH mediated by super oxide radical (Paoletti *et al.*, 1986). The presence of SOD in the reaction mixture inhibits the rate of oxidation of NADH by scavenging the super oxide radicals. In brief, reaction mixture included 100 µl of TDB (Triethanolamine–diethanolamine) buffer, 100 µl of NADH (100 µM), 200 µl EDTA–$MnCl_2$ (1 mM) and liver homogenate as well as blood plasma samples (2 µg protein). The reaction mixtures were thoroughly mixed and read at 340 nm for a stable baseline. Finally, 5 µl of 2-mercaptoethanol was added, mixed and the decrease in absorbance was monitored at 340 nm for 20 min. A blank was run in the absence of the sample. The amount of SOD activity was calculated with respect to the blank value. One unit of SOD is the amount of enzyme required to inhibit NADPH oxidation of blank by 50%.

Glutathione (GSH) content in the liver homogenates was determined according to the

method described by Ellman (1959). In brief, 0.1 mg protein of liver homogenate and blood plasma in 1 ml of distilled water were added with 1.0 ml sulfosalicylic acid (10%) followed by centrifugation at 1,000xg for 10 min. Blank and standards were prepared by taking 1 ml of distilled water and 1 ml of GSH standard (1-100 µmol). About 0.5 ml of supernatant was added with 4.5 ml of Tris-buffer (10 mM, pH 8.23). Color was developed by adding 0.5 ml of DTNB solution and absorbance was recorded at 412 nm.

5.2.5 Morphological and histopathological study on protective effects of GAM and PEH against CCl_4 induced toxicity.

It has been widely observed that CCl_4 intoxication results into severe morphological and histological alterations indicating hepatic oxidative stress injury (Recknagel, 1967). The H&E staining light microscopy and scanning electron microscopy were conducted to evaluate the histopathological alterations during CCl_4 induced toxicity.

5.2.5.1 Histopathological study using H&E staining light microscopy

For the histopathological study using H&E staining light microscopy, rat liver tissues obtained from different treatment were processed as per the method of Krajain (1963) with slight modification. Liver sections were evaluated for steatosis, inflammation, necrosis, and apoptosis. After sacrifice of rats, liver was excised and cut to approximately 3–5 mm thick pieces and immediately submerged in 10% neutral buffered formalin for fixation and embedding in paraffin. Formalin fixation of tissue specimens was performed over night (20–24 hour) at room temperature, unless otherwise noted. Specimens were dehydrated in 70%, 90%, and 100% ethanol, followed by xylene for 2 hour twice and then embedded in Paraplast Plus (Sherwood Medical Co., St. Louis) for 2–3 hour at 60°C. For histopathology, 4 micron meter

thick paraffin-embedded liver sections were cut and stained with H&E for light microscopy histopathological observations.

The procedure for H&E staining has been standardized and performed by placing the deparaffinized slide in the distilled H_2O for 30 second twice, followed by Hematoxylin for 1 min twice, H_2O for 30 second twice, Blueing reagent 30 for twice, Eosin solution for 10 second twice, 70% ethanol for 30 second twice, 95% ethanol for 30 seconds twice and then in 100% ethanol for 30 seconds or air-dry at room temperature twice. Hematoxylin stains the nuclei and eosin stains the cytoplasm. Results from H&E distinguish nuclei with blue-black and cytoplasm with varying shades of pink. The light microscopy observation of H&E stained liver tissue sections was performed at 100X and 400X magnifications.

5.2.5.2 Protective effects of GAM and PEH pretreatment on surface morphology study of rat liver under toxicity by scanning electron microscopy

For scanning electron microscopy (SEM), liver tissue samples were prepared according to standardized protocol. Briefly, liver tissue was fixed into glutaraldehyde and cut to 1 mm^3 different tissue blocks. Tissue blocks were post fixed in 1% osmium tetroxide and dehydrated in graded ethanol solutions. The liver tissue was then dried with hexamethyldisilazane and subsequently broken in liquid nitrogen, mounted on stubs and sputter coated with a thin layer of 20 nm gold. The different treatment SEM samples were examined in a Philips SEM 505 at 30 kV. Imaging and morphometric analysis were performed on randomly acquired digitized SEM images at magnifications of 2000X.

5.2.6 Detection of ROS in CCl_4 intoxicated rat liver using DCFH-DA probe.

ROS were measured using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) probe, as previously described by Rabindranath *et al.,* (2001). The DCFH-DA is

hydrolyzed by cellular esterases to nonfluorescent 2', 7`-dichlorofluorescin (DCFH) which is rapidly oxidized in the presence of reactive oxygen species to 2', 7'-dichlorofluorescein (DCF). The DCF is estimated by fluorescent spectrometer. The ten µl of liver tissue homogenate (prepared above in section 5.2.3.2 from 2.5 mg of liver tissue) was incubated with 10 µl DCFH-DA (5mM, prepared fresh in methanol) at 37°C for 15 min. The reaction is terminated by chilling the assay mixture in ice, and the volume was made up to 2.0 ml by adding ice-cold phosphate buffer phosphate buffer (0.1 M, pH 7.4) containing Triton X-100 (0. 1%, v/v). The fluorescence of the oxidized derivative (DCF) is measured at an excitation wavelength of 488 nm and emission wavelength of 525 nm. Assay of a blank without the addition of DCFH-DA is carried out with each sample. The concentration of the ROS is expressed as arbitrary fluorescent intensity (UF) per g liver tissue.

5.2.7 Protective effects of GAM and PEH pretreatment on oxidative damage to DNA under CCl_4 induced toxicity in rat liver

The CCl_4 intoxication has been reported to cause extensive DNA damage during the liver oxidative stress disease condition. To determine the extent of oxidative damage to DNA during CCl_4 induced damage, several experiments have been used earlier including DNA fragmentation assay, DNA laddering experiment and single cell gel electrophoresis (COMET assay).

5.2.7.1 Quantitative DNA fragmentation assay

Quantitative estimation of DNA fragmentation was determined by colorimetric diphenylamine assay as described Burton (1968). Liver samples from different groups were homogenized in chilled lysis buffer (10 mmol Tris-HCl, 20 mmol EDTA, 0.5% Triton X-100, pH 8.0). Homogenates (1 ml) were then centrifuged at 27,000Xg for 20 min to separate intact DNA in the pellet from

fragmented/damaged DNA in the supernatant fractions. Perchloric acid (to reach a final concentration of 0.5 M) was added separately to both the pellets and supernatant samples. Samples were heated at 90°C for 15 min then centrifuged at 1500Xg for 10 min to remove proteins. Resulting supernatants, whether containing whole or fragmented DNA, were left to react with diphenylamine (0.088 M) for 16-20 hour at room temperature. Afterwards absorbance was measured at 600 nm. DNA fragmentation was expressed as a percentage of total DNA to fragmented DNA. The protective effects of GAM and PEH are expressed as percentage of DNA fragmentation observed in comparison to CCl_4 treated control group.

5.2.7.2 DNA laddering experiment on rat liver DNA under CCl_4 intoxicated rat liver

The total genomic DNA was isolated and purified from rat liver by the standard protocol described (Gupta, 1984). Liver samples (3 g) were thawed at room temperature and homogenized in 15 ml buffer, pH 7.4 (250 mM mannitol, 70 mM sucrose and 5 mM HEPES) in a 30 ml potter. The liver tissue homogenate was centrifuged at 1000Xg for 10 min. The supernatant was removed to waste and the pellet resuspended in 15 ml of buffer (1% SDS, 1 mM EDTA). Protein was removed by addition of Proteinase K (7.5 mg) with incubation overnight at 37°C. After that, the 1.5 ml of Tris–HCl, (pH 7.4), was added to the aqueous phase which was sequentially extracted with 15 ml of phenol and phenol: $CHCl_3$: 2-propanol (25:24:1). DNA was precipitated from the aqueous phase with addition of 1 ml of 5.0 M NaCl and 20 ml of ethanol (-20°C) overnight at -20°C. The DNA pellet was collected by centrifugation at 1000X*g* for 10 min then washed with 70% ethanol (1 ml). The DNA was re-dissolved in 6 ml buffer (1.5 mM NaCl, 0.15 M sodium citrate, 1 mM EDTA) and then 300 µl of 1.0 M Tris–HCl pH 7.4 and RNase A (600 µg) were added. The mixture incubated for 30 min at 37°C. The mixture was extracted once with an equal volume of

$CHCl_3$:2-propanol (24:1). The DNA was re-precipitated from the aqueous phase as described above in this section. The DNA was redissolved in water at the concentration of 2 mg per ml and stored at -20°C. The concentration and purity of each extracted DNA samples from different rat liver tissue was determined by UV spectrometry. The DNA concentration was calculated at absorbance $A_{260/280}$ assuming 50 µg per ml = 1.0 absorbance unit at 260 nm. The concentration of DNA isolated from liver samples (3 g) was 1.9 ± 0.82 mg per g liver. The purity of DNA was determined from absorbance ratios $A_{230/260}$ and $A_{260/280}$.

5.2.7.3 Single cell gel electrophoresis studies on DNA damage induced by CCl_4 toxicity in rat liver tissue

The rat liver was excised and immediately used for the cell suspension preparation in Merchant's solution (0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 0.53 mM Na_2EDTA (pH 7.4). Liver was briefly minced and then homogenized using a loosely fitting Potter–Elvehjem homogenizer. After sedimentation of the large tissue fragments, single cells remaining in the supernatant were centrifuged at 50×*g* for 4 min. All cell types were finally resuspended in a suitable volume of Merchant's solution and counted in a hemocytometer. The fraction of viable cells was determined with the trypan blue-exclusion method as described by Brambilla *et al.*, (1978).

The extent of DNA damage during CCl_4 induced damage and their protection in the presence of GAM and PEH constituents was evaluated using the single cell gel electrophoresis (Comet assay) technique. The procedure was performed as described by Singh *et al.*, (1988). Ten µl of liver cellular suspension (>10 000 cells) were mixed with 75 µl of low melting point agarose at 37°C and then added to normal melting point agarose-coated microscope slides. The slides were immersed in cold lysing

solution overnight, and then placed in an electrophoresis tray with an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA; pH 13) for 20 min to allow DNA to unwind. Electrophoresis was conducted at room temperature for 20 min at 25 V and 300 mA. Immediately before analysis, the slides were stained with 50 µl of 20 µg per ml ethidium bromide and examined at 400X magnification using (Olympus-Bx60, excitation filter: 515-560 nm; barrier filter: 590 nm) attached to a color CCD video camera and connected to an image analysis system (Comet II, Perspective Instruments, UK). Image analysis was performed with the software COMETA (New Generis Ltd., Norway) on 50 randomly chosen cells from two slides. The extent of DNA damage was quantified by increase of tail moment. The tail moment was defined as the product of the tail length and the amount of DNA in the tail (Olive *et al.*, 1990).

5.2.8 Protective effect of GAM and PEH on lipid peroxidation level in rat liver intoxicated with CCl_4

The importance of lipid peroxidation liver tissue induced by CCl_4 is extensively studied. To demonstrate the protective effects of GAM and PEH against CCl_4 induced toxicity, it has been considered to determine the level of lipid peroxidation as a parameter for study. The lipid peroxidation level is usually assessed by spectrophotometric method estimating MDA content and *in vivo* lipid peroxidation detection using Schiffs staining.

5.2.8.1 Spectrophotometric determination of total MDA in rat liver tissue

Total MDA content as lipid peroxidation end products in rat liver tissue intoxicated with CCl_4 was determined using the modified method of Esterbauer and Cheeseman (1990). Liver tissue homogenate (1 mg protein) prepared in material and method section (5.2.3.2) was incubated at 37°C for 1 hour. Then 1 ml 20% TCA and 2

ml 0.67% TBA were added and heated for 30 min at 100°C. Precipitate was removed by centrifugation at 1000Xg for 10 min. The absorbance of the supernatant was measured at 535 nm against a blank that contains all the reagents except the sample. The total MDA content was calculated with the help of calibration MDA (1,1 Tetraethoxy propane) given in 5.7b and expressed in terms of nmol of MDA per mg protein.

5.2.8.2 Histochemical detection of lipid peroxidation

This histological study on lipid peroxidation was performed as described by Pompella *et al.*, (1987). In brief, tissue sections were stained with Schiff's reagent for 20 min, which detects aldehydes that originate from lipid peroxides. After the reaction with Schiff's reagent, tissue sections were rinsed with a sulfite solution (0.5% [w/v] $K_2S_2O_5$ in 0.05 M HCl). Tissue sections were visualized under light microscope to observe the extent of tissue staining. The percentage of area on liver tissue slide showing positive Schiffs staining was taken as representation of lipid peroxidation level.

5.2.9 Protective effect of GAM and PEH on oxidative damage to protein induced by CCl_4 toxicity in rat liver

The involvement of free radicals and toxic products generated during CCl_4 induced toxicity has been suggested to inflict oxidative damage to rat liver protein. The extent of reduction in oxidative damage to protein in the presence of protective agents like GAM and PEH could be a approach to assess the protective potential against hepatic damage. The following methods have been considered to be suitable for the evaluation of oxidative damage to protein.

5.2.9.1 Spectrophotometric determination of protein carbonyl formation in rat liver homogenate

The 200 µg liver homogenate protein in 500 µl of 50 mM 500 phosphate buffer, pH 7.4) from different treatment were reacted with 500 µl DNPH reagent as per the standard protocol described (Levine *et al.,* 1990) and detail of this assay is described in material method section 4.2.4.2.

5.2.9.2 Study on protein carbonyl formation using western blot technique

In this experiment, rat liver homogenate (50 µg protein) prepared in material method section ( ) from different treatment group were separated electrophoretically on a 12 % SDS-PAGE gel using a MINI-PROTEIN II electrophoresis cell (Bio-RAD) as per standard method Laemmli (1970). Electrophoretic transfer to 0.45 µm nitrocellulose sheets was then carried out as described by Towbin *et al.*, (1979). The membrane was first derivatized with DNPH reagent according to (Levin *et al.,* 1990) with slight modification. The DNPH derivatized membrane was immunochemically stained with a rabbit polyclonal anti-DNP primary antibody (Sigma Chemical Co, St. Louis, MO; 1:2000) and HRP conjugated goat anti- rabbit IgG (Sigma Chemical Co, St. Louis, MO; 1:5,000 dilution) for 1 hour. The blots were visualized using DAB/H_2O_2 chromogenic substrates.

5.2.9.3 Immunohistochemical study on detection of protein carbonyl in rat liver

In vivo detection of protein carbonyl was performed on rat liver tissue sections of different treatment group receiving CCl_4 toxicity with or without pretreatment of GAM and PEH constituent. The procedure was adopted as per the standard immunohistochemical method. In brief, the rat liver tissue section was first derivatized with DNPH reagent to expose protein carbonyl groups on oxidized protein (Levin *et al.,* 1990). Immunohistochemical staining on derivatized rat liver tissue

section was performed with a rabbit polyclonal primary anti-DNP antibody and HRP conjugated goat anti-rabbit IgG. The rat liver tissue sections were observed in fluorescent microscope as per the standard method.

5.2.10 Assessment of CYP2E1 activity, protein level and preparation of microsome

The CYP2E1 enzyme activity and expression in liver microsome of experimental rats pretreated with GAM and PEH were studied employing spectrophotometric, western blot and immmunohistochemical methods.

5.6.10.1 Preparation of rat liver microsomes

The rat liver microsome was prepared as described by Slater and Sawyer, (1971). The 2 g frozen liver tissues from different experimental rats were homogenized in 10 ml ice-cold Tris-acetate buffer (pH 7.4) (1:5 w/v) containing 1.15% KCl. The liver tissue homogenate was centrifuged at 10,000X*g* for 30 min at 4°C. The supernatant was then centrifuged at 100,000X*g* for 60 min at 4^0C. Microsomal pellets were resuspended by homogenization in a Potter Elvehjem glass homogenizer. Protein content was determined as per standard method described by Bradford (1976). The liver microsomes were quick frozen, and stored at −80°C for later use.

5.2.10.2 p-Nitrophenol assay for CYP2E1 enzyme activity determination

The catalytic activity of microsomal CYP2E1 was measured by *p*-nitrophenol (PNP) hydroxylation as described by Koop (1990) with minor modifications. Reaction mixtures of 1 ml contained 250 mM PNP, 0.1 M potassium phosphate buffer (pH 6.8) and 1 mg of microsomal protein. After the reaction mixtures were pre equilibrated at 37°C for 3 min, then 1 mM NADPH was added to the above reaction mixtures to initiate the reaction. At the end of 20 min, the reaction was stopped by the

addition of 0.5 ml perchloric acid (0.6 N). The reaction mixtures were then centrifuged at 1,000X*g* for 5 min and 1 ml of supernatant was added to tubes containing 0.1 ml of 10 N NaOH. The amount of 4-nitrocatechol formed was determined by reading the absorbance at 546 nm using a Perkin Elmer UV/Visible spectrophotometer (USA). The PNP activity was calculated as per the (pmol/min/mg of protein) = $OD_{546}/9.53/0.2/60/7.1 \times 10^6$.

