EVALUATION OF ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIAL OF *FICUS BENGALENSIS*

A THESIS

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**EVALUATION OF ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIAL OF *FICUS BENGALENSIS***" in 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 September 2009 under the supervision of **Dr. Ramasare Prasad,** Associate Professor and **Dr. Pravindra Kumar**, 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.

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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 **Sreela Dey**, Research Scholar, has been held on.....

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LIST OF ABBREVIATIONS

1O2	Singlet oxygen
ABTS	2, 2'- Azinobis (3-ethylbenzothiazoline-6 sulphonic acid)
BHA	Butylated hydroxy anisole
BHT	Butylated hydroxy toluene
BSA	Bovine serum albumin
GSH	Glutathione
ESI-MS	Electrospray ionisation-mass spectrometry
NMR	Nuclear Magnetic Resonance
FTIR	Fourier Transformation Infra Red spectroscopy
MDA	Malondialdehyde
CYP450	Cytochrome P-450
DNPH	2.4-Dinitrophenylhydrazine
m/z	Mass to charge ratio
H_2O_2	Hydrogen peroxide
CCl_4	Carbon tetrachloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NO^{\cdot}$	Nitric oxide
$OH^{\cdot}$	Hydroxyl radical
ROS	Reactive oxygen species
SDS- PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TPTZ	Tripyridyltriazine
TBARS	Thiobarbituric acid
min	minutes

Chapter 1

Introduction

Liver being a vital organ also plays a pivotal role in detoxification and metabolic control of many toxins which are further excreted out of the body (Lee and Senior, 2005). However, during such detoxification processes, liver itself faces a load of free radical that is generated from various sources like detoxification system, oxidative enzymes and immune system (Britton and Bacon, 1994; Lykkesfeldt *et al.*, 2007). Under normal physiological condition, hepatic aerobic metabolism results in a steady state production of pro-oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are balanced by a similar rate of their consumption by antioxidants. Imbalance in the pro-oxidant/antioxidant equilibrium in favor of pro-oxidant generates the oxidative stress phenomenon, a condition that may induce a number of pathophysiological events in the liver. Hepatotoxicity by oxidative stress maybe achieved through ROS, such as hydroxyl radical, superoxide radical anion and nitric oxide that causes cell membrane damage through lipid peroxidation (Halliwell and Chirico, 1993). It also modifies or damage biomolecules, like proteins, lipids, carbohydrates and DNA both *in vitro* and *in vivo* (Halliwell and Aruoma, 1991; Bandyopadhyay *et al.*, 1999; Manibusan *et al.*, 2007; Halliwell, 2007; Loft *et al.*, 2008).

Several attempts have been made to study 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), which is reported to show great similarity with most of the chronic liver diseases (Cesaratto *et al.*, 2004). Mechanisms like increase in fatty acid ß oxidation as well as peroxisomal fatty acid oxidation, mitochondrial dysfunctioning and the biotransformation of CCl_4 by CYP2E1 enzyme to a more toxic free radical (Ingelman-Sundberg, 1988; Lieber, 1997) results in a consequent increase in intracellular oxidant load that ultimately leads to liver cell damage (Parola and Robino, 2001). Once hepatocellular function is impaired, accumulation of bile acid causes additional stress and toxicity (Jaeschke *et al.*, 2002). Therefore, it is evident that ROS play an important role in pathological changes in the liver (Dianzani, 1987; Poli, 1993; Poli and Parola, 1997), particularly in case of liver cirrhosis (Natarajan *et al.*, 2006). In view of above implications and importance of liver as an organ, there is need to protect it from oxidative stress toxicity by external supply of antioxidative agents, when endogenous protective mechanisms evolved to limit ROS and the damage caused by them is unable

to cope up with excessive free radical generation and subsequent oxidative stress damage (Sies, 1997; Serafini, 2000; Valko *et al.*, 2007).

Therefore, antioxidants with free radical scavenging property could have much relevance as prophylactic and therapeutic agents in diseases in which oxidants or free radicals are implicated (Vitaglione *et al.*, 2004; Meghana *et al.*, 2007). A number of synthetic antioxidants such as BHA (Butylated hydroxy anisole) and BHT (Butylated hydroxy toluene) and tertiary butylhydroxyquinone (TBHQ) have also been developed that can assist in coping with oxidative stress. But some of their physical properties such as high volatility and instability at elevated temperatures, toxicity, higher manufacturing cost, strict legislation on the use of synthetic food additives and consumer preferences aroused the need to find alternatives to synthetic antioxidants (Rice-Evans, 1998; Abdalla *et al.*, 1999). Besides, the conventional drugs used in the treatment of liver diseases are often inadequate. It is therefore necessary to search for alternative drugs for the treatment of liver diseases to replace the currently used drugs of doubtful efficacy and safety. Consequently, there is considerable interest in preventive medicine and food industry in the development of natural antioxidants.