5.2.10.3 Western Blot Analysis for CYP2E1 expression

Liver microsomes (20 µg protein) prepared in above section (5.6.7.1) from different treatment groups were separated electrophoretically on 10% SDS-PAGE gel using a MINI-PROTEIN II electrophoresis cell (Bio-RAD) as per standard (Laemmli, 1970). Electrophoretic transfer to 0.45 µm nitrocellulose sheets was then carried out according to Towbin *et al.*, (1979). The membrane was immunochemically stained with a rabbit polyclonal primary anti-CYP2E1 antibody (a kind gift from Dr. Ingleman Sunderberg, Sweden (1:1000 dilution) and HRP conjugated goat anti- rabbit IgG (1:5,000 dilutions) for 1 hour. The β-actin was used an internal standard to correct CYP2E1 band intensities for differences in protein concentration. The blots were visualized using DAB/H_2O_2 chromogenic substrates. The density of immunblot band was quantified by densitometric Quantity one software (Bio-Rad, Hercules, CA, USA).

5.2.10.4 Immunohistochemistry study on CYP2E1 enzyme level in rat liver tissue

Immunohistochemical study was performed on rat liver tissue section using primary polyclonal anti-CYP2E1 antibody and visualized by DAB/H_2O_2 developing agents and whole procedure was adopted as per the standardized protocol.

5.2.11 Statistical analysis

All the hepatoprotective evaluation studies were carried out in triplicate and six replicates respectively, experimental results represents the mean of three/six identical studies. Standard deviation (SD) and standard error (SE) were calculated using following formula:

$$SD = \frac{\sqrt{\sum X^2}}{N}$$

$$SE = \frac{SD}{\sqrt{N}}$$

5.3 Results

There is accumulating evidence that oxidative stress plays a considerable role in the development of acute liver toxicity by CCl_4 mediated free radical generation and different pathways. Because of free radicals mediated oxidative stress condition play pivotal role in liver diseases pathology and progression, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Hensley *et al.*, 2000; Vitaglione *et al.*, 2004). Therefore, many natural antioxidants derived from dietary and medicinal plants possessing antioxidative properties have been demonstrated to prevent and treat hepatopathies induced by CCl_4 mediated oxidative stress in liver tissue (Lieber 1997; Luper, 1998; Bhaduria *et al.*, 2008).

5.3.1 Effects of GAM and PEH pretreatment on CCl_4 induced toxicity in relation to body weight, liver weight and total lipid profile

The protective effect against CCl_4 induced toxicity in liver was studied using two different concentrations of GAM and PEH (50 & 100 mg per kg.bw). These two concentrations were chosen on basis of standard hepatoprotective agent silymarin which found to show good efficacy at this range. As summaried in Table 5.2, CCl_4 group rats had no significant change on mean body weight as compared to that of

control group but relative liver weight was increased (45.70%) in comparison to normal control group. The pretreatment with GAM (50 and 100 mg per kg.bw) and PEH (50 and 100 mg/kg.bw) along with CCl_4 administration reduced the increase in liver weight with dose dependent manner (40.00% and 64.42%, at 50 and 100 mg per kg.bw of GAM, respectively) and (44.28% and 68.57% at 50 and 100 mg per kg.bw of GAM, respectively) compared to CCl_4 intoxicated group. The standard hepatoprotective agent silymarin showed protective effects with 72.85% reduction in the increased relative liver weight under CCl_4 induced toxicity. The animals fed with GAM and PEH extract alone had a similar mean body weight and relative liver weight to that of normal control group.

Changes of liver lipid profile among different treatment groups under CCl_4 toxicity pretreated with GAM and PEH are presented in Table 5.3. The CCl_4 administered group showed three to four fold increase in total lipid profile including lipid, triacylglycerol and cholesterol content. On the other hand GAM pretreated animal group, increase in lipid profile was diminished up to 40-45% (50 mg/kg.bw/day) and 73-80 (100 mg/kg.bw/day) percent. The PEH pretreated animal group demonstrated reduction in lipid profile up to 45-52 (50 mg/kg.bw/day) and 81-85 (100 mg/kg.bw/day) percent in comparison to the CCl_4 treated group. The standard hepatoprotective agent silymarin pretreatment (75 mg/kg.bw/day) indicated hepatoprotection with 83-90% reduction in increased lipid profile during CCl_4 intoxication.

5.3.2 Protective effects of GAM and PEH constituents on serum oxidative stress markers levels in CCl_4 intoxicated experimental rat

The serum level of various liver marker enzymes of control and GAM and PEH pretreated animal group was determined and the result is summarized in Table 5.4.

The data interpretation revealed that there was many fold increase in all these markers enzymes in serum of CCl_4 group compared to normal group. There found to be significant reduction in the level of these marker enzymes in serum of GAM and PEH pretreated group animals compared to CCl_4 group. Similar trend was also observed with bilirubin level.

5.3.3 Effects of GAM and PEH constituents on antioxidant status in serum and liver from different CCl_4 toxicity groups

The status of antioxidative enzymes like SOD and catalase, antioxidant capacity (level of glutathione and reducing power) in liver homogenate and serum of CCl_4 treated, normal and GAM and PEH treated group, were determined and results are presented in Table 5.5 and Table 5.6. There found to be a concentration dependent increase in the liver tissue SOD (148.93 and 158.45 at 50 mg per kg.bw; 198.65 and 213.57 at 100 mg per kg.bw) and catalase activity (128.47 and 137.76 at 50 mg per kg.bw; 145.86 and 163.48 at 100 mg per kg.bw) in GAM and PEH treated group, respectively compared to CCl_4 treated (SOD 106 U per mg protein and catalase 80.82 U per mg protein (Table 5.5). Significant increases were also found in level of glutathione and reducing power capacity. Similar trend for all these parameters was also found in serum of CCl_4 and GAM and PEH pretreated group (Table 5.6).

5.3.4 Protective potential of GAM and PEH constituents in ROS production during CCl_4 toxicity

The protective effects against liver toxicity was also studied by measuring the level of ROS production using DCFH-DA fluorescent probe *in vivo* CCl_4 intoxicated rat liver without or pretreated with GAM and PEH. The result is depicted in Fig 5.1.

There found to be significant reduction in ROS in GAM (993.47 UF per mg protein at 50 mg per kg.bw and 582.16 UF/mg protein at 100 mg per kg.bw) and PEH

(872.73 UF per mg protein at 50 mg per kg.bw and 489.18 UF per mg protein at 100 mg per kg.bw) treated animal group liver homogenate compared to CCl_4 treated alone (1593.65 UF/mg protein).

5.3.5 Effects of GAM and PEH pretreatment on histopathological changes in rat liver under CCl_4 toxicity

Hepatoprotective effect of GAM and PEH was also investigated by study of liver histological parameters. Light microscope photomicrograph of H&E stained rat liver tissue showing protective effects of GAM and PEH on CCl_4 intoxicated rat is presented in Fig 5.2. The severity scores for CCl_4 induced hepatic damage in rat liver tissue is shown in Table 5.7. The severity scores indicate the extent of hepatocellular necrosis, ballooning degeneration and inflammatory infiltration in different treatment group under CCl_4 induced toxicity with or without pretreatment of GAM and PEH constituents. Severity scores were much lower in case of GAM and PEH treated group compared to control confirming their protective effect.

Scanning electron microscope (SEM) photograph of rat liver surface is presented in Fig 5.3. High level of hepatocellular disorganization and necrosis was observed in CCl4 treated group and it was significantly reduced to normal in treated animal groups.

5.3.6 Protective role of GAM and PEH constituents against oxidative damage to DNA during CCl_4 induced toxicity

The spectrophotometric method based quantitative determination of DNA fragmentation presented in Fig 5.4 under CCl_4 toxicity along pretreated with or without GAM and PEH constituents. A concentration dependent reduction in DNA fragmentation was observed compared to CCl_4 treated group and at 100 mg per kg.bw it was almost similar to normal group.

DNA damage was also tested by agarose gel electrophoresis and the extent of DNA fragmentation pattern on rat liver genomic DNA under CCl_4 toxicity is depicted in Fig 5.5. Similar trends of protection of DNA damage were observed and almost no damage was seen at 100 mg per kg.bw GAM and PEH treatment group.

Protective effect of GAM and PEH on DNA damage induced due to CCl_4 toxicity was also studied by comet assay and the results are shown in Fig 5.6. The fluorescent photograph of comet assay depicts the TM (Tail Moments) pattern from rat liver single cell gel electrophoresis under CCl_4 toxicity. The tail moment's pattern was significantly reduced in treated group compared to CCl_4 and at 100 mg concentration it was found to be near normal. On the basis of different parameters, results revealed that both constituents GAM and PEH indicated their ability to reduce oxidative damage to DNA up to 74.36% and 78.73%, respectively under CCl_4 toxicity.

5.3.7 Effects of GAM and PEH pretreatment on lipid peroxidation inhibtion during CCl_4 induced toxicity

The protective effect of GAM and PEH on lipid peroxidation under CCl_4 induced oxidative stress was tested. The result presented in Fig 5.7a & b, indicates MDA generation as an index of extent of lipid peroxidation under CCl_4 induced toxicity and their protection on pretreatment with GAM and PEH constituents. As clear from Fig. 5.7a, the enhancement in total MDA production was observed up to 8 fold (4.12 nmol per mg protein) under CCl_4 induced lipid peroxidation in compared to normal animal group. The generation of MDA production was found to be diminished in the GAM (3.62), PEH (1.98) and silymarin (3.34) pretreated group. Lipid peroxidation level of different treatment group was also studied by liver tissue Schiffs staining. Schiffs stained liver tissue section is presented in Fig 5.8. There observed to

be much reduction in staining in treated group compared to CCl_4 group, indicating the reduced lipid peroxidation in treated animal group.

5.3.8 Effects of GAM and PEH pretreatment on oxidative damage to protein under CCl_4 induced toxicity

The protective effect of GAM and PEH were also tested against CCl_4 induced protein damage. The carbonyl formation due to oxidative damage of protein in rat liver tissue during CCl_4 intoxication is presented in Fig 5.9. A concentration depend effect of GAM and PEH pretreatment on protection against oxidative damage to protein was observed by reduction in the carbonyl index. The protective effect was also studied by checking the extent of carbonyl formation under different treatment groups by immunobloting using anti-DNP antibody and result is presented in Fig 5.10a. The different lanes represent the different treatment group indicating pretreatment with GAM and PEH to demonstrate their hepatoprotective effects as less staining were observed in these lanes. The densitometric band intensity analysis is given in Fig 5.10 b with their corresponding different treatment group to quantify the extent of reduction in protein carbonyl formation during pretreatment with GAM and PEH constituents under CCl_4 toxicity condition. The immunohistochemical detection of protein carbonyl formation under CCl_4 induced oxidative damage is depicted in Fig 5.11. On the basis of formation protein carbonyl content during CCl4 toxicity, results indicated that both constituents GAM (50 and 100 mg per kg) and PEH (50 and 100 mg per kg.bw) on pretreatment to CCl_4 induced liver toxicity, showed the ability to reduce enhanced protein carbonyl formation. The ability expressed in terms of percentage reduction in protein carbonyl formation was found to be 71.54 and 65.74%, which is statically equivalent with the protective ability of silymarin.

5.3.9 Effects of pretreatment with GAM and PEH constituents on CYP2E1 enzyme activity and expression under CCl_4 induced toxicity

Effects on CYP2E1 enzyme activity under CCl_4 toxicity pretreated with GAM and PEH constituents are presented in Table 5.8. The effects of GAM and PEH treatment on the major isozyme of cytochrome P-450 family (CYP2E1) which is involved in carbon tetrachloride bioactivation, were investigated. It was observed that both GAM and PEH markedly decreased the P4502E1 specific activities in a dose-dependent manner, as revealed from p-nitrophenol hydroxylation.

The immunoblot photograph for CYP2E1 enzyme expression is shown in Fig 5.12a. The densitometric band intensity analysis of immunoblot with their corresponding treatment lane is presented in Fig 5.12b. There found to be reduction in expression level of enzyme in presence of GAM (lane 3 &4) and PEH (lane 5 & 6) compared to normal group (lane 1). The expression of CYP2E1 enzyme expression was inhibited in the pretreated group by both GAM and PEH constituents up to 45.65 and 53.64 percent. The immunohistochemical study on CYP2E1 enzyme expression is was also studied and result is shown in Fig 5.13. Intensity found to be low in GAM and PEH treated tissue compared to the normal group tissue. All these observations and results confirm the protective role of GAM and PEH against CCl_4 induced hepatotoxicity.

5.4 Discussion

Hepatotoxicity due to prolong exposure to drug or environmental factors has now become a significant cause of acute liver damage. Carbon tetrachloride is widely used as a hepatotoxic compound for screening the hepatoprotective activity of several natural antioxidants in experimental model systems, because CCl_4 induced hepatotoxicity is regarded as an analogue for most of liver diseases (Cessarato *et al.*,

2004; Manibusan *et al.,* 2007). A numbers of biomarkers or products have been identified, which level significantly altered during liver damage (David *et al.*, 2000). These include the elevated level of liver enzymes (ALT, AST, ALP, LDH etc.) in serum or liver homogenate; reduction in anti-oxidative enzymes SOD and catalase activities and reduced glutathione level and the elevation of MDA level which is one of the end products of lipid peroxidation in the liver tissue; and are important indicators of generation of oxidative stress condition in CCl_4 intoxicated rats (Brent and Rummack, 1993; Cessareto *et al.*, 2004). Thus hepatoprotective potential of any compound or agent could be assessed by measuring the effect on level of these parameters.

In present study the hepatoprotective potential of GAM and PEH was investigated in CCl_4 induced toxicity in rat by measuring the levels of various biomarker indicators in serum and liver tissue. It has been observed that during CCl_4 induced oxidative stress condition, there is accumulation of fatty substances and found to increase in relative liver weight in comparison to normal treatment group (Aiyar *et al.*, 1964; Castro, 2004). There was slight increase in relative liver weight of CCl_4 alone treated animal group. On the other hand no change observed in relative liver of weight of antioxidant (GAM and PEH) treated animal compared normal and silymarin (well known hepatoprotective agent), show the hepatoprotective effect of GAM and PEH. It has been suggested that disturbances of hepatic lipid homeostasis are one of the multifaceted alterations caused due to CCl_4 toxicity in liver organ (Cessarato, 2004). The fatty degeneration and accumulation is one of the clear symptom developed in CCl_4 intoxicated rat liver and possibly may be due the alteration in fatty metabolism by free radical toxicity condition (Ara *et al.*, 2005; Manibusan *et al.*, 2007). The observed reduction in lipid profile in the presence of

GAM and PEH could be attributed to hepatoprotective role of GAM and PEH antioxidant constituents. The hepatoprotective ability of GAM and PEH was demonstrated to be equivalent in terms of dose and percent recovery in alterations in lipid profile as well as relative liver weight.

In the present investigation, the dose of CCl_4 used, caused significant hepatic damage in rats which was observed through a substantial increase in the concentration of serum parameters. Pre treatment of the rats with GAM and PEH at (50 and 100 mg per kg.bw) for 7 days before CCl_4 administration resulted in a significant protection against CCl4 induced elevation of serum marker enzymes (ALT, AST, ALP, LDH) and was found to be comparable with the recognized plant derived hepatoprotective agents. An obvious sign of hepatic injury is the leaking of cellular enzymes into the plasma due to the disturbance caused in the transport function of hepatocytes (Castro *et al.*, 1997). When liver plasma membrane is damaged a variety of enzymes located normally in cytosol is released into the blood, thereby causing increased enzyme level in the serum. The estimation of enzymes in the serum is a useful quantitative marker of the extent and types of hepatocellular damage (Sun *et al.*, 2001). Thus significant reduction in the level of liver marker enzyme in serum of GAM and PEH treated animal group is clear indication of stabilization of plasma membrane, as well as repair of hepatic tissue damage caused by CCl_4. The results are in agreement with the commonly accepted view that serum level of transaminase returns to normal with normalization of hepatic parenchyma organization and the regeneration of hepatocytes (Drotman and Lawhorn, 1978; Jaschke *et al.*, 2004). The tendency of these enzymes to return towards a near normal level in groups treated with silymarin, GAM, PEH constituents is a clear manifestation of their protective effects against CCl_4 induced oxidative stress liver pathologies.

Further the pretreatment with GAM and PEH demonstrated increased activity of antioxidant enzymes in blood plasma and rat liver tissue compared to CCl_4 treated animals, indicating the potentiality of both GAM and PEH to act as an antioxidant by preventing the oxidative damage inflicted on antioxidant system under CCl_4 induced toxicity. The results also indicated that treatment with GAM and PEH alone exhibited an increase in the activity of antioxidant enzymes and total antioxidant activity of blood plasma and liver tissue. The Reactive oxygen species (ROS), such as superoxide anions and H_2O_2, are produced throughout cells during normal aerobic metabolism (David *et al.*, 2000). The intracellular concentration of ROS is a consequence of both their production and their removal by various antioxidants. A major component of the antioxidant system in mammalian cells consists of three enzymes, namely, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These enzymes work in concert to detoxify superoxide anion, H_2O_2 and toxic metabolites produced under CCl_4 induced oxidative damage in rat liver tissue. Recent studies on the antioxidant properties of flavonoids from various plant extracts reveal their stimulatory action on antioxidative enzymes (Pavanato *et al.*, 2003; Xi *et al.*, 2008). It has been well known that SOD, CAT and glutathione constitute a mutually supportive team of defense against reactive oxygen species (ROS). Similar to the observation in the present study, increased activity of antioxidant enzymes by pretreatment with hepatoprotective agent found to prevent the peroxidative damage caused by CCl_4 treated animals in earlier study (Fraga *et al.*, 1987). Decline in GSH content in the liver of CCl_4 intoxicated rats and its subsequent recovery towards near normalcy in GAM, PEH and silymarin treated rats once again revealed the antioxidant and hepatoprotective effects. It has been reported in earlier studies that various natural antioxidant constituents obtained from dietary and

medicinal plant sources could protect liver organs against CCl_4 induced oxidative stress by enhancing the decreased activities of antioxidant defense components like superoxide dismutase (SOD) and catalase (CAT) as well as enhanced the decreased level of the hepatic reduced glutathione (GSH) (Bleibel *et al.*, 2007).