Among several sources of antioxidant and hepatoprotective agent, the importance of plants as a natural source have been well established due to its wide diversity and its ability to synthesize a wide array of phytochemicals as part of its defense strategy. Natural antioxidants from plant sources have better antioxidant activity and are safer to health, reliable and compatible with human diet without any side effects as caused by synthetic antioxidants. Conventional medicine is now pursuing the use of natural products such as herbs/ plant extracts to provide the support that the liver needs on a daily basis. For example, Liv.52, an Ayurvedic preparation (mixture of several herbal extracts) is a well prescribed liver tonic and provides protection to liver from the hepatotoxicity (Dhawan and Goel, 1994; Kataria and Singh, 1997). Presently, in spite of an increasing need for agents to protect the liver from damage, modern medicine lacks a reliable liver protective drug. Therefore, search for natural antioxidants showing hepatoprotective role in liver oxidative stress toxicity has been gaining momentum and considered to be thrust areas in biomedical sciences globally (Abalea *et al.*, 1999; Seeff *et al.*, 2001; Lee *et al.*, 2008).

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, though relatively little knowledge about their mode of action is available (Matthews *et al.*, 1999). Recently, the number of cases with

drug-induced liver injury has been increasing (Saad, 2006; Stickel *et al.*, 2009), parallel to the growing number of drugs including health food and 'natural' foods (Seeff, 2009). Further clarifying the role of oxidative stress in drug hepatotoxicity is needed for useful therapy of drug-induced liver injury, and many drugs and treatments now being investigated are directed toward preventing the damage from oxidative stress (Medina and Otero, 2005; Antoine, 2008).

India due to its geographical location and climatic conditions is blessed with a widely diversified plant flora and are endowed with diversified classes of plant phytochemicals, which has been found to deliver preventive role in several oxidative stress involved human diseases including liver diseases (Dahanukar *et al.*, 2000; Samarth *et al.*, 2008). It is more likely that some of these maybe valuable sources of natural antioxidants. A large number of plants of dietary and medicinal importance in India have been evaluated for their antioxidant potential (Aqil *et al.*, 2006; Kumar *et al.*, 2008; Ali *et al.*, 2008). However, a large number of these plants with diversified medical potential still remains unexplored; it is likely that some of these maybe valuable source of potent antioxidant and hepatoprotective agent. Therefore, there is need to explore the rich diverse flora to search new antioxidant and hepatoprotective agents and identify the active constituents. The present work was emphasized to search a potential source of antioxidant and identify its active constituents.

This study was undertaken with the following objectives:

1. Screening of selected Indian medicinal plants for their antioxidant activity.
2. Extraction and bioactivity guided fractionation of active antioxidant constituents from selected plant using various biochemical leading techniques (liquid-liquid partitioning, Thin layer chromatography, column chromatography and suitable *in vitro* antioxidant assays).
3. Identification of components in active fraction using various analytical techniques like UV- Visible spectrophotometry, FTIR, ESI-MS and H^1NMR.
4. Determination and evaluation of antioxidant activity and protective potential against oxidative damage to biomolecules including DNA, protein and lipid using various *in vitro* biochemical and molecular biology based assays.
5. Evaluation of hepatoprotective role against CCl_4 intoxicated liver oxidative stress model employing biochemical, histological and molecular biology techniques.

Chapter 2

Review Literature

2.1 OXIDATIVE STRESS AND HUMAN DISEASES

It is well established that molecular oxygen is vital for all living organism. However, strict anaerobes cannot survive in the presence of oxygen as they lack sufficient defenses against the multiple secondary reactions induced by oxygen. All other organisms are provided with an efficient battery of antioxidant defenses with the ability to trap reactive intermediates before they can cause potential damage to biomolecules (Blokhina *et al.*, 2003; Serafini, 2006). At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune functions (Valko *et al.,* 2007). However, when produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can produce serious alterations in the membranous structure of the cell and cause extensive damage to biomolecules such as proteins, lipids, and deoxyribonucleic acid (DNA) (Slater, 1984; Aruoma, 1998; Bandopadhyay, 1999). Normally there is a balance between the amount of free radicals generated inside the body and the antioxidant defense systems that scavenge/quench these free radicals preventing them from causing deleterious effects inside the body (Finkel and Holbrook, 2000). The antioxidant defense system in the body can only protect the body when the amount of the free radicals is within the normal physiological level. But when this balance is shifted towards more of free radicals, increasing their burden in the body either due to environmental condition or uncontrolled production within the body, it leads to oxidative stress, which may result in tissue injury and subsequent diseases (Slater, 1984; Halliwell, 2007). Therefore, one can say that oxidative stress occur when the production of free radicals increases, when quenching of free radicals or repair of damaged macromolecules decreases, or when both these changes occur simultaneously. According to Sies (1991), oxidative stress corresponds to a general disturbance in the prooxidant/antioxidant balance in favour of the former causing potential damage. Most of the present day diseases occur due to shift in the balance of pro-oxidant and antioxidant homeostatic phenomenon in the body; pro-oxidant conditions dominate either due to unregulated production of the free radicals as a result of increased oxidative stress or due to poor scavenging/quenching of these radical species caused by depletion of the dietary antioxidants (Tiwari, 2001; Govindarajan *et al.,* 2005). Oxidative stress plays a major role in the development of chronic and degenerative diseases like cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases (Naidu *et al.,* 2007; Pham Huy *et al.*,

2008). Over about 100 disorders like rheumatoid arthritis, hemorrhagic shock, cardiovascular disorders, cystic fibrosis, metabolic disorders, neurodegenerative diseases, gastrointestinal ulcerogenesis and AIDS have been reported as ROS mediated. Some specific examples of ROS mediated diseases include Alzheimer's disease, Parkinson's disease, Atherosclerosis, Cancer, Down's syndrome and Ischemic reperfusion injury in different tissues including heart, liver, brain, kidney and gastro intestinal tract.

Cancer: It is well established that oxidative DNA damage is responsible for cancer development (Olinski *et al.,* 2002). Oxidative damage in case of cancer is mostly mediated by hydroxyl radicals that produce a multiplicity of modifications in the DNA structure including base and sugar lesions, strand breaks, DNA protein cross links and base free sites (Chen *et al.,* 2002; Valko *et al.,* 2004; Halliwell, 2007).

Diabetes: The etiology of the complications of diabetes involves oxidative stress perhaps as a result of hypoglycemia. Hyperglycemia-related increased protein glycosylation are important sources of free radicals (Wolff and Dean, 1987; Kar and Chakraborti, 2001). Glucose auto-oxidation in the presence of transition metal ions also generate oxygen free radicals, which make the membrane vulnerable to oxidative damage. Other possible sources include elevated plasma lipids leading to increased lipid oxidation and decreased levels of antioxidant defense systems (Maritim *et al.,* 2003).

Parkinson's disease: Usually appears in the middle to old age and is often characterized by rhythmic tremor in a foot or hand especially when the limb is at rest. Comparison of the brain of Parkinson's disease with that of the neurologically normal brain shows several parameters consistent with increased oxidative stress and defective mitochondrial function. Damaged mitochondria may generate more ROS than usual and ROS/RNS (including $O^{2\bullet-}$, $OH^{\bullet}$, ONOO−) can inactivate complex I. Hence it is possible that oxidative stress and mitochondrial defects form a vicious cycle (Onyango, 2008; Nikam *et al.,* 2009).

Rheumatoid arthritis: An autoimmune disease characterized by chronic inflammation of the joints and tissue around the joints along with infiltration of macrophages and activated T cells. Pathogenesis is due to generation of ROS and RNS by activated phagocytes at the site of inflammation (Tak *et al.,* 2000).

2.2 LIVER DISEASE AND OXIDATIVE STRESS

It is well established that the liver is the first line of protection against damage by ingested agents, including xenobiotics and drugs. Hence it is evident that the liver is subjected to toxic injury more often than any other organ (Adams and Linder, 2007). It being one of the most important detoxifying organ of our body, cleanses the blood of toxins either ingested or produced by the body itself (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.

Oxidative stress has recently been recognized as major contributing factor in the deterioration of various liver diseases (Tanikawa and Torimura, 2006); especially in alcoholic liver disease (ALD) (Wu and Cederbaum, 2003; Cederbaum *et al.*, 2009), nonalcoholic steatohepatitis (NASH) (Marra *et al.*, 2008) and hepatitis type C (Choi and Ou, 2006). Oxidative stress also plays an important role in the pathophysiological changes that progress to liver cirrhosis and finally to hepatocellular carcinoma (HCC) (Sasaki, 2006; Olaya, 2007). However, the role of oxidative stress in these liver diseases are not yet fully understood, and further studies on this subject are required. The liver hepatocytes play a pivotal role in the metabolism of alcohol or drugs. Alcohol abuse or increased drug intake enhances ROS production in these metabolic processes (Wu and Cederbaum, 2003). In obese patients, a large amount of free fatty acids (FFAs) from the visceral fat tissues, as well as from dietary glucose and fat, flows directly into the liver. Such inflow of FFAs to the hepatocyte also enhances the fatty acid beta-oxidation capacity of the mitochondria and other organelles in the hepatocyte (Qureshi and Abrams, 2007), and simultaneously exceeds the excretion capacity of very low density lipoproteins (VLDL) from the hepatocyte, resulting in development of fatty liver or NASH. In the process mitochondria, peroxisomes, and endoplasmic reticulum metabolize the excessive amount of fatty acid, resulting in overproduction of reactive oxygen intermediates and oxidative stress in the hepatocytes. Excessively high level of iron accumulates in the hepatocytes of patients suffering from NASH, alcoholic hepatitis, or hepatitis type C. Such overaccumulation of iron also generates oxidative stress in the hepatocytes (Pietrangelo, 2003). Therefore, liver hepatocytes are richly endowed with antioxidant defense mechanisms compared with the cells of other organs.

It has also been observed that oxidative stress is easily induced in the hepatocyte as a result of lifestyle-related factors such as alcohol abuse or obesity. The hepatocytes need a high antioxidant function to cope with such oxidative stress (Tanikawa and Torimura, 2006).