The role of CCl_4 intoxication to develop cellular necrosis and morphological alteration is well known. Such morphological alterations in during CCl_4 induced oxidative damage have been suggested due to fatty deposition, hepatocytes structural disorganization and cellular swelling (Cullen *et al.*, 2005; Manikbusan *et al.*, 2007). The observations of histopathological study of control and GAM and PEH treated group employing light microscopy and SEM analysis in present study clearly exhibited fewer incidences of alterations in rat liver surface morphology, hepatocellular necrosis, fatty degeneration and inflammatory cell infiltration in GAM and PEH pretreated groups. The results of histopathological and morphological study supported the hepatoprotective activity of GAM and PEH constituents against CCl_4 induced toxicity as revealed from biochemical aspects of study. In animal experiments CCl_4 has been shown to produce liver damage including hepatocellular necrosis and fatty degeneration in various species (Cessarato *et al.*, 2004). It has been considered that lipid peroxidation of hepatocyte membranes and thereafter changes in hepatic architecture is one of the principal causes of CCl4 induced oxidative stress damage and is mediated by the production of free radical derivatives of CCl4 (Recknagel *et al.*, 1989; Basu, 2003; Weber *et al.*, 2003). The histopathological observations of the liver of rats pretreated with GAM and PEH and subsequently CCl_4 intoxication showed a more or less normal architecture of the liver demonstrating recovery to a large extent and the protective potential was almost comparable to the silymarin pretreated groups. It is suggested that GAM and PEH may play a role in stabilizing

the plasma membrane as was reported in case of several plant derived hepatoprotective agents (Bhadauria *et al.*, 2008).

The toxic metabolites produced during CCl_4 induced toxicity have been suggested to produce cellular injury by damaging acts on target biomolecules inside liver cells. The extent of oxidative damage done on these target biomolecules including DNA, protein and lipid determines the cellular survival or the ability of liver cell to recover from CCl_4 induced toxicity (Castro *et al.*, 1997; Sundari *et al.*, 1997; Choi and Ou *et al.*, 2006). In various earlier studies oxidative damage to biomolecules has been as parameter to evaluate the ability of hepatoprotective agents to protect liver cells against hepatotoxin induced oxidative damage including CCl_4 toxins (Orhan *et al.*, 2007; Lin *et al.*, 2008).

The ability of GAM and PEH to protect oxidative damage to DNA induced due to CCl_4 toxicity was more or less equivalent to standard silymarin. In the present study, different evaluating methods including spectrophotometric, agarose gel electrophoresis and comet assay have been to assess the protective effect of GAM and PEH on DNA. It has been well established from the fact that CCl_4 induced toxicity condition generates a number of deadly free radicals and there by induce damage on DNA molecules which has been observed in all the methods used and also have been used in earlier studies (Cook *et al.*, 2003; Halliwell, 2007). To evaluate the hepatoprotective effects of antioxidant agents in terms of their ability to protect oxidative damage to DNA has been suggested to be suitable parameters (Castro et al., 1997; Amin and Hamza, 2007). In the present results, both GAM and PEH exhibited significant reduction in DNA damage, so it could be suggested that they have exerted their hepatoprotective activity.

Elevated levels of TBARS observed in CCl_4-treated rats indicate excessive formation of free radicals and activation of the lipid peroxidation system, resulting in hepatic damage. Similar to the earlier studies malondialdehyde (MDA) level in liver tissue increased substantially upon CCl_4 intoxication in present study. However, it was observed that that if the liver were pretreated with GAM and PEH, MDA level decreased distinctly as evident from spectrophotometric determination and schiffs staining on liver tissue sections. There are reports that free radicals combines with the cellular lipid and proteins to produce lipid peroxidation, measured through its catabolite, malondyaldehyde (MDA), resulting in structural changes of endoplasmic reticulum and other biomembranes and loss of metabolic activity leading to liver damage (Kadiiska *et al.,* 2000; Zhu and Fung, 2000). Consistent with the serum AST and ALT activities, pretreatment with GAM and PEH resulted in a significant dose-dependent decrease in the concentration of hepatic MDA when compared with the CCl_4 group. Lipid peroxidation, a reactive oxygen species mediated mechanism, has been implicated in the pathogenesis of various liver injuries and subsequent liver fibrogenesis in experimental animals and humans (Lee *et al.*, 2005). MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid. Therefore, the hepatic content of MDA is used as an indicator of liver tissue damage involving a series of chain reactions (Ohkawa *et al.*, 1979; Choudhary *et al.,* 1994). In the present study, the significant increase in hepatic MDA concentration observed in the CCl_4 group is an indicator of increased lipid peroxidation caused by administration of CCl_4. Here the study clearly demonstrates that GAM and PEH treatment ameliorated the deleterious effects of CCl_4 on lipid peroxidation in whole liver and microsomal fraction by acting as an antioxidant.

In the present study, administration of CCl_4 resulted in a significant increase in the hepatic MDA concentration and indicating increased Schiffs stained rat liver tissue. The high antioxidant activities of innate defense system like superoxide dismutase and catalase were correlated with the less incidence of lipid peroxidation. The significant dose dependent decrease in the hepatic MDA concentration confirms that pretreatment with both GAM and PEH constituents could effectively protect liver against the lipid peroxidation induced by CCl_4. Most of the hepatotoxic chemicals including CCl_4 inflict oxidative damage to liver mainly by enhancing lipid peroxidation directly or indirectly. In the present study, GAM and PEH pretreatment was effective in reducing the increases amount of MDA during CCl_4 induced toxicity. They reported the strongest inhibition of malondialdehyde (MDA), MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid (Abd Ellah *et al.*, 2007). Therefore, the hepatic content of MDA is considered to be an indicator of CCl_4 induced liver tissue damage involving a series of chain reactions (Ohkawa *et al.*, 1979).

The scientific facts indicate that in due course of CCl_4 toxicity in rat liver, the formed free radicals inside liver is capable of inflict damage on many important protein targets. Such free radical mediated oxidation to proteins lead to increase in the production of protein carbonyl (Berlett and Stadtman, 1997; Wilson, 1998) and in the present attempt results revealed three to four fold increases in protein carbonyl formation. As results demonstrated the ability of GAM, PEH and silymarin agents to reduce amount of protein carbonyl increased due to CCl_4 toxicity, it could be suggested that these agents protected liver from damage.

The mechanism of cellular damage caused by CCl_4 is its bioactivation by the cytochrome P-450 isozyme system (CYP2E1) in smooth endoplasmic reticulum into

trichloromethyl free radical (Slater, 1984; Weber *et al.*, 2003). Once the trichloromethyl radical is formed, it reacts with molecular oxygen to form the highly toxic trichloromethyl peroxy radical (Packer *et al.*, 1978; Connor *et al.*, 1990; (Manibusan *et al.*, 2007). The increased expression of the CYP2E1 during CCl_4 intoxication enhances generation of superoxide, hydroxyl and hydroxyethyl radicals, and peroxisomal β-oxidation of free fatty acids, which generates hydrogen peroxide. These free radicals covalently bind and cause extensive damage to DNA, proteins and lipids resulting into oxidative stress toxicity to liver. Since the cytochrome P-450 isozyme CYP2E1 plays central role in transformation of CCl_4 into its free radical intermediate (Castro *et al.*, 1997; Weber *et al.*, 2003). Therefore, the effect GAM and PEH on this enzyme was studied, both GAM and PEH constituents showed its ability to inhibit the CYP2E1 enzyme activity in dose dependent manner. On the basis of such ability, it could be suggested that both GAM and PEH may protect against CCl_4 induced damage by lowering down the CYP2E1 enzyme activity. As results revealed that under CCl_4 toxicity, amount of CYP2E1 enzyme is also reduced and such facts have been suggested by earlier findings (Wong *et al.*, 1998; Neve and Ingelman-Sundbeard, 2008). Consistent with these observations, the P4502E1 expressions were also decreased, as determined by immunoblot analysis. These results demonstrate that pretreatment of rat with GAM and PEH decreases the expression of P4502E1 enzyme, and reduces biotransformation of carbon tetrachloride, and diminished carbon tetrachloride-induced liver injury. The further insights implies that Fe^{2+} ions play a role as mediators of CCl_4 induced hepatotoxicity due to their ability to produce free radicals *in vivo* and *in vitro* condition (Younes, 1985; Aust and Svingen, 2002).

Since it is clear from the above observation and discussion that free radicals mediated oxidative stress condition play pivotal role in liver diseases pathology and

progression therefore the compounds or agents which have ability to scavenge free radical or inhibit there formation and able to chelates metal ions particularly Fe^{2+}, could be an effective hepatoprotective agents. Antioxidant due to their free radical scavenging and metal chelating ability could also be a potent hepatoprotectant. Many natural antioxidants derived from dietary and medicinal plants possessing antioxidative properties have been demonstrated to prevent and treat hepatopathies induced by CCl_4 mediated oxidative stress in liver tissue (Hensley *et al.*, 2000; Vitaglione *et al.*, 2004; Bhaduria *et al.*, 2008). A number of plants hepatoprotective activity was found to be due to their glycosides (Xi *et al.*, 2008; Lin *et al.*, 2008); flavonoids (Hanje *et al.*, 2006; Rivera *et al.*, 2008) and phenolic compounds (Zhen *et al.*, 2007; Orhan, 2007; Dani *et al.*, 2008). Since the GAM and PEH found to have strong free radical scavenging and metal chelating ability and their active constituents found to be glycol sides and phenolics. Thus it is suggested that potential hepatoprotective activity observed in the present study is also due to their glycosides and phenolics constituents.

CONCLUSION

The hepatoprotective potential of GAM and PEH in CCl_4 intoxicated rats was tested by the level of serum liver marker enzyme, antioxidative status, reduction in biomolecules damage and liver tissue histological parameters. All these observations clearly indicate that both the GAM and PEH found to have strong hepatoprotective effect, as there strong reduction in the oxidative stress induced damage of liver in GAM and PEH pretreated animals and it was almost similar to the normal rat in contrast to CCl_4 treated which showed maximum liver damaged. The efficacy was almost similar to the well known hepatoprotective agent silymarin. It is suggested that the strong hepatoprotective effect of GAM and PEH is due to their high free

radical scavenging and metal chelating abilities. Since the active constituents of GAM and PEH are identified to be glycosides and phenolics, respectively, as described in Chapter-3, it is further suggested that the hepatoprotective ability of GAM and PEH is due to their glycosides and phenolic constituents. When both GAM and PEH constituents were fed alone, there was no sign of any *in vivo* toxicity effects at biochemical, histological and behavioral level. Thus is it strongly suggested that GAM and PEH from *A. marmelos* and *E. hirta,* respectively could serve as a source of antioxidant and hepatoprotective agent without any *in vivo* toxicity effects.

Table 5.1 Group design and different treatment group

Group/ Treatment	Week 1	Week 2	Week 3	Week 4
Group 1 (N=6)	Only Vehicle (VEH) (Olive oil)	Only VEH	Only VEH	Only VEH
Group 2 (N=6)	VEH+CCl_4 (1:1) (1 ml per kg. bw, i.p) first dose on 7th day	VEH+CCl_4 (1:1) (1 ml per kg. bw, i.p) Second dose on 14th day	VEH+CCl_4 (1:1) (1 ml per kg. bw, i.p) third dose on 21st day	Fourth dose on 28th day
Group 3 (N=8)	GAM (50 mg per kg.bw p.o) per day+CCl_4 dose given in Group 2	GAM (50 mg per kg.bw p.o) per day+CCl_4	GAM (50 mg per kg.bw p.o) per day+CCl_4	GAM (50 mg per kg.bw p.o) per day+CCl_4
Group 4 (N=8)	GAM (100 mg per kg.bw p.o) per day+CCl_4 Dose given in Group 2	GAM (100 mg per kg.bw p.o) per day+CCl_4	GAM (100 mg per kg.bw p.o) per day+CCl_4	GAM (100 mg per kg.bw p.o) per day+CCl_4
Group 5 (N=8)	PEH (50 mg per kg.bw p.o) per day+CCl_4 Dose given in Group 2	PEH (50 mg per kg.bw p.o) per day+CCl_4	PEH (50 mg per kg.bw p.o) per day+CCl_4	PEH (50 mg per kg.bw p.o) per day+CCl_4
Group 6 (N=8)	PEH (100 mg per kg.bw p.o) per day+CCl_4 Dose given in Group 2	PEH (100 mg per kg.bw p.o) per day+CCl_4	PEH (100 mg per kg.bw p.o) per day+CCl_4	PEH (100 mg per kg.bw p.o) per day+CCl_4
Group 7 (N=6)	Silymarin (75 mg per kg.bw p.o) per day+CCl_4 Dose given in Group 2	Silymarin (75 mg per kg.bw p.o) per day+CCl_4	Silymarin (75 mg per kg.bw p.o) per day+CCl_4	Silymarin (75 mg per kg.bw p.o) per day+CCl_4
Group 8 (N=6)	VEH+GAM (200 mg per kg.bw) per day	VEH+GAM (200 mg per kg.bw) per day	VEH+GAM (200 mg per kg.bw) per day	VEH+GAM (200 mg per kg.bw) per day
Group 9 (N=6)	VEH+PEH	PEH (200 mg per kg.bw) per day +VEH	PEH (200 mg per kg.bw) per day+VEH	PEH (200 mg per kg.bw) per day+VEH

Table 5.2 Study on liver body weight, absolute liver weight and relative liver weight of CCl_4 treated rats with or without the pretreatment of GAM and PEH constituent.

Group/Treatment	0 days of treatment	28th day of treatment		
	Body weight (g) a	Body weight (g) a	Absolute liver weight (g) a	Relative liver weight (g %)
Normal Control	210.48±13.26	250.14±16.12	4.01±0.56	1.60±0.18
CCl_4 Control	219.28±4.87	255.18±14.28	5.96±0.68	2.3±0.21*
GAM (50 mg per kg.bw)+CCl_4	201.38±3.78	246.21±3.86	4.98±0.32	2.02±0.14 ** (40.00%)
GAM (100 mg/kg bw)+CCl_4	228.21±5.34	275.32±7.54	5.16±0.43	1.85±0.12** (64.42%)
PEH (50 mg per kg bw)+CCl_4	211.23±5.38	260.27±6.43	5.19±0.51	1.99±0.16** (44.28%)
PEH (100 mg per kg bw)+CCl_4	232.18±4.21	280.31±3.87	5.12±0.29	1.82±0.11** (68.57%)
Silymarin (75 mg per kg.bw)+CCl_4	209.46±5.56	250.21±5.32	4.52±0.24	1.79±0.13** (72.85%)
Vehicle+GAM (200 mg per kg .bw)	241.29±8.69	287.32±4.38	4.65±0.35	1.61±0.13
Vehicle+ PEH (200 mg per kg .bw)	206.37±3.85	251.21±3.85	4.11±0.27	1.63±0.14

a Data presented are given as mean±SEM, n=6

* Significantly different from Normal control group, $p < 0.05$.

** Significantly different from the CCl_4 treated group, $p < 0.05$.

Table 5.3 Total lipid profile of rat liver tissue during CCl_4 toxicity and their protection in the presence of GAM and PEH constituent.

Treatment Group	Total lipid profile of rat liver (mg/g fresh wt. liver tissue) a		
	Total lipid	Total triacylglycerol	Total Cholesterol
Normal Control	65.18±4.27	5.83±0.27	28.65±3.12
CCl_4 Control	274.13±8.63*	28.19±2.18*	104.82±5.91*
GAM (50 mg per kg.bw)+CCl_4	184.28±10.7≠ (43.00%)	19.23±1.78≠ (40.07%)	70.18±3.82≠ (45.47%)
GAM (100 mg/kg bw)+CCl_4	105.58±8.43≠ (80.66%)	11.86±2.85 ≠ (73.03%)	46.15±3.56≠ (77.02%)
PEH (50 mg per kg bw)+CCl_4	175.49±12.84≠ (47.20%)	17.82±1.05 ≠ (46.37%)	65.23±3.42≠ (51.57%)
PEH (100 mg per kg bw)+CCl_4	100.29±11.09≠ (83.19%)	9.98±0.08 ≠ (81.44%)	40.27±2.74≠ (84.74%)
Silymarin (75 mg per kg.bw)+CCl_4	82.81±9.93≠ (91.56%)	9.51±0.05≠ (83.54%)	38.64±3.83≠ (86.88%)
Vehicle+GAM (200 mg per kg .bw)	69.07±5.65	6.02±0.31	32.89±2.85
Vehicle+ PEH (200 mg per kg .bw)	68.95±6.76	6.11±0.43	31.72±3.76

a Data presented are given as mean±SEM, n=6

* Significantly different from Normal control group, $p < 0.05$.