2.2.1 Liver pathophysiological changes

Liver histology in NASH is characterized by steatosis, necrosis, degeneration of hepatocytes, inflammation, and pericellular and intralobular fibrosis. The spectrum of histological changes varies from mild steatohepatitis to bridging fibrosis and cirrhosis. Hepatic steatosis in NASH is macrovesicular and primarily centrilobular (zone 3), although it may be diffuse in severe forms of the disease. Inflammation, which must be present for the diagnosis of NASH, is usually low grade, lobular, and mixed neutrophilic and mononuclear. Carbon tetrachloride widely accepted as a model hepatotoxin induces functional and morphological changes in the cell membrane hydropic degeneration, centrilobular necrosis, fatty changes, cirrhosis and hepatoma (Nagano *et al.*, 2007). The centrilobular region of the hepatic lobule is by far highly prone to toxic effects of the chemical toxin and necrosis in this region is believed to be a major cause of CCl_4 induced acute liver injury (Wilson, 1998; Treadway, 1998). One of the earliest, most frequent and most conspicuous changes seen in liver injured by CCl_4 administration is the ballooning effect of hepatocytes with Mallory body accumulation; however small irregularly shaped Mallory bodies are observed in case of NASH. Thus, injury to liver hepatocytes may start a cascade of necroinflammatory changes that include the accumulation of mixed inflammatory cells.

2.2.2 Liver pathogenesis and oxidative stress mechanism in CCl_4 toxicity

It has been widely accepted that oxidative stress plays a major role in the pathogenesis of liver diseases. The understanding of the cellular response to oxidative stress condition in non-alcoholic fatty liver diseases (NAFLD), also known as hepatic steatosis or fatty liver, refers to a medical condition that arises as a direct consequence of excessive triglyceride accumulation in the absence of significant alcohol consumption. Due to oxidative stress and lipid peroxidation, hepatic steatosis can cause inflammation and hepatocellular damage, leading to non-alcoholic steatohepatitis (NASH), which can progress to liver injury (Liao and Yin, 2000). There is increasing evidence that the alteration of the cellular redox state with production of ROS plays a

crucial role in the steps involved in the progression of liver diseases, independent of the type of etiological agents. The major sources of ROS generation during CCl_4 intoxication in the liver are represented by the mitochondrial enzymes particularly cytochrome P450 of damaged hepatocytes and the activated inflammatory cells (Kuffer cells, neutrophils and macrophages). Reactive oxygen species (ROS) binding to macromolecules such as DNA, protein and lipids and resulting in physiologic dysfunction; have been implicated in the initiation of various liver pathological processes, such as fibrogenesis, cirrhosis and steatosis (Halliwell, 1987; Poli and Parola, 1997). Thus the most remarkable pathological characteristics of CCl_4 induced hepatotoxicity are fatty liver, cirrhosis and necrosis, which have been thought to result from the formation of reactive intermediates such as trichloromethyl free radicals ($CCl_3^{\cdot}$) metabolized by cyt P450 in endoplasmic reticulum. 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).

2.2.3 Free radical pathway in CCl_4 toxicity

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 damage by CCl_4 is regarded as the analogue of liver damage caused by a variety of hepatotoxins in humans (Cessaratto *et al.*, 2004). The molecular mechanisms underlying CCl_4 toxicity are rather well known today. The principle theory behind the mechanism of cellular damage caused by CCl_4 states that the toxin is bioactivated by the drug metabolizing system (mixed function oxidase system utilizing the NADPH-cytochrome P-450 electron transport chain) inside the smooth endoplasmic reticulum; cyt P450 enzyme mediates one electron reduction of the halogenated hydrocarbons like CCl_4 resulting in free radical intermediates that can damage cell membranes via lipid peroxidation, or can target nucleophilic DNA residues. Also it is thought that the homolytic cleavage of the carbon-chlorine bond occurring during its metabolism may yield haloalkane free radicals, the most important of which are the trichloromethyl radicals (Recknagel, 1967; Slater, 1984), or possibly the more toxic trichloromethylperoxy radicals formed by further reaction of the former with molecular oxygen (Packer *et al.*, 1978; Slater, 1984). The formation of these

radicals has been then demonstrated both *in vitro* and *in vivo* with the use of free radical traps that render free radicals more stable and therefore detectable by the electron spin resonance technique (Tomassi *et al.*, 1987). These free radicals can act in two ways: either in a direct way (direct attack), by covalent binding to membrane lipids and protein, in particular to those of endoplasmic reticulum with resulting alkylation reactions and possible enzyme inactivation; or in an indirect way, through interactions with membrane unsaturated fatty acids and consequent promotion of lipid peroxidation. Trichloromethyl free radicals also react with sulfhydryl groups such as reduced glutathione (GSH) and the protein thiols, and the covalent binding of the trichloromethyl free radicals to the cell protein is considered to be the initial step in the chain of events that eventually lead to membrane lipid peroxidation and finally to cell necrosis (Recknagel *et al.*, 1973, 1989; William and Burke, 1990). The potential for the chemical to modify cellular protein through covalent modification may be another established mechanism of chemical toxicity. These chains of events further result in the breakdown of membrane structure and disruption of 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.

2.2.4 Oxidative markers in CCl_4 intoxicated rat liver injury

Elevation of MDA levels, one of the end products of lipid peroxidation in the liver tissue, and the depletion of hepatic glutathione (GSH) content are significantly marked indicators of generation of oxidative stress condition in CCl_4 intoxicated rats (Tirkey *et al.*, 2005; Mehmetcik *et al.*, 2008). CCl_4 is a commonly used hepatotoxin to induce lipid peroxidation and toxicity. CCl_4-induced hepatic damage also produces alterations in the antioxidant status of the tissues, which is manifested by abnormal level of antioxidant marker enzymes. Reduction in hepatic antioxidant defenses is indicated by a more global inhibition of SOD, catalase and glutathione thus suggesting that the decreased efficiency of antioxidant systems maybe a direct consequence of oxidative stress phenomenon (Rajesh and Lata, 2004; Lieberta *et al.*, 2009). Lipid peroxidation is also thought to be at least one of its toxic principles during CCl_4 hepatotoxicity (Basu, 2003). The further insight 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 and Siegers, 1985). Earlier studies on the

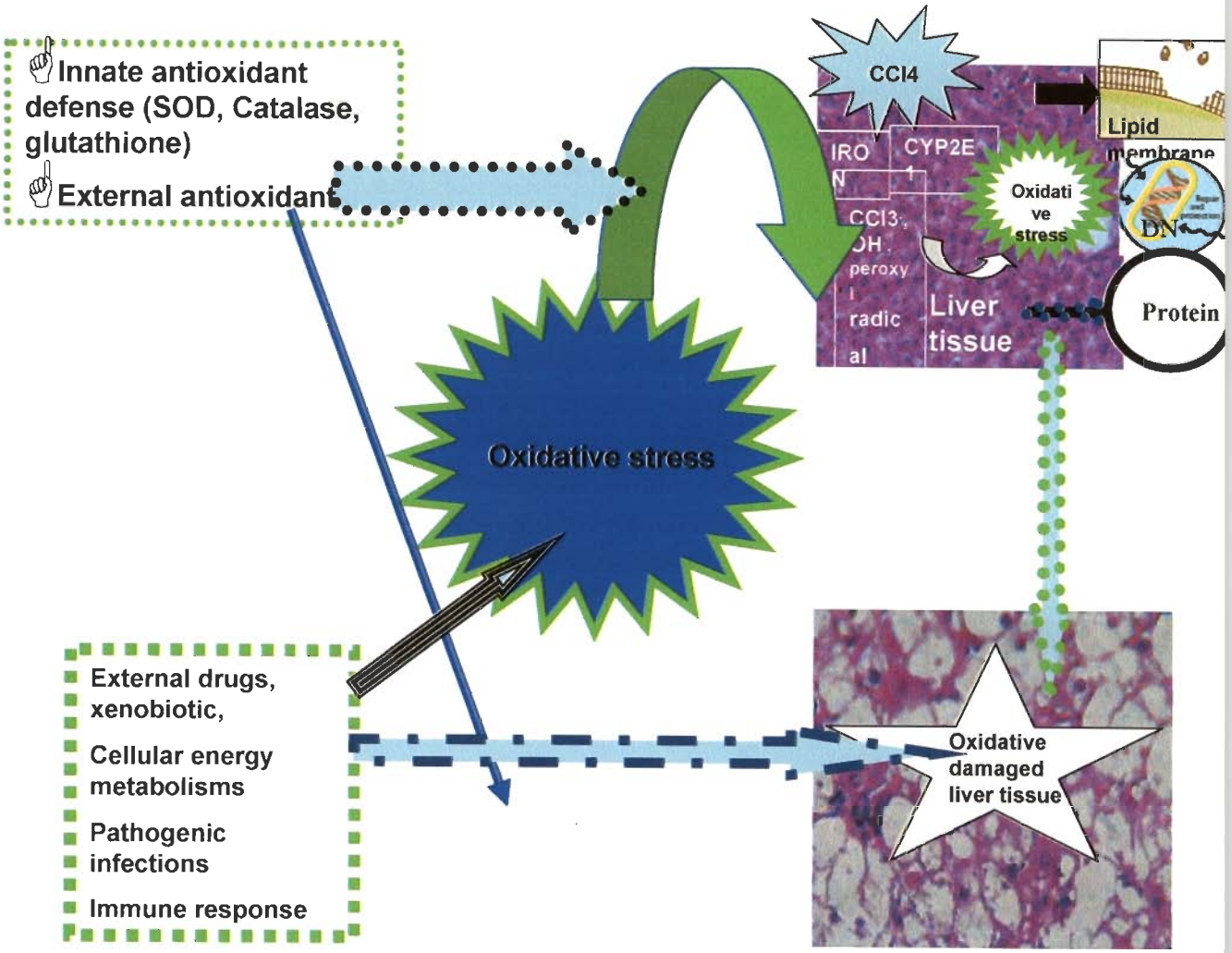


Fig. 2.1 Schematic representation for free radical mediated oxidative stress toxicity induced by CCl_4 in rat liver model system.