** Significantly different from the CCl4 treated group, $p < 0.05$.

Table 5.4 Protective effect of GAM and PEH constituents on serum oxidative stress markers AST, ALT, ALP, LDH and Bilirubin levels of CCl_4 intoxicated in experimental rat.`

Group/treatment	Serum oxidative stress marker level				
	ALT a (U/l)	AST a (U/l)	ALP a (KA units)	LDH a (IU/dl)	Bilirubin a (mg/dl)
Normal Control	45.38±5.26	54.18±4.98	50.84±3.18	97.18±4.89	0.95±0.1
CCl_4 Control	1201.28±20.65*	855.32±16.8*	386.21±4.98*	805.28±18.3 *	4.21±0.23 *
GAM (50 mg per kg.bw)+CCl_4	678.23±15.38 (45.25%)**	563.27±16.4 (36.45%)**	231.98±12.6 (45.98%)**	517.91±18.2 (40.58%)**	2.65±0.17 (47.85%)**
GAM (100 mg/kg bw)+CCl_4	362.18±12.51 (72.59%)**	293.31±13.8 (70.15%)**	144.71±11.8 (72.01%)**	306.81±16.91 (70.39%)**	1.56±0.15 (81.28%)**
PEH (50 mg per kg bw)+CCl_4	628.29±8.69 (49.57%)**	517.32±14.3 (42.18%)**	218.65±13.1 (49.96%)**	512.47±23.52	2.47.59±0.35 (53.37%)**
PEH (100 mg per kg bw)+CCl_4	306.37±3.85 (77.42%)**	251.21±13.8 (75.40%)**	126.48±8.83 (77.44%)**	254.79±18.9 (77.74%)**	1.31±0.38 (88.95%)**
Silymarin (75 mg per kg.bw)+CCl_4	225.84±5.34 (84.37%)**	132.62±12.4 (90.20%)**	88.28±9.28 (88.83%)**	164.47±17.1 (90.49%)**	1.28±0.26 (89.87%)**
Vehicle+GAM (200 mg per kg .bw)	56.82±6.85	67.96±5.92	59.81±7.92	109.56±4.39	1.03±0.05
Vehicle+ PEH (200 mg per kg .bw)	61.38±5.58	62.18±3.84	53.82±4.72	105.29±6.18	1.02±0.03

a Data presented are given as mean±SEM, n=6

* Significantly different from Normal control group, $p < 0.05$.

** Significantly different from the CCl4 treated group, $p < 0.05$.

Table 5.5 Effects of GAM and PEH pretreatment on antioxidant status of rat liver tissue showing oxidative stress induced CCl_4 toxicity.

Group/treatment	Total antioxidant enzyme and antioxidant capacity of liver tissue [a]				
	SOD (U/mg protein)	Catalase (U/mg protein)	Glutathione (µmol/mg protein)	FRAP (µmol Trolox eq. /mg protein)	Total protein mg protein per g liver tissue
Normal Control	240.78±14.38	189.27±12.7	48.12±3.75	87.29±6.54	178.77±12.8
CCl_4 Control	106.85±10.86*	80.82±8.96*	15.63±1.83*	38.78±2.18*	165.92±11.9*
GAM (50 mg per kg.bw)+CCl_4	148.93±13.37**	128.47±9.25**	28.41±2.17**	51.27±4.45**	168.33±14.4**
GAM (100 mg/kg bw)+CCl_4	198.65±12.47**	145.86±14.7**	36.32±3.37**	64.85±5.45**	171.3±14.3**
PEH (50 mg per kg bw)+CCl_4	158.45±14.34	137.76±12.7	31.43±4.83	58.28±5.49	169.8±12.1
PEH (100 mg per kg bw)+CCl_4	213.57±16.48**	163.48±13.3**	42,28±3.68**	71.47±7.65**	180.3±15.9**
Silymarin (75 mg per kg.bw)+CCl_4	207.57±14.38**	165.57±14.6**	40.34±4.58**	73.21±7.43**	182.7±16.3**
Vehicle+GAM (200 mg per kg .bw)	398.63±17,32	323.65±16.3	104.56±8.32	186.43±12.5	180.3±14.2
Vehicle+ PEH (200 mg per kg .bw)	416.35±19.45	387.45±18.4	114.47±6.57	283.32±15.32	178.32±14.5

a Data presented are given as mean±SEM, n=6

* Significantly different from Normal control group, $p < 0.05$.

** Significantly different from theCCl4 treated group, $p < 0.05$.

Table 5.6 Protective effects on blood plasma antioxidant status using in CCl_4 induced toxicity experiments.

Group/treatment	Total antioxidant enzyme level and antioxidant capacity in plasma [a]				
	SOD (U/mg protein)	Catalase (U/mg protein)	Glutathione (μmol/mg protein)	FRAP (μmol Trolox eq. /mg protein)	Total protein (mg protein per ml)
Normal Control	125.73±12.64	154.18±14.98	38.68±2.91	197.18±14.89	0.75±0.12
CCl_4 Control	29.48±4.76*	46.32±5.74*	6.93±0.86*	46.48±5.17*	0.63±0.10
GAM (50 mg per kg.bw)+CCl_4	57.34±5.19**	62.32±6.38**	14.86±1.38**	69.32±6.18 **	0.68±0.08
GAM (100 mg/kg bw)+CCl_4	86.85±7.37**	94.48±7.57**	26.65±3.92**	94.32±5.29 **	0.71±0.14
PEH (50 mg per kg bw)+CCl_4	63.46±4.51**	68.58±3.47**	17.93±1.76**	74.37±6.68**	0.65±0.13
PEH (100 mg per kg bw)+CCl_4	97.57±9.37**	118.46±3.87**	29.87±2.75	125.75±7.32**	0.76±0.16
Silymarin (75 mg per kg.bw)+CCl_4	93.41±5.65**	125..21±8.46**	31.54±3.78**	137.29±10.45**	0.73±0.12
Vehicle+GAM (200 mg per kg .bw)	286.94±13.57	313.48±18.32	98.68±6.67	359.65±21.75	0.81±0.16
Vehicle+PEH (200 mg per kg .bw)	315.34±18.57	402.1±22.47	105.18±7.46	423.7±25.32	0.78±0.11

a Data presented are given as mean±SEM, n=6

* Significantly different from Normal control group, $p < 0.05$.

** Significantly different from the CCl4 treated group, $p < 0.05$.

Table 5.7 Severity scores for histopathological alteration in CCl_4 intoxicated rat liver hepatocytes showing ballooning degeneration, inflammatory infiltration and hepatocellular necrosis.

Group*	N	Ballooning degeneration [a]				Avg.	Inflammatory infiltration [a]				Avg.	Hepatocellular necrosis [a]				Avg.
		0	1	2	3		0	1	2	3		0	1	2	3	
A	4	0	0	0	0	NA	0	0	0	0	NA	0	0	0	0	NA
B	4				4	3.0			3	1	2.25				4	4.0*
C	4		2	2		1.5±0.4*		3	1		1.25*		4			1.0*
D	4		4			1.0±0.3*	2	2			0.5*	1	3			0.75*
E	4		2	2		1.5+0.7*		4			1.0*		4			1.0*
F	4	3	1			0.25*	3	1			0.25*	2	2			0.5*
G	4	4				NA	4				NA	3	1			0.25
H	4	0	0	0	0	NA	0	0	0	0	NA	0	0	0	0	NA
I	4	0	0	0	0	NA	0	0	0	0	NA	0	0	0	0	NA

a Data presented are given as mean±SEM, n=6

Significantly different from Normal control group, $p < 0.05$.

Table 5.8 Effects on CYP2E1 p- nitrophenyl and aniline 4-hydroxylase activity under CCl_4 toxicity and pretreatment with GAM and PEH antioxidant constituents.

Treatment group	Effects on CYP2E1 activity under CCl_4 toxicity and pretreatment with GAM and PEH
	p- nitrophenyl activity PNP activity (pmol/ min/mg protein)
Normal Control	298.32±23.41
CCl_4 Control	143.85±11.36 *
GAM (50 mg per kg.bw)+CCl_4	187.19±12.82
GAM (100 mg per kg bw)+CCl_4	236.21±16.46
PEH (50 mg per kg. bw)+CCl_4	195.48±13.81
PEH (100 mg per kg .bw)+CCl_4	253.31±19.72
Silymarin (75 mg/kg.bw)+CCl_4	260.34±21.53
VEH+GAM (50 mg per kg .bw) for one week	228.26±17.4
VEH+GAM (100 mg per kg.bw) one week	187.57±12.65
VEH+ PEH (50 mg per kg .bw) one week	219.43±16.4
VEH+PEH (100 mg per kg .bw) one week	176.39±12.86

a Data presented are given as mean±SEM, n=6

* Significantly different from Normal control group, $p < 0.05$.

** Significantly different from the CCl4 treated group, $p < 0.05$.

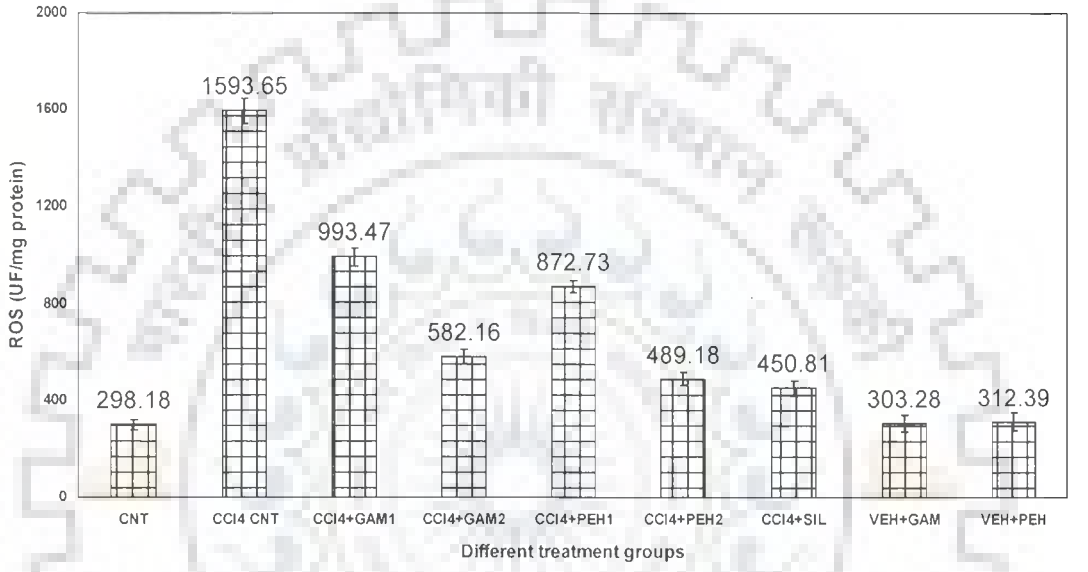

Fig 5.1 Depicts the ROS production using DCFH-DA fluorescent probe *in vivo* CCl_4 intoxicated rat liver. In the graph CNT (Normal control, vehicle only); CCl4 CNT (CCl_4 treated control); CCl_4+GAM1 (50 mg per kg.bw); CCl_4+GAM2 (100 mg per kg.bw); CCl_4+PEH1 (50 mg per kg.bw); CCl_4+PEH2 (100 mg per kg.bw); CCl_4+SIL (Silymarin 75 mg per kg.bw). The ROS measurement is expressed in terms of fluorescent unit per mg rat liver protein.

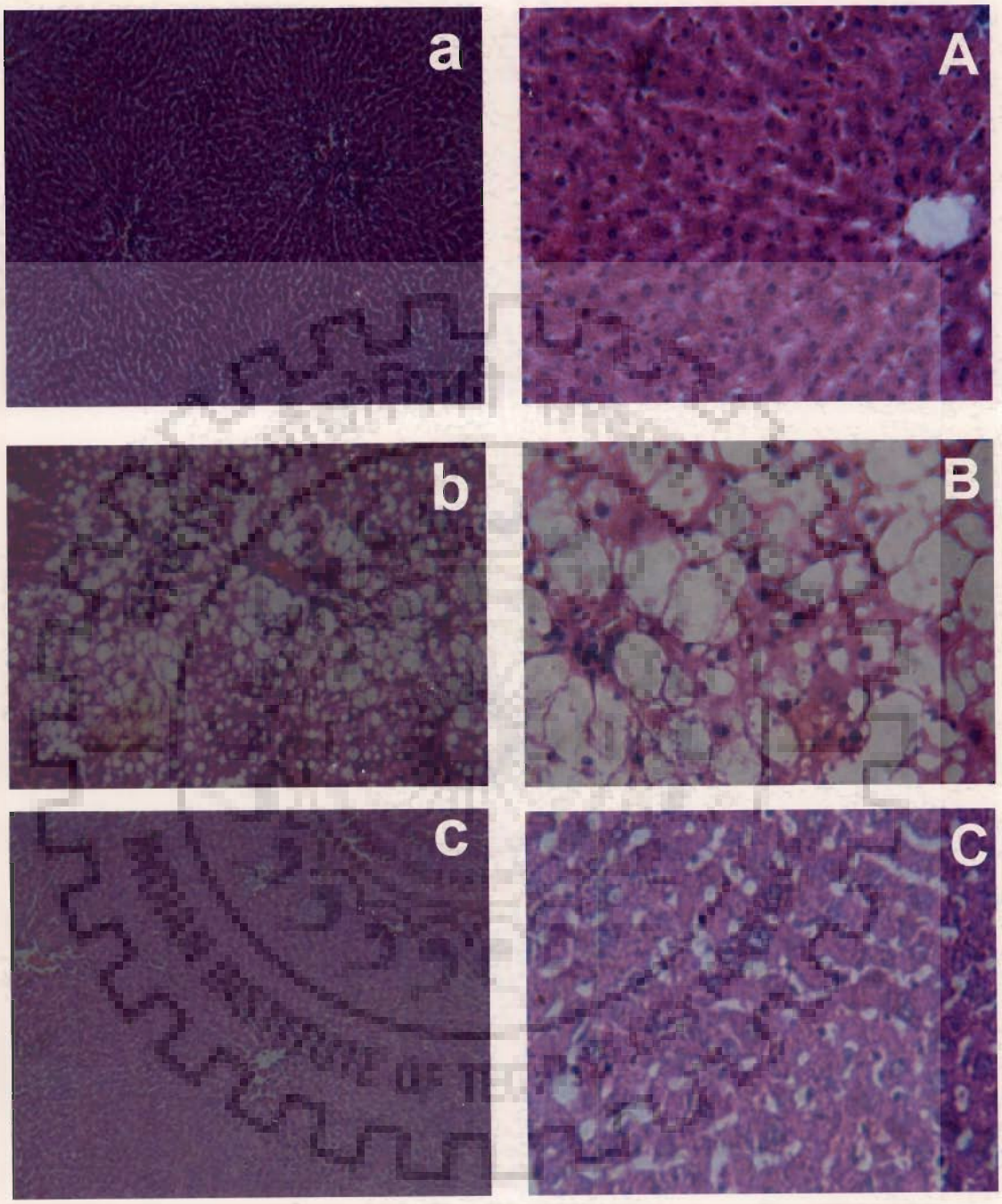

Fig 5.2 Light microscope photomicrograph of H&E stained rat liver tissue showing protective effects of GAM and PEH on CCl_4 intoxicated rat. Photograph [a, A (Normal control) 100X, 400X)] Normal arrangement of hepatocytes and sinusoids; [b, B (CCl_4 treated control) 100X, 400X] centrilobular necrosis, fatty deposition, hepatocellular ballooning degeneration with multiple vacuolation, enlargement and collapse of sinusoidal walls, and inflammatory cell infiltration; [a, A (CCl_4+silymarin 75 mg per kg.bw 100X, 400X] less symptom of hepatocellular damage with improved hepatocellular arrangement.