mechanism of CCl_4-induced hepatotoxicity reported that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl_4 and depleted GSH stores may lead to liver necrosis (Casini *et al.*, 1984; William and Burke, 1990). In addition to release of cytotoxic molecules, oxidative stress may result from derangement of antioxidant defenses such as glutathione and antioxidant enzymes, causing a shift in the oxidant–antioxidant balance. Reactive aldehydes, especially 4-hydroxynonenal and MDA among the degradation products of fatty acids are regarded as important oxidative markers in liver injury; binds easily to functional groups of proteins and inhibit important enzyme activities (Poli *et al.*, 2008; Grotto *et al.*, 2009). Thus, in brief well-known markers of chronic oxidative stress in liver include enzymes such as superoxide dismutase, catalase, glutathione peroxidase and nonenzymatic compounds, such as 4-hydroxynonenal, glutathione, malondialdehyde. Identification of these markers represents a principal outcome of liver disease research since it not only enables early detection of liver diseases but also allow monitoring the degree of liver damage, the response to pharmacological therapies and the development of new therapeutic approaches (Cesaratto *et al.*, 2004).

2.3 INTRODUCTION TO FREE RADICALS AND THEIR MECHANISM OF GENERATION

2.3.1 Role of free radicals and their mode of generation

Free radicals are natural by-products formed inside the body as a result of metabolic processes. These are electrically charged molecules capable of independent existence. They contain one or more unpaired electron in their outer atomic orbital. The presence of an unpaired electron makes the atom or molecule more reactive by increasing their chemical reactivity (David *et al.*, 2000). They are able to donate an electron to other molecules, therefore behaving as oxidants. They are continuously produced inside body of organism and when generated in tightly regulated manner they maintain homeostasis at cellular level in healthy tissues and also some of them serve as important signaling molecules (Ames *et al.*, 1993; Valko *et al.*, 2007). They are also generated through external sources such as environmental pollutants, cigarette smoke, automobile exhaust, radiation, air-pollution, pesticides, etc (Li and Trush, 1994). Free radicals contain oxygen such as hydroxyl (OH•), superoxide ($O2^{\bullet -}$), nitric oxide (NO•), peroxyl (ROO•) and lipid peroxyl (LOO•). Also, hydrogen peroxide (H_2O_2), ozone

(O3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH), are not free radicals and generally called oxidants, but can easily lead to free radical reactions in living organisms. Free radicals are not always harmful metabolic byproducts; at low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. However, when produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can produce serious alterations in the membranous structure of the cell and other biomolecules such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA) (Halliwell, 2007).

Superoxide anion radical- can be formed by one electron reduction of molecular oxygen or by one electron oxidation of hydrogen peroxide (Rielski, 1978). It is generated via the electron transport systems in either the endoplasmic reticulum or mitochondria via electron leakage from intermediate electron carriers onto oxygen (Halliwell and Gutteridge, 2000). Though it is less reactive than hydroxyl radical, it is potentially more damaging because of its ability to diffuse at a distance before encountering a possible target. It vigorously seeks to remove an electron from biological molecules to pair up the lone electron in its outer orbit, thus it has ability to attack biomolecules like sugars, proteins, polyunsaturated fatty acids and DNA. It can also react with nitric oxide and form peroxynitrite.

Hydroxyl radical- is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper or iron. $OH^{\cdot}$ is highly reactive oxygen centred radical with an extremely short half life. Thus when produced *in vivo* it reacts with almost any biomolecule it comes across like protein, DNA, PUFA in membranes. The hydroxyl radical is an initiator of lipid peroxidation; it removes a hydrogen atom from the unsaturated fatty acids of membrane phospholipids resulting in the formation of lipid free radical.

$$LH + OH^{\cdot} \longrightarrow L^{\cdot} + H_2O$$

The lipid radical in turn, reacts with molecular oxygen and forms a lipid peroxyl radical.

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$$

Like $OH^{\cdot}$, the lipid peroxide radical can function as an initiator of oxidation by removing another hydrogen atom from a second unsaturated fatty acid.

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$

Thus, a chain reaction is initiated.

Hydroxyl radical can also modify cell membrane proteins by formation of disulphide bonds resulting in aggregation of membrane proteins, forming ion channels and finally disruption of membrane structure and function. Hydroxyl radicals can interact with DNA and inhibit its replication. They can attack the purine and pyrimidine bases like thiymine and guanine resulting in mutation (Ashok and Ali, 1999).

Hydrogen peroxide – Unlike others, hydrogen peroxide is not a free radical. It is least reactive molecule among all ROS species and is stable under physiological pH and temperature. However, it can diffuse across biological membranes. Hydrogen peroxide is considered a key oxygen free radical because of its high stability, diffusion and involvement in cell signaling cascades. It can be formed by a direct two electron reduction of molecular oxygen or by an electron reduction of superoxide enzymatically by superoxide dismutase (Chance *et al.*, 1979). Enzymes such as amino acid oxidase and xanthine oxidase also produce hydrogen peroxide from superoxide anion (Mates and Sanchez, 2000).