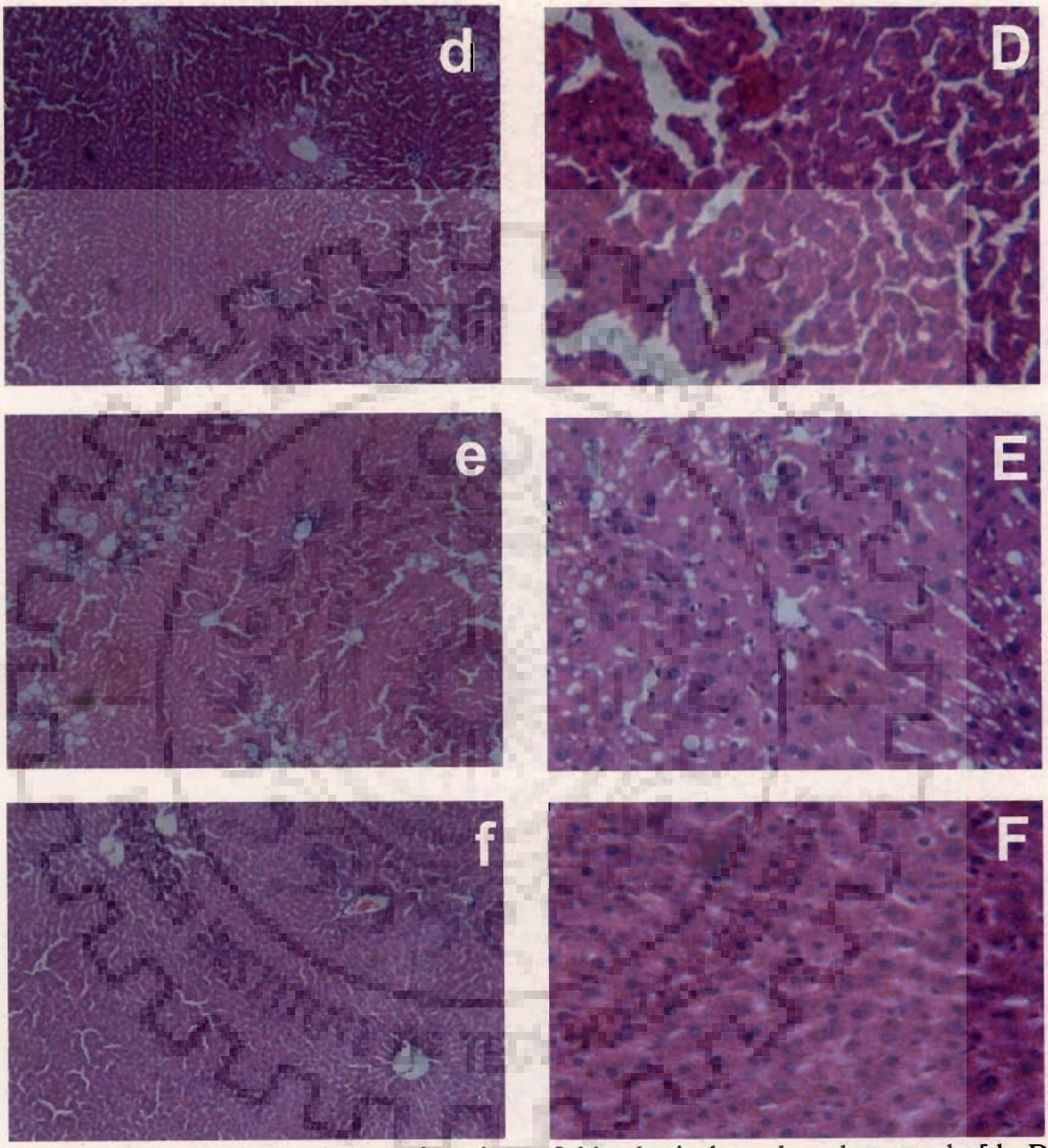

Fig 5.2 Continued. In the continuation of histological study, photograph [d, D (CCl_4+GAM 50 mg per kg.bw) 100X, 400X] mild ballooning, moderate necrosis and mild inflammatory infiltration; [e, E (CCl_4+GAM 100 mg per kg.bw) 100X, 400X] improved hepatocytes arrangement and mild observations of cellular necrosis and ballooning and fatty deposition; [f, F (Only GAM+ vehicle 200 mg per kg,.bw) 100X, 400X] Normal hepatic architecture without any hepatocellular damage.

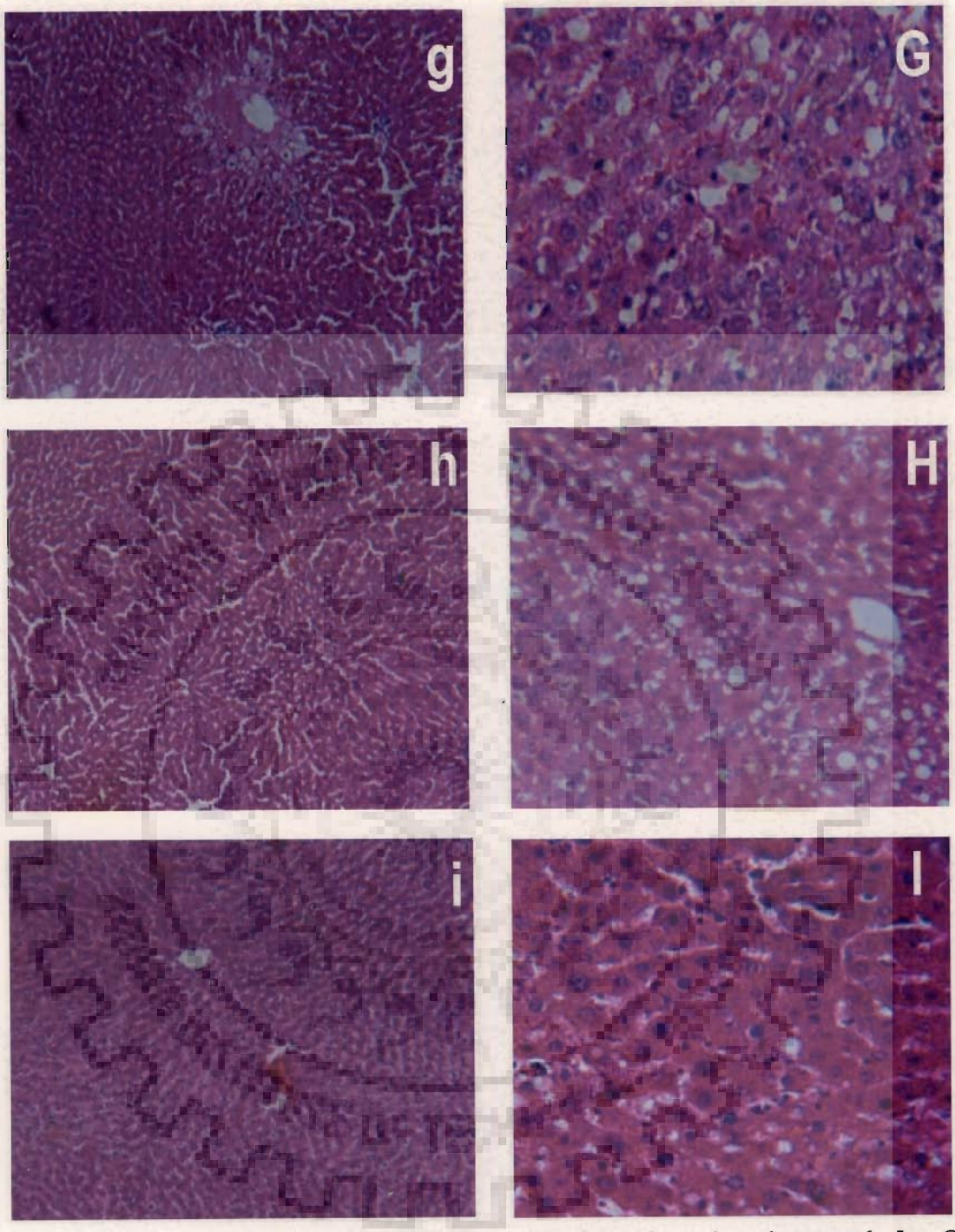

Fig 5.2 Continued.. In the continuation of histological study, photograph [g, G (CCl_4+PEH 50 mg per kg.bw) 100X, 400X] mild ballooning, moderate necrosis and mild inflammatory infiltration ; [h, H (CCl_4+PEH 100 mg per kg.bw) 100X, 400X] improved hepatocytes arrangement and mild observations of cellular necrosis and ballooning and fatty deposition; [i, I (Only vehicle+ PEH 200 mg per kg.bw) 100X, 400X] Normal hepatic architecture without any hepatocellular damage.

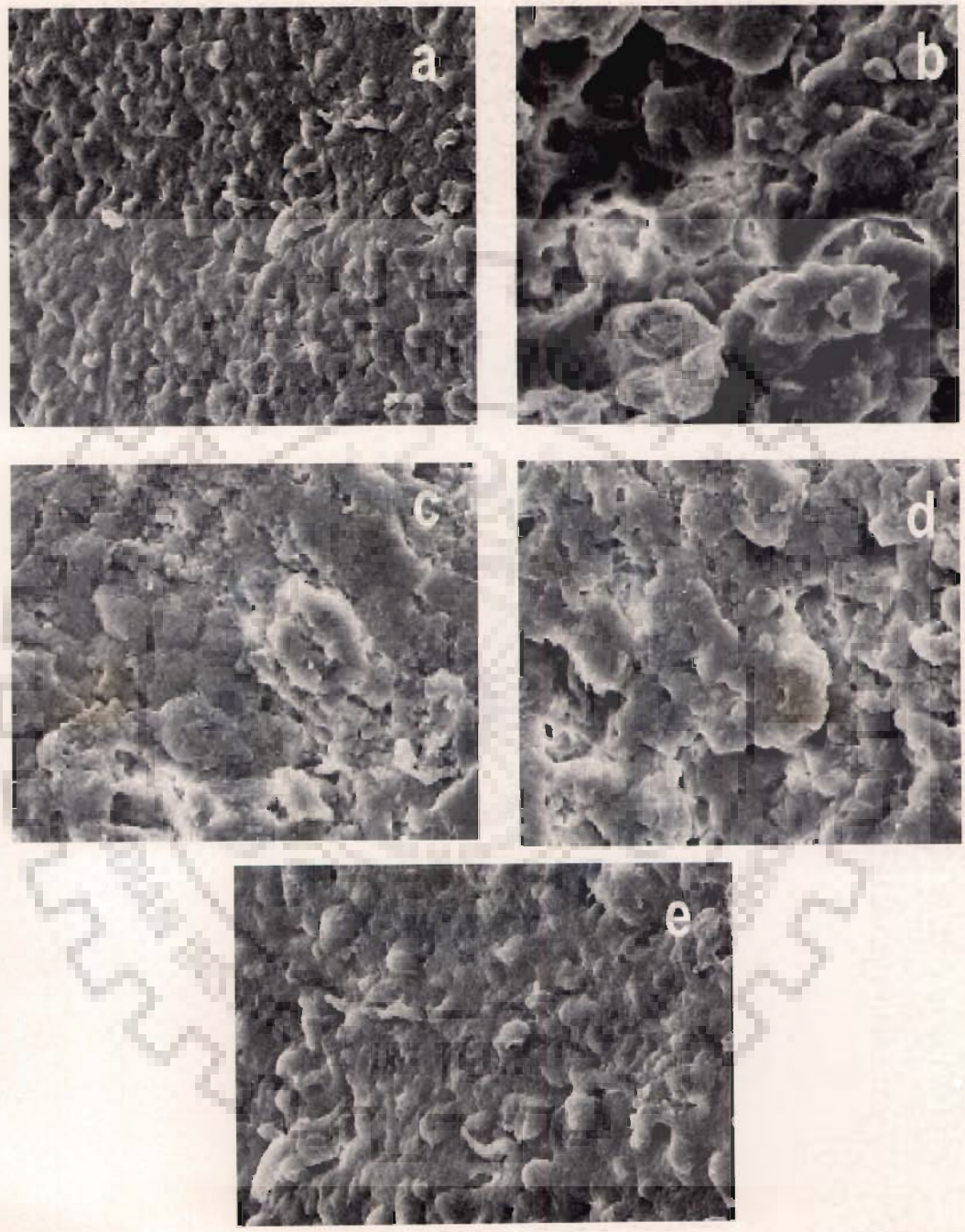

Fig. 5.3 Scanning electron microscope photograph of rat liver surface under different CCl_4 toxicity model experiment at 2000X magnification. In photograph [a, Normal control)]; [b (CCl_4 treated control)]; [c (CCl_4+GAM 50 mg per kg.bw); [d, CCl_4+GAM 100 mg per kg.bw) 2000 X,]; [e, only GAM 200 mg per kg.bw).

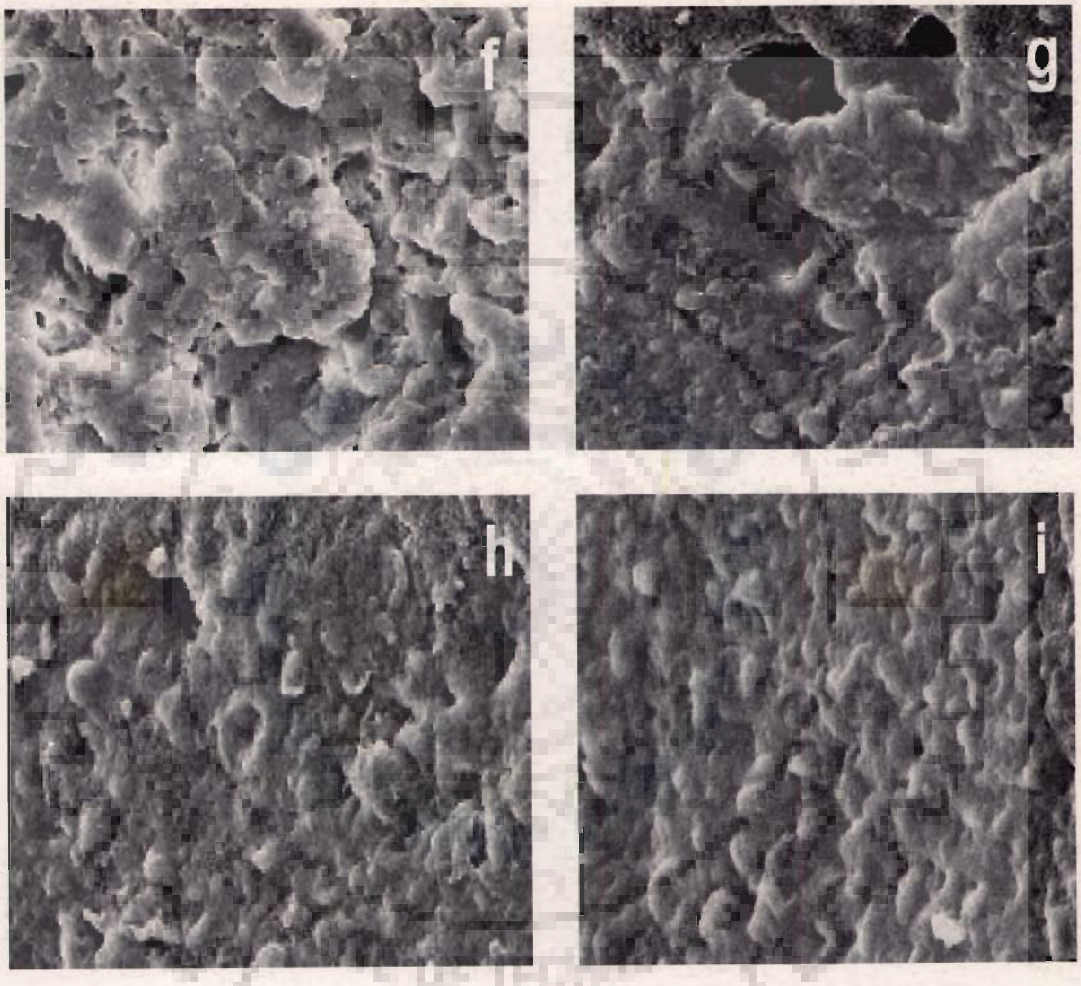

Fig 5.3 Continued. In the photograph SEM photograph at 2000X: [f, (CCl_4+PEH 50 mg per kg. bw)]; [g, CCl_4+PEH 100 mg per kg. bw)]; [h, only PEH 200 mg per kg. bw)]; [i, CCl_4+Silymarin 75 mg per kg.bw)].

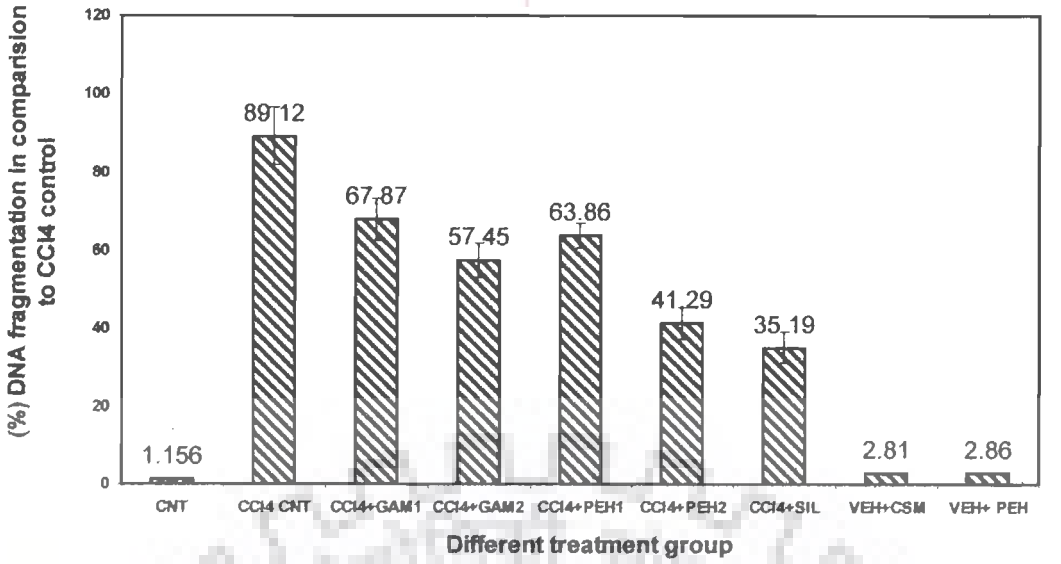

Fig 5.4 Quantitative determination of DNA fragmentation using diphenylamine spectrophotometric method in different rat liver tissue under CCl_4 toxicity along with or without GAM and PEH constituents. In brief, (Normal control, vehicle only); CCl_4 CNT (CCl_4 treated control); CCl_4+GAM1 (50 mg per kg.bw); CCl_4+GAM2 (100 mg per kg.bw); CCl_4+PEH1 (50 mg per kg.bw); CCl_4+PEH2 (100 mg per kg.bw); CCl_4+SIL (silymarin 75 mg per kg.bw. VEH+GAM (vehicle and GAM (200 mg per kg.bw), VEH+PEH (vehicle and PEH (200 mg per kg.bw)

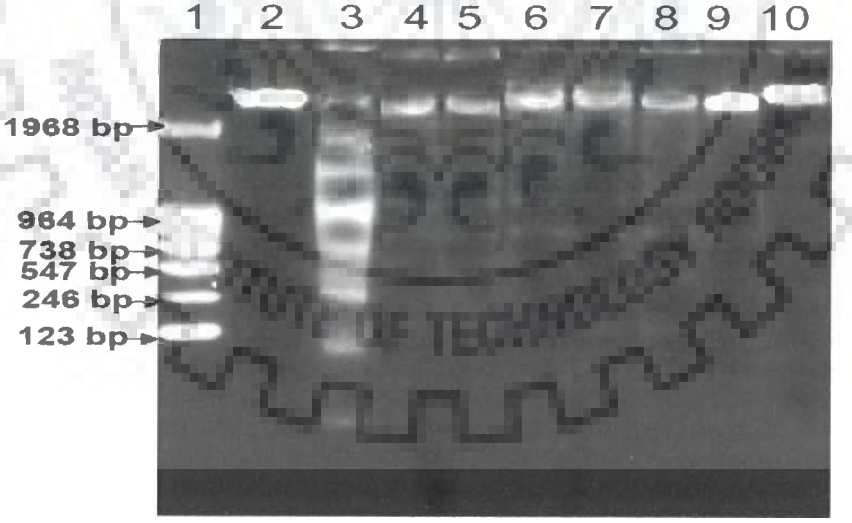

Fig 5.5 Shows the DNA fragmentation pattern on rat liver genomic DNA under CCl_4 toxicity and pretreatment with GAM and PEH constituents. Lane 1- marker, Lane 2- Normal control, Lane3 CCl_4 treated control, Lane 4 CCl_4+GAM (50 mg per kg.bw), Lane 5: CCl_4+GAM (100 mg per kg.bw), Lane 6 CCl_4+PEH (50 mg per kg.bw), Lane CCl_4+PEH (100 mg per kg.bw), Lane 8 CCl_4+Silymarin (75 mg per kg.bw), Lane 9: vehicle+GAM (200 mg per kg.bw.), Lane 10: vehicle+PEH (200 mg per kg.bw).

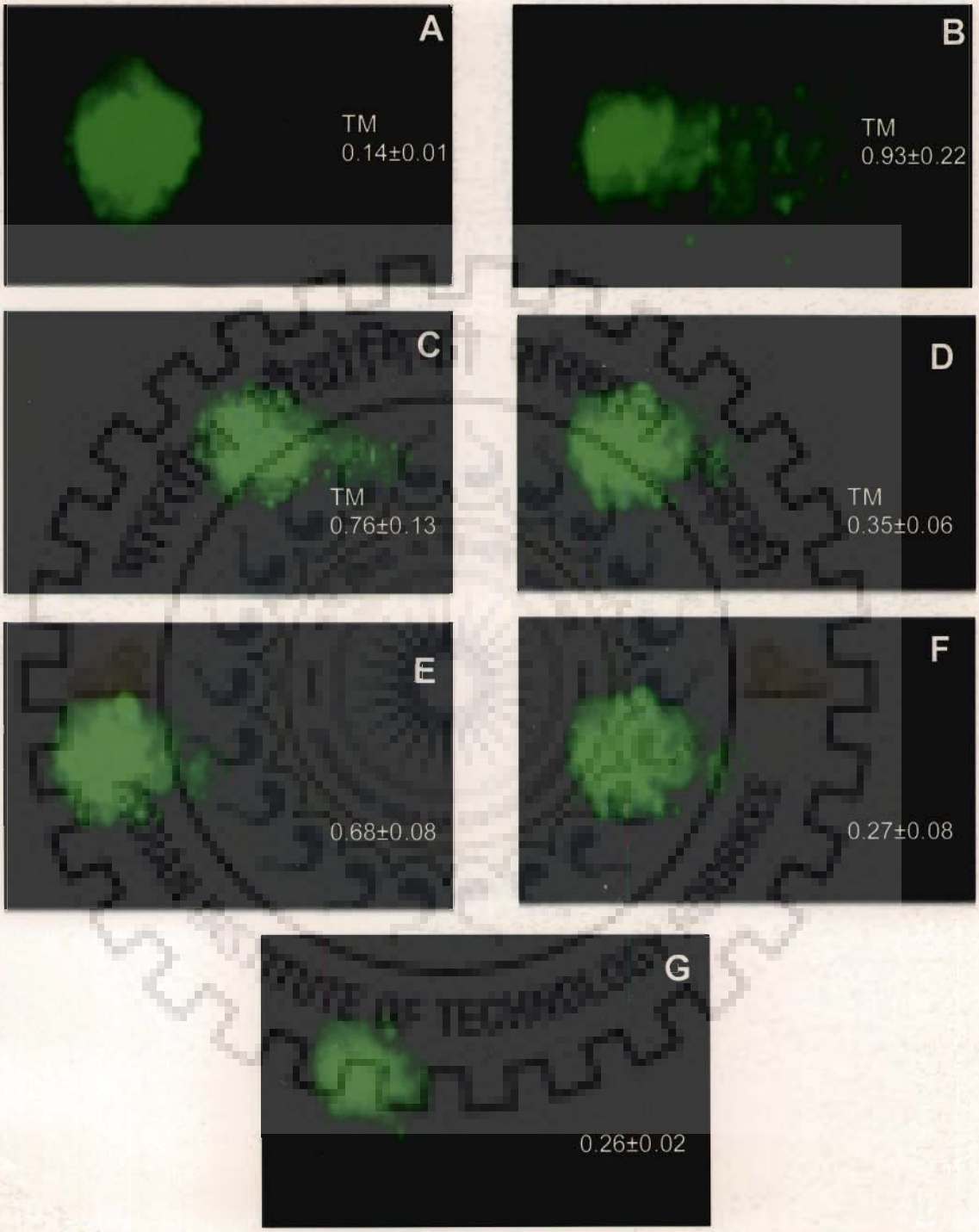

Fig 5.6 Shows the Comet assay photograph with TM (Tail Moments) pattern from rat liver single cell gel electrophoresis under CCl_4 toxicity and pretreatment GAM and PEH constituents. A-Normal control, B-CCl_4 treated control, C-CCl_4+GAM (50 mg per kg.bw), D-CCl_4+GAM (100 mg per kg.bw), E-CCl_4+PEH (50 mg per kg.bw), F-CCl_4+PEH (100 mg per kg.bw) and G-CCl_4+Silymarin (75 mg per kg.bw).

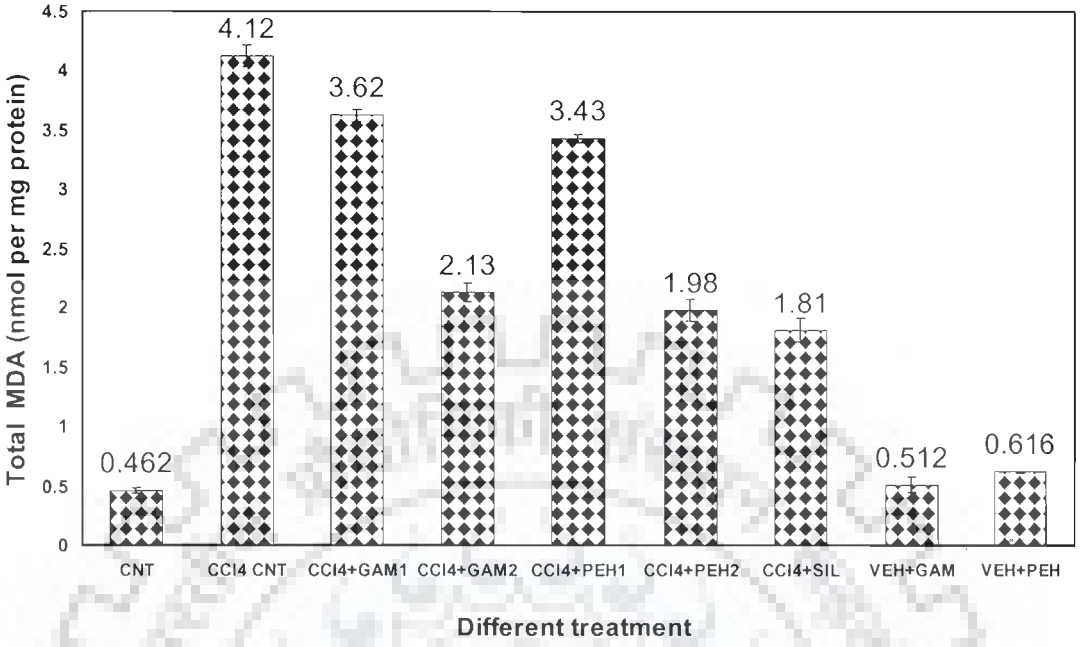

Fig 5.7a shows the total MDA production during lipid peroxidation in different rat liver tissue under CCl_4 toxicity and pretreatment with GAM and PEH constitunets. In the graph CNT (Normal control); CCl_4 CNT (CCl_4 treated control); CCl_4+GAM1 (50 mg per kg.bw); CCl_4+GAM2 (100 mg per kg.bw); CCl_4+PEH1 (50 mg per kg. bw); CCl_4+PEH2 (100 mg per kg.bw); CCl_4+SIL (Silymarin 75 mg per kg.bw); VEH+GAM (Vehicle+GAM 200 mg per kg.bw); VEH+GAM (Vehicle+GAM 200 mg per kg.bw).

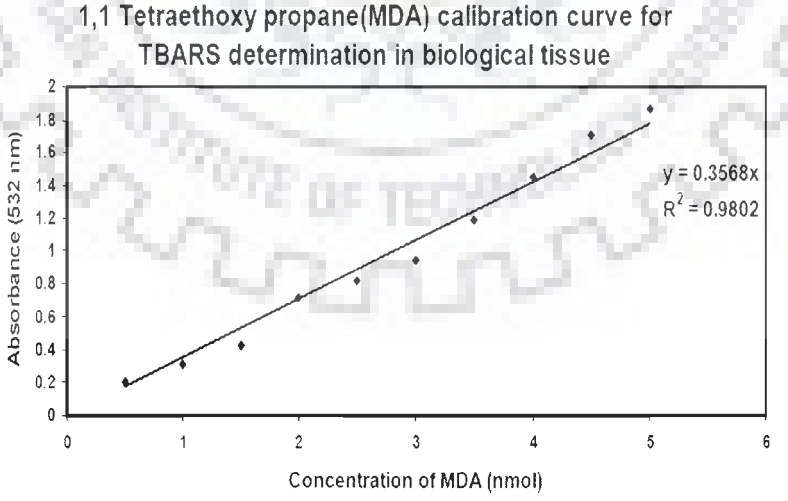

Fig 5.7b depicts the 1,1 tetraethoxypropane (MDA) calibration curve for total MDA determination in biological samples. The absorbance was recorded at 532 nm for MDA-TBA complex formation.

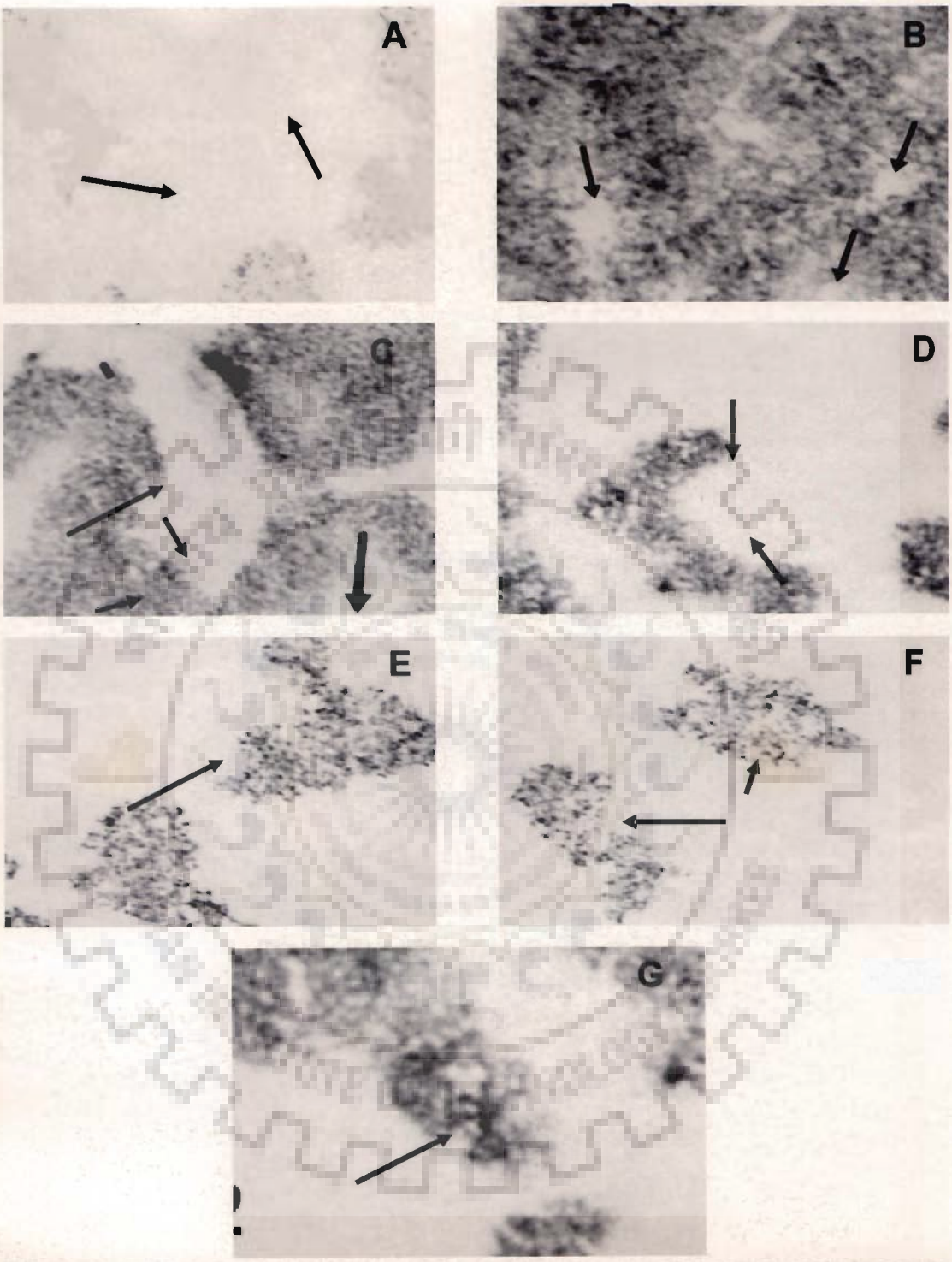

Fig 5.8 Schiffs stained liver tissue section showing extent of lipid peroxidation under CCl_4 intoxication with or without treatment with GAM and PEH constituents. A-Normal control, 8.92±1.2, B-CCl_4 treated control, 88.92±6.82, C-CCl_4+GAM (50 mg per kg.bw, 58.73±5,42), D-CCl_4+GAM (100 mg per kg.bw, 36.28±3.21), E-CCl_4+PEH (50 mg per kg.bw, 48.36±4.28), F-CCl_4+PEH (100 mg per kg.bw, 28.29±3.7), G-CCl_4+SIL (75 mg per kg.bw, 25.73±2.8).