Peroxyl radical - These radicals are intermediate species formed during lipid oxidation chain reactions, such as oxidation of PUFA, resulting in deterioration of lipid containing foods. They are formed by a direct reaction of oxygen with alkyl radicals ($R^{\cdot}$), for example, the reaction between lipid radicals and oxygen. Decomposition of alkyl peroxides (ROOH) also results in peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals. Both peroxyl and alkoxyl radicals are good oxidizing agents (Decker, 1998). However, they are less reactive than respective open chain radicals due to delocalization of electrons in the ring.

Nitric oxide – is a free radical with a single unpaired electron. NO is formed from L- arginine by NO synthase (Fang *et al.*, 2002). Nitric oxide itself is not a very reactive free radical but its overproduction is involved in ischemia reperfusion, neurodegenerative and chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease.

2.3.2 Role of free radicals in oxidative damage to biomolecules

An imperative and direct consequence of oxidative stress phenomenon is oxidative modification of biomolecules that are involved in a number of physiological

and pathophysiological processes such as aging, atherosclerosis, inflammation, carcinogenesis and drug toxicity. The major biological targets of free radical insult are mainly protein, lipids and DNA although carbohydrates may also be subjected to free radical damage (Aruoma, 1998; Dalle-Donne *et al.,* 2006). The degree of oxidative damage suffered by an organism, tissue and organ maybe evaluated by the measurement of a number of molecules which are indexes of oxidative stress. Oxidative damage to DNA may lead to mutagenesis and carcinogenesis (Thompson, 2006). Free radical damage to protein may result in loss of enzyme activity. Similarly, lipids are most susceptible to attack by ROS resulting in extensive peroxidation.

2.3.2.1 Oxidative damage to protein

Proteins can be oxidatively modified in three ways: by oxidative modification of amino acid residues, by free radical mediated peptide cleavage and by reaction with lipid and carbohydrate oxidation products (Dean *et al.,* 1986; Stadman and Levine, 2003; Park and Xiong, 2007). Proteins with amino acids containing unsaturated or sulphur groups such as cysteine, methionine, arginine, histidine, tryptophan and tyrosine are most susceptible to oxidation (Freeman and Crapo, 1982). 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 (Meucci, 1991), increase in susceptibility to enzyme proteolysis, adverse effects on heat stability, alteration of signal transduction mechanisms and decrease or loss of biological function. Oxidative modification also introduces carbonyl groups into amino acid side chains of the protein. The level of carbonyl groups in proteins is widely used as a marker of oxidative protein damage. An increase in protein carbonyl content in tissues has been observed in a number of pathological disorders like rheumatoid arthritis, alzheimer's disease, atherosclerosis, Parkinson's disease (Aksenov *et al.,* 2001; Lemarechal *et al.,* 2006).

2.3.2.2 Lipid peroxidation

Lipid peroxidation is one of the major consequences of free radical mediated oxidative stress (Halliwell and Chirico, 1993). 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, 1992). Polyunsaturated fatty acids are highly susceptible to reactions with free radicals like hydroxyl and

hydroperoxyl radicals but not $O^{2\bullet-}$ and H_2O_2. Several experimental evidences have suggested that extensive peroxidation causes impairment of biological membrane functioning like decrease in membrane fluidity, inactivation of membrane bound enzymes and receptors and may change non-specific calcium ion permeability (Ohyashiki *et al.,* 1995). Thus, it maybe concluded that lipid peroxidation is both a free radical mediated process and a source of secondary free radicals, some of which may serve as second messengers and others can directly react with surrounding molecules or diffuse before further reaction, thereby spreading the biochemical lesion. As a consequence of lipid peroxidation, a number of compounds like alkanes, malonaldehyde, HNE and isoprostanes are produced. These compounds serve as markers in lipid peroxidation assays and an increase in level of these products has been observed in diabetes, atherosclerosis, liver disease, apoplexy and inflammation (Esterbauer *et al.,* 1993; Watanabe *et al.,* 2001; Wiswedel *et al.,* 2005).

2.3.2.3 Oxidative damage to DNA

DNA is considered one of the major targets of free radical mediated oxidative damage and this damage may occur in any cell exposed to an oxidant. Oxidative damage to DNA includes single or double strand breaks, sister chromatid exchange, DNA-DNA cross links, damage to the deoxyribose-phosphate backbone as well as specific modifications of purine and pyrimidine bases (Lloyd and Phillips, 1999; Cooke *et al.,* 2003). The components of DNA most susceptible to free radical attack are the thymine and cytosine bases, followed by adenine and guanine and finally the deoxyribose sugar. Mitochondrial DNA is more susceptible to oxidative damage due to lack of histone proteins and close locations to the ROS producing systems (Ames *et al,* 1993). However, lately 8-Hydroxy-2-deoxyguanosine has been acknowledged as a biological marker for oxidative stress (Kadioglu *et al.,* 2004); further used for estimation of DNA damage in humans.