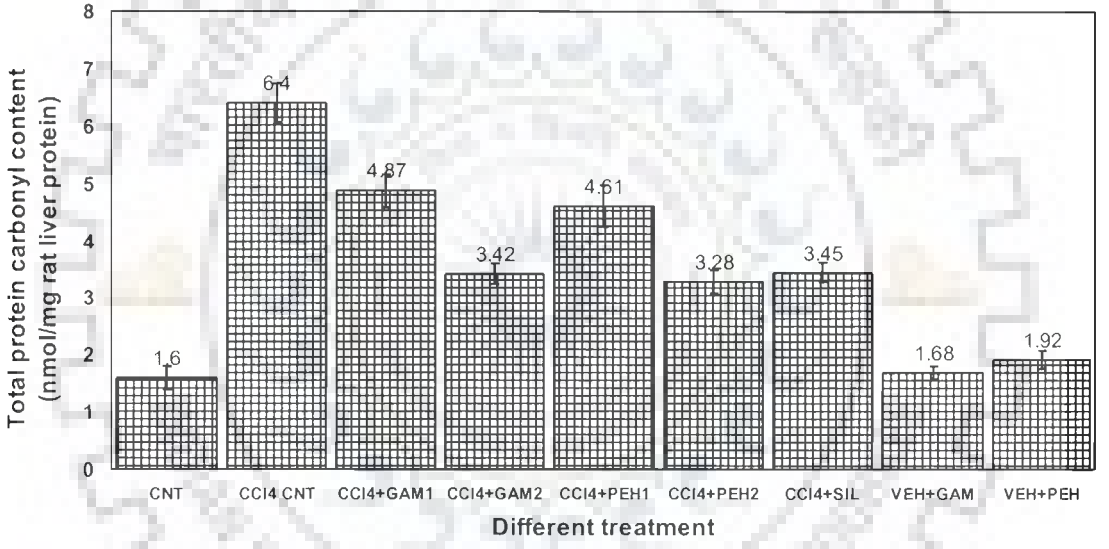

Fig 5.9 Total protein carbonyl estimation using DNPH spectrophotometric method under CCl_4 toxicity and their protection pretreated with GAM and PEH constituents. In the graph CNT (Normal control, vehicle only); CCl_4 CNT (CCl_4 treated control); CCl_4+GAM1 (50 mg per kg.bw); CCl_4+GAM2 (100 mg/kg.bw); CCl_4+PEH1 (50 mg/kg.bw); CCl_4+PEH2 (100 mg per kg.bw); CCl_4+SIL (75 mg per kg.bw). VEH+GAM (vehicle and GAM (200 mg per kg.bw), VEH+PEH (vehicle and PEH (200 mg per kg.bw)

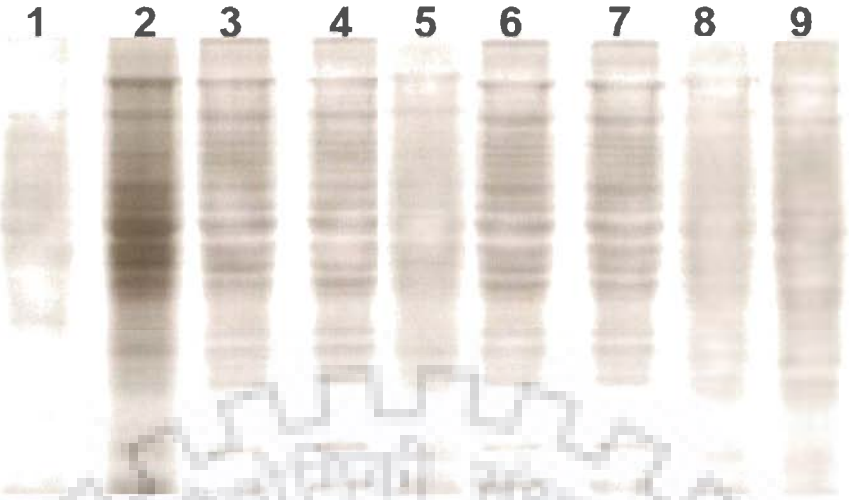

Fig 5.10a Total protein carbonyl content in rat liver homogenate using western blot technique under CCl_4 mediated oxidative stress. Lane 1: Normal control, Lane2:CCl_4 treated control, Lane 3:CCl_4+GAM (50 mg per kg.bw), Lane 4: CCl_4+GAM (100 mg per kg.bw), Lane 5: vehicle+GAM (200mg per kg.bw.), Lane 6: CCl_4+PEH (50 mg per kg.bw), Lane 7: CCl_4+PEH (100 mg per kg.bw), Lane 8: vehicle+PEH (200mg per kg.bw). Lane 9: CCl_4+Silymarin (75mg per kg.bw).

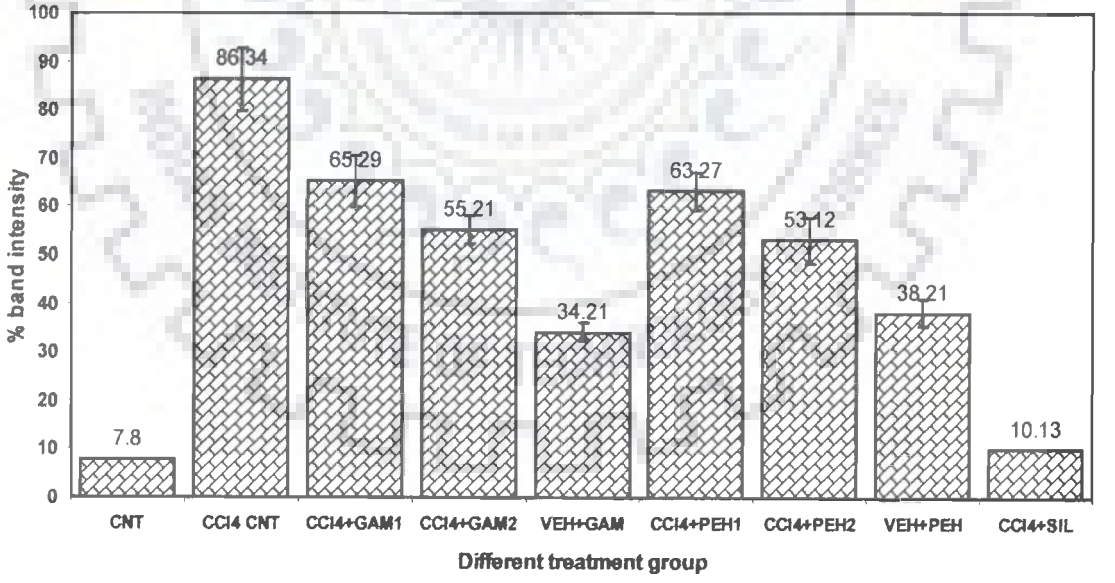

Fig 5.10b Band intensity analysis of immunoblot for total protein carbonyl content shown in Fig 5.10a. CNT: Normal control, CCl_4 CNT: CCl_4 treated control, CCl_4+GAM1: CCl_4+GAM (50 mg per kg.bw), CCl_4+GAM2: CCl_4+GAM (100 mg per kg.bw), VEH+GAM (vehicle+GAM (200 mg per kg.bw), CCl_4+PEH1 (CCl_4+PEH (50 mg per kg.bw), CCl_4+PEH2: CCl_4+PEH (100 mg per kg.bw), VEH+PEH: vehicle+PEH (200 mg per kg.bw). Lane 8: CCl_4+Silymarin (75mg per kg.bw).

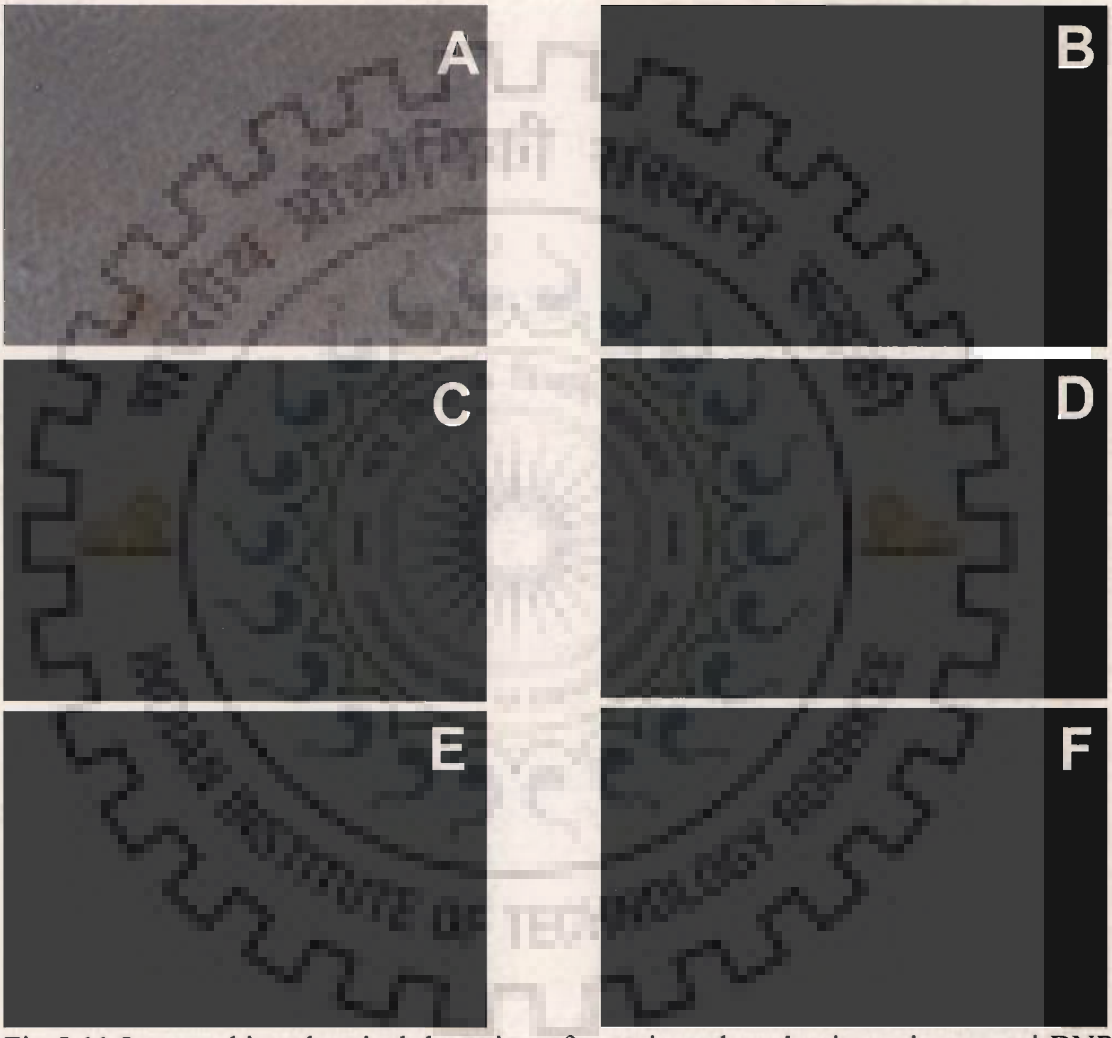

Fig 5.11 Immunohistochemical detection of protein carbonyl using primary anti-DNP antibody on rat liver tissue under CCl_4 toxicity and pretreatment with GAM and PEH. A -Control normal, B-CCl_4 control, C-Vehicle+GAM 50 mg per kg.bw), D-Vehicle+GAM 100 mg per kg.bw), E-Vehicle+PEH 50 mg per kg.bw), F-Vehicle+PEH 100 mg per kg.bw).

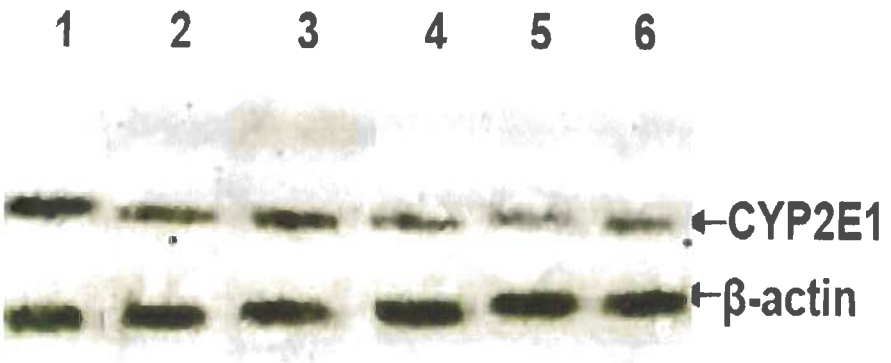

Fig 5.12a Immunoblot photograph for CYP2E1 protein under CCl4 toxicity and pretreatment with GAM and PEH constituents. Lane 1-Control normal, Lane 2-CCl_4 control, Lane 3-Vehicle+GAM 50 mg per kg.bw), Lane 4-Vehicle+GAM 100 mg per kg bw), Lane 5-Vehicle+PEH 50 mg per kg.bw), 3-Vehicle+PEH 50 mg per kg.bw), Lane 6-Vehicle+PEH 100 mg per kg.bw).

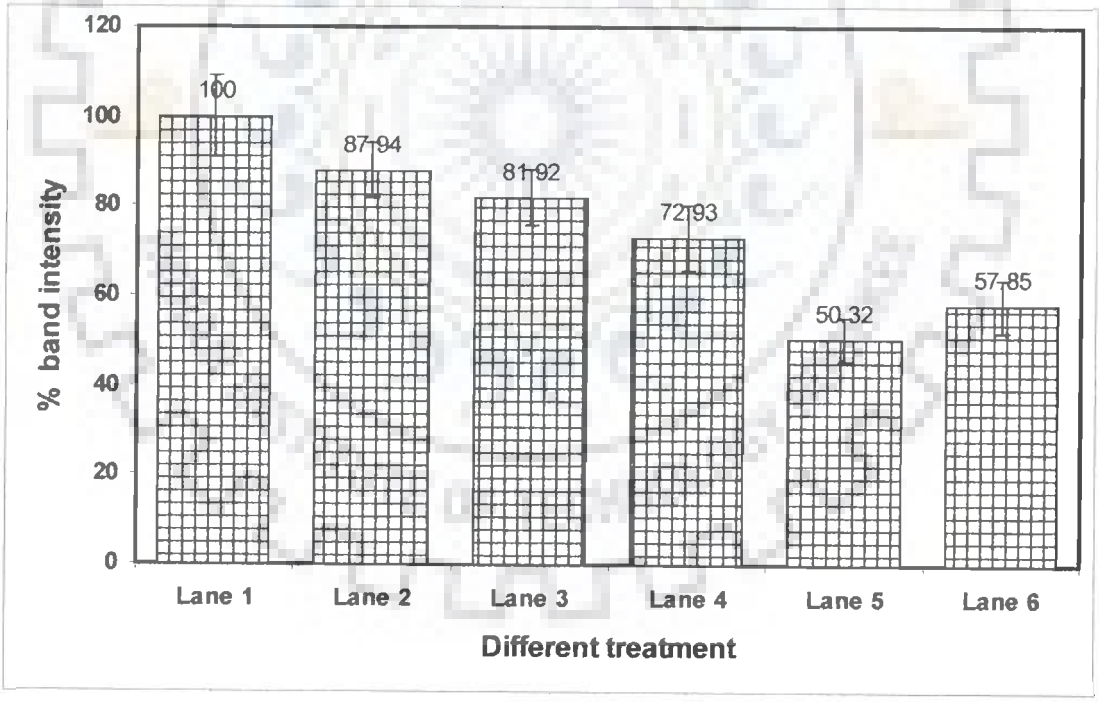

Fig 5.12b Band intensity analysis of Immunoblot for CYP2E1 protein under CCl_4 toxicity and pretreatment with GAM and PEH constituents. Lane 1-Control normal, Lane 2-CCl_4 control, Lane 3-Vehicle+GAM 50 mg per kg.bw), Lan4-Vehicle+GAM 100 mg per kg.bw), Lane 5-Vehicle+PEH 50 mg per kg.bw), Lane 6-Vehicle+PEH 100 mg per kg.bw).

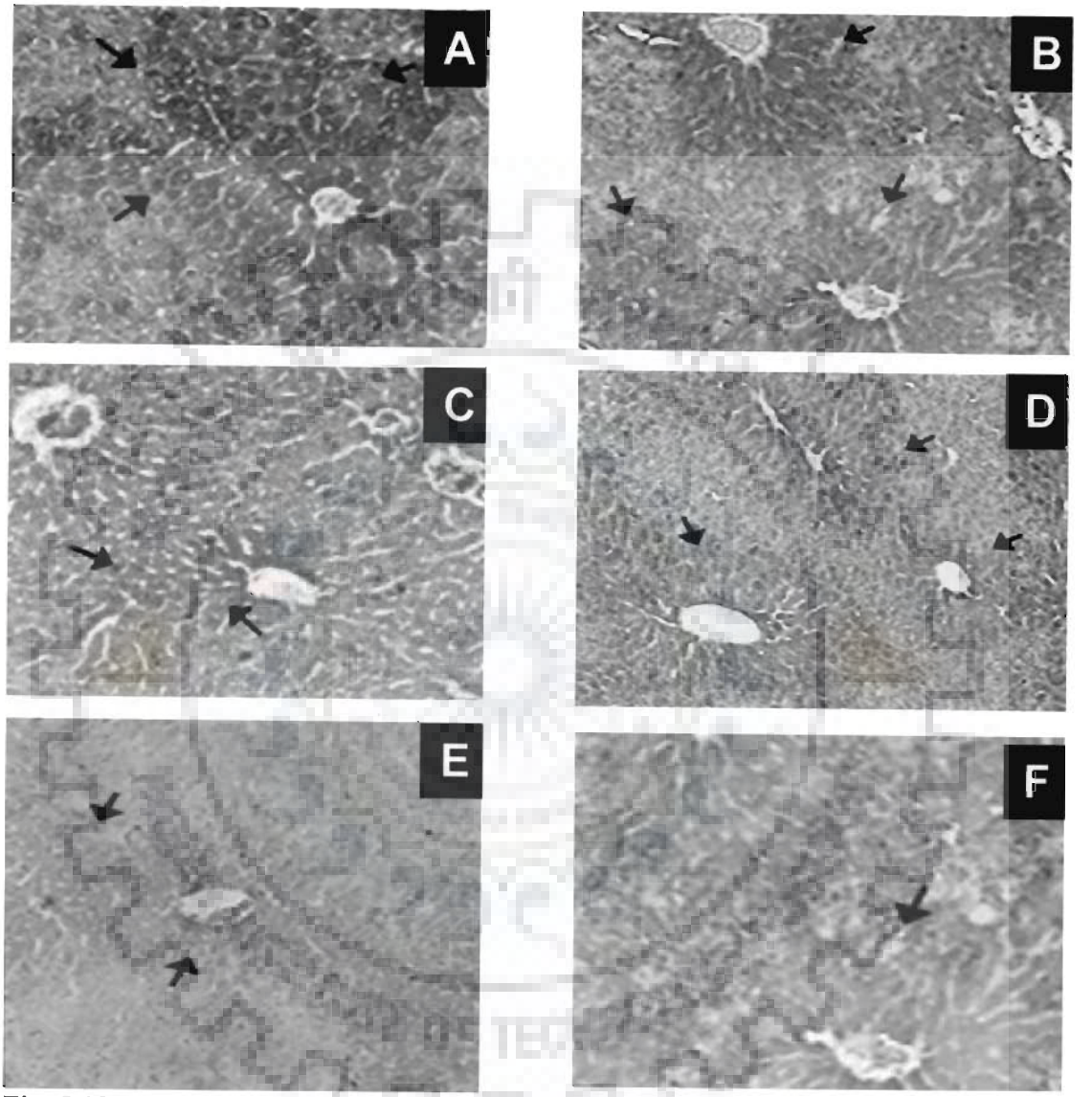

Fig 5.13 Immunohistochemical study on CYP2E1 enzyme expression on rat liver tissue under CCl_4 toxicity and pretreatment with GAM and PEH. A - normal Control, B-CCl_4 treated control, C-Vehicle+GAM 50 mg per kg.bw), D-Vehicle+GAM 100 mg per kg.bw), E-Vehicle+PEH 50 mg per kg.bw), F-Vehicle+PEH 100 mg per kg.bw).

Summary

The use of antioxidants has been widely considered as one of promising approach in the prevention and treatment of many oxidative stress mediated human diseases including liver pathophysiological conditions. Antioxidant based drugs/formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, cancer and liver diseases have appeared during the last few decades.

Though it is difficult to truly define the antioxidants, however, antioxidants are those compounds or substances which cause reduction in oxidative damage. Antioxidants act as reducing agents (free radical terminators), metal chelating and singlet oxygen quenchers. Due to their protective role against oxidative stress related diseases, aging and prevention of oxidative deterioration of food materials, antioxidant has been one of the thrust areas of research in biomedical sciences and neutraceuticals. Some synthetic antioxidant drugs/agents have been developed and are in use but they are not safe due to toxicity and other side effects. Attempts have been to develop suitable anti-oxidants or search potential anti-oxidant compounds from natural sources. Among available source of external antioxidant agents, dietary and medicinal plants derived antioxidant agents are gaining edge globally over the other sources. Attempts have been made by several research groups to screen and evaluate antioxidant potentials of various dietary fruits, vegetables and medicinal plants in different part of world.

India due to its geographical location and climatic conditions are blessed with a widely diversified plant flora and are endowed with diversified classes of

phytochemicals, which has been demonstrated to deliver preventive role in many traditional as well as therapeutic medicine. Therefore in order to search a good source of potential antioxidant agents/compounds there is need to evaluate the antioxidant potential of unexplored flora and identify their active constituents to validate their ethnical use in medicine and to develop a new antioxidant agent. In the present work, an attempt has been made to screen 20 different dietary and medicinal plants for their antioxidant activity on the basis of reliable *in vitro* based assays. Efforts have also been made for extraction, fractionation and identification of active fraction/constituents from *A. marmelos* and *E. hirta* plants conferring high antioxidant activity. The hepatoprotective potential of these purified materials has also been investigated. The whole study is divided into three main parts and described below.

Screening, extraction and fractionation of active antioxidant constituents

In view of wide array of phytochemical antioxidants available in dietary and medicinal plants, there is a need to find plants with maximum antioxidant activity and identification of their active antioxidant constituents. In order to find valuable source of antioxidant twenty different medicinal and dietary plants were screened for their antioxidant activity. Aqueous and methanolic extract of these plants were prepared and screened for antioxidant activity and for total phenolics, glycosides, flavonoids etc. Out of twenty plants *A. marmelos* and *E. hirta* showed high antioxidant activity in their leaves aqueous and methanolic extracts. Since these two plants showed high antioxidant activity further studies were focus on these two plants only. It was observed that these two plants also had high glycoside and phenolic content. Though it was not clear at this stage whether the high antioxidant activity of these two plant are associated with their high glycoside and phenolic content. However, based on the earlier observations of plant derived antioxidant that their antioxidant activity is

mostly linked to their phenolic and glycoside, it was logical to assume that most probably the observed high antioxidant activity in these plants may be associated with their glycosides and phenolic constituents.

Therefore, attempts were made to purify the active constituents of these plants. A bioactivity guided extraction and fractionation of these two plants aqueous (*A. marmelos*) and aqueous: methanolic (*E. hirta*) extracts (having maximum antioxidant activity) were performed by liquid-liquid partitioning using hexane, dichloromethane and chloroform and Sephadex LH-20 column chromatography. The antioxidant activity, glycosides and phenolic content of each fraction were obtained and checked. Fractions of respective *A. marmelos* and *E hirta* with high antioxidant activity were pooled. It was observed that fractions of *A. marmelos* which showed high antioxidant activity also has maximum glycosides content while the fractions of *E. hirta* with high antioxidant activity showed high phenolic content. Thus it was very much clear after fractionation that antioxidant activity of *A. marmelos* and *E. hirta* are due to their glycosides and phenolic content, respectively. The pooled active fractions of *A. marmelos* and *E. hirta* were named glycoside enriched fraction of *A. marmelos* (GAM) and phenolic enriched fraction of *E. hirta* (PEH), respectively. Further identification of antioxidant constituents present in GAM and PEH were performed using various analytical tools such as TLC, HPLC, FTIR, GC-MS and ESI-MS analytical techniques. The identification of components in PEH active fraction confirmed the presence of gallic acid, ferulic acid and quercetin as three major constituents for antioxidant activity. On the other hand identification of active constituents in GAM fraction showed rutin (flavonoid glycoside), β-sitosterol glucoside and lupeol (triterpenoids) as three major active components. Therefore, it was clear that the high antioxidant activity of GAM and PEH were due to their

identified glycoside and phenolic constituents, as the antioxidant potential of various other plants has been established due to their glycosides and phenolic components. Though the medicinal importance of these two plants has been reported earlier, however, so far, there has been lack of scientific evidences regarding antioxidant potential of these two plants. Thus this is the first of its kind giving scientific evidence of their antioxidant potential and also purification and identification of the active constituents.

Validation of antioxidant potentials of GAM and PEH using multiple assays and evaluation of their protective effect against oxidative damage to biomolecules.

There found to be much variation in the antioxidant activity of a given compound depending on the nature of assay and antioxidant agent. True antioxidant potential of any preparation could not be assessed based on a single assay. Therefore, to validate further the antioxidant activity of GAM and PEH were evaluated using multiple assay systems. Different *in vitro* antioxidant assays were carried out to demonstrate antioxidant activity and their possible mechanism. Both GAM and PEH antioxidant constituents showed promising levels of antioxidant activity displaying scavenging efficacy against DPPH, ABTS, hydroxyl and super oxide free radicals and their minimum inhibitory concentration was equivalent with standard antioxidants like BHA and Trolox. The both GAM and PEH also showed strong metal chelating ability. All these assays showed strong antioxidant activity from both GAM and PEH. Thus it is clear that the strong antioxidant potential of both GAM and PEH fractions is due to their free radical scavenging and metal chelating ability.

The antioxidant potential GAM and PEH was also evaluated by their protective effect against oxidative damage to DNA, protein and lipid. To study protective mechanism of GAM and PEH constituents against oxidative damage to

DNA, calf thymus DNA and pUC18 plasmid were used as oxidizing substrate and metal catalyzed (MCO) oxidation system were employed to produce oxidative damage to DNA. The extent of protection against oxidative damage to DNA was checked by employing spectrophotometric and agarose gel electrophoresis technique. Protective role against oxidative damage to proteins was studied using bovine serum albumin as a model protein and metal catalyzed oxidation (MCO) system was used to induce oxidation. The protective effect was studied using spectrophotometric, SDS-PAGE and western blot technique (primary anti-DNP antibody) to evaluate protective effects against protein oxidation. Protection of GAM and PEH against lipid peroxidation was checked by extent of inhibition of ascorbate/Fe^{2+} and CCl_4/NADPH induced lipid peroxidation in rat liver homogenate. Both GAM and PEH antioxidant constituents demonstrated significant level of concentration dependent protection against oxidative damage to DNA, protein and lipid, which is statistically equivalent in comparison to standard compounds like Trolox and glutathione. The protection against biomolecules found to be equivalent or even better than those reported by other plant derived antioxidants like procyanidin-rich extract from pine *(Pinus maritima)* bark and sage (*Salvia officinalis*). It is suggested that the strong protective effect of GAM and PEH in the present study against oxidative damage to DNA, proteins and lipid, are both due to their free radical scavenging and metal chelating ability.

***In vivo* hepatoprotective effect of GAM and PEH constituents against CCl_4 induced rat liver oxidative damage**

Since free radical mediated liver hepatotoxicity is implicated in various liver diseases. Therefore, there been great demand for effective and safer hepatoprotective agents. Antioxidant compounds due to their free radical scavenging and metal

chelating ability could also be or rather also found to be potent hepatoprotective agents against oxidative stress related liver diseases. Therefore, the hepatoprotective effects of GAM and PEH antioxidant constituents was evaluated in CCl_4 intoxicated rat liver toxicity model as this depict the typical free radical mediated damage to liver. The protective effect was monitored by employing biochemical, histological and molecular biology technique. To demonstrate the hepatoprotective effects in biochemical aspects, the level of marker enzyme level, antioxidant status and total lipid profile were analyzed in different treatment animals group. Protection effect of GAM and PEH was also investigated by monitoring effect on histological and morphological parameters using light microscopy and scanning electron microscopy (SEM) on rat liver tissue sections of different treatment groups. At molecular level, the protective effect of GAM and PEH against CCl4 induced oxidative damage to protein, DNA and lipid was evaluated using various techniques. To study protective effects on oxidative damage to protein, DNPH based spectrophotometric, western blot and immunohistochemistry (using primary anti-DNP) technique were used. The extent of protection against oxidative damage to DNA among different treatment group of rat liver was checked by employing spectrophotometric, DNA laddering and single cell gel electrophoresis (COMET) methods. The extent of GAM and PEH on inhibition of lipid peroxidation was determined using TBARS and schiffs staining methods. The protective was also determined by studying the CPY2E1 activity and expression among different treatment group including pretreated with GAM and PEH constituents employing spectrophotometric, immunoblot and immunohistochemical technique using primary anti-CYP2E1 (a kind gift from Prof. Ingleman-Sundberg, KI, and Sweden). Study on *in vivo* toxicity of both GAM and PEH constituents were

performed with higher dose of (200 mg per kg.bw) along with vehicle only for one month.

In biochemical aspects the increased level of marker enzymes ALT, AST, ALP, LDH and bilirubin were reduced up to 40% after treatment at the dose of (50 mg per kg bw) and up to 80% at the dose of (100 mg per kg bw) pretreated with GAM and PEH constituents in comparison to CCl_4 treated animal group. On the other hand the reduced antioxidant enzymes SOD and catalase level in CCl_4 treated group was improved markedly in GAM and PEH pretreated groups. The histological data showed that there was clear dose dependent recovery in hepatocellular damage indicating reduced necrosis, ballooning of hepatocytes, fatty degenerations, inflammatory infiltration and sinusoidal enlargement in comparison to the condition observed in CCl_4 treated animal group. On the basis of severity scores from light microscopy and SEM observations, GAM and PEH constituents demonstrated significant hepatoprotection with recovery up to 75 and 80%, respectively, which further substantiates the biochemical aspects of hepatoprotective results. The protection was remarkable as it was almost similar to the well known hepatoprotective agent silymarin.

The pretreatment with GAM and PEH showed strong hepatoprotective effect against oxidative damage of biomolecules as indicated reduction in protein carbonyl formation, fragmentations of DNA and MDA production in comparison to CCl_4 intoxicated animal group. It was also observed that GAM and PEH pretreatment caused inhibition of CPY2E1 enzyme metabolizing activity and expression. On the basis above discussed parameters, the hepatoprotective efficacy of GAM and PEH was found to be equivalent to standard silymarin (75 mg per kg.bw) and several other reported hepatoprotective constituents like *Phyllanthus amarus* and glycerrizhin in

terms of percentage recovery in CCl_4 induced liver damage. At 100 mg per kg.bw dose of GAM and PEH the recovery of CCl_4 induced damage was reduced to almost normal liver condition and protective potential was equivalent to standard hepatoprotective agents. Therefore, both the GAM and PEH are potential hepatoprotective agents. The hepatoprotective effects are suggested due to their free strong antioxidant against free radicals, ability to chelate transition metal ion, strengthening of antioxidant enzymes and inhibitory role in CYP2E1 enzyme activity. Such hepatoprotective role of both GAM and PEH attributed to their glycosides and phenolic constituents, respectively.

Conclusion: The aqueous and methanolic extracts of leaves of *A. marmelos* and *E. hirta* found to have strong antioxidant activity among twenty different Indian dietary and medicinal plants tested in the present study. There found to be strong correlation between antioxidant activity and their glycoside and phenolic content suggesting that antioxidant activity may be due to their glycoside and phenolic constituents. A glycosides enriched fraction (GAM) from *A. marmelos* and phenolic enriched fraction (PEH) from *E. hirta* having strong antioxidant activity were obtained from their aqueous extract by liquid-liquid partitioning and column chromatography. On further analysis using various analytical tools showed rutin (flavonoid glycosides), β-sitosterol glucosides and lupeol (triterpenoids) as major components in GAM while quercetin, gallic acid and ferulic acid identified as major phenolic components. Both the GAM and PEH demonstrated strong antioxidant activity that was almost equivalent to the well recognized synthetic antioxidant agents and reported plant sources. Both GAM and PEH demonstrated strong free radical scavenging and metal chelating ability and these activities are attributed to their glycoside and phenolic constituents, respectively. The both GAM and PEH showed a significant

concentration dependent protection against oxidative damage to DNA, protein and lipid *in vitro* conditions. Both the GAM and PEH also showed a dose dependent hepatoprotection against CCl_4 induced hepatic oxidative damage. It is suggested that strong antioxidant potential, protection against oxidative damage to biomolecules and strong hepatoprotective effects of GAM and PEH are due to their free radical scavenging, metal chelating ability, strengthening antioxidant enzyme status and inhibition to CYPE1 enzyme activity and attributed to their glycoside and phenolic constituents. Though the medicinal importance of *A. marmelos* and *E. hirta* are indicated earlier however, this is first report showing direct evidence for their antioxidant and hepatoprotective potential and identification of active constituents.

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List of Publications

Based upon the research work carried out, following papers are published/in press/accepted/under review/communicated for publications to International Journals and Conferences.

A. Published/Communicated

1. Sharma NK and Prasad R. 2008. Oxidative protein damage and their inhibition by phenolic acid antioxidants from *Euphorbia hirta* leaves. Journal of Biotechnology. 136S:S717-S742.
2. Sharma NK, Dey S, Prasad R. 2007. *In vitro* antioxidant potential evaluation of *Euphorbia hirta* L. plant. PharmacologyOnline 1:91-98.
3. Sharma NK and R Prasad. 2008. *In vivo* study on hepatoprotective role of antioxidant saponin from *A. marmelos* against CCl_4 mediated oxidative damage to liver. Journal of Hepatology. Communicated
4. Sharma NK and R Prasad. 2008. Lipid, DNA and protein oxidative damage inhibition by glycoside fraction from *Aegle marmelos* leaves. Molecular and Cellular Biochemistry. Communicated.

B. Book Chapter.

1. Sharma NK, Dey Sreela, R Prasad. 2007. A chapter "Antioxidants from medicinal plants and their impact on human health" in book "*In Recent Advances in Plant Biotechnology and Its Applications*" Ed. by Ashwani Kumar and Sudhir K.Sopory, Publisher I.K. International, New Delhi, Chapter 29. pp 470-496.

C. Papers and Presentations in Conferences.

1. Sharma NK, Prasad R. 2008. Protective potential of hydroxy-cinnamic acid derivative natural antioxidants from *Euphorbia hirta* against DNA oxidation. Annual Symposium. DNA damage: from causes to cures Robinson College, Cambridge, UK. 15-17 December 2008. Abstract. P-75.

2. Sharma NK, Prasad R. Synergistic interaction of phenolic acid constituents from *Euphorbia hirta* leaves and their protective on oxidative injury to protein. Oxygen Club California World Congress 2008. Santa Barbara, California USA. P-35.

3. Sharma NK, Dey S, Prasad R. 2008. *In vitro* Antioxidant potential evaluation of *Euphorbia hirta* L. Plant.
International conference on oxidative stress HAVANA REDOX 2007. Organized by University of Havana and Cuban Society for free radical research and sponsorship by International Society for free radical research. January 25-27, 2007. pp 58.

4. Sharma NK, Prasad R. Protective effect of crude extract fraction of *Euphorbia hirta* L. leaves on oxidative damage to biomolecule. 2nd symposium nutrition, oxygen biology and medicine 2007: nutritional modulation of oxidative stress, anti-oxidants, anti-inflammatory, anti-aging strategies in health. Organized by Universite de Paris-i, pantheon-sorbonne, 12 place du pantheon, Paris, France. April 11-13, 2007. pp. 35.