2.4 ANTIOXIDANT DEFENSE SYSTEM IN HUMAN BODY AND THEIR CLASSIFICATION

Biological systems have developed sophisticated antioxidant mechanisms to combat the damaging effects of free radicals and other ROS species.

Antioxidants can be divided into three classes:

- Primary antioxidants prevent formation of new ROS. They include antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and metal binding proteins such as ceruloplasmin (Cu binding protein), transferrin (Fe binding protein), and ferritin.
- Secondary antioxidants also called chain breaking antioxidants, include Vitamin E and A, ascorbic acid, uric acid and albumin. These antioxidants remove newly formed free radicals before they can initiate a chain reaction.
- Tertiary antioxidants include repair enzymes as they repair cell structures, damaged by free radicals.

2.4.1 Enzymatic antioxidants

The superoxide anion has been implicated in inflammation, hyperoxic cell damage and reperfusion injury (Mc Cord, 1983) and is involved in wide range of diseases. The enzyme superoxide dismutase's are the first line of defense against toxicity of superoxide radical and their radical derivatives (Fridovich, 1974). Although superoxide once formed, undergoes dismutation to peroxide and oxygen, the presence of SOD increases the reaction rate by 10^9 fold. The non-enzymatic dismutation of $O^{2\cdot-}$ results in the production of singlet oxygen, and it has therefore been proposed that the function of SOD is to protect cell from the toxic effects of not only $O^{2\cdot-}$ but singlet oxygen as well (Halliwell and Gutteridge, 1989). There are atleast three superoxide dismutase isozymes in the mammalian body; Copper-Zn-superoxide dismutase in the cytosol of cells (Liou *et al.*, 1993), manganese superoxide dismutase (Mn-SOD) in the mitochondrial matrix (Weisiger and Fridovich, 1973) and extracellular space (Marklund, 1984).

H_2O_2 is damaging in living system as it can give rise to formation of toxic reactive hydroxyl radicals. Two types of enzymes exist to remove H_2O_2 within the cells; Catalase and Glutathione peroxidase (Halliwell and Gutteridge, 1989). Catalase has a double function, because it catalyzes the following reactions.

(1) Decomposition of H_2O_2 to give H_2O and O_2,

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

(2) Oxidation of H^+ donors for example methanol, ethanol, formic acid, phenols with

consumption of 1 mol of peroxide.

$$ROOH + AH_2 \longrightarrow H_2O + ROH + A$$

The predominating reactions depend on the concentration of H^+ donor and the steady state concentration or rate of production of ROH in the system. In both cases the active catalase $-H_2O_2$ complex 1 is formed first. The decomposition of H_2O_2 in which a second molecule of H_2O_2 serves as H+ donor for complex 1, proceeds exceedingly rapidly, whereas peroxidative reactions proceed relatively slowly (Aebi, 1984).

In animals, catalase occurs as a heme protein and is present in all major body organs. However, its activity in tissues varies greatly, being predominantly concentrated in liver and erythocytes. The brain, heart and skeletal muscle contain only low amount of catalase. In tissues, it is mainly particle bound (in mitochondria and peroxisomes), whereas it exists in a soluble state in erythrocytes (Matkovics *et al.*, 1982, Halliwell and Gutteridge, 1989).

Glutathione Peroxidase has a key role in the enzyme defense system against oxygen derived free radicals (Raes *et al.*, 1987). It detoxifies H_2O_2 or any hydroperoxide utilizing reduced (GSH) as a reductant and results in the formation of H_2O and oxidized glutathione (GSSG) as shown below:

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2\ H_2O$$

GPx is present as both a selenozyme and a selenium independent form. The selenoenzyme is capable of catalyzing the reduction of both H_2O_2 and lipid peroxides (Asayama *et al.*, 1996).

H_2O_2 is produced within the cells as result of various metabolic processes. Under normal conditions, it is mostly destroyed by catalase and in part by GPx, its accumulation within the cells is thus prevented (Mavelli *et al.*, 1982), however, it has been demonstrated that both catalase and GPx pathway are dependent on NADPH for function and both systems are actively involved in the disposal of H_2O_2 and that a failure in the generation of NADPH as with glucose-6-phosphate dehydrogenase deficiency impairs both mechanisms for detoxification of H_2O_2.

2.4.2 Non enzymatic antioxidants

2.4.2.1 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.

2.4.2.2 Glutathione

GSH is the main storage form of sulphur, and it acts as a potent detoxifier of xenobiotics through GSH-conjugation and can serve as a precursor of phytochelatins. Together with its oxidized form (GSSG) glutathione maintains a redox balance in the cellular compartment. 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). Functioning of GSH as antioxidant under oxidative stress has received much attention during last decade. A central nucleophilic cysteine residue is responsible for high reducing potential of GSH. It scavenges cytotoxic H_2O_2, and reacts non-enzymatically with ROS; singlet oxygen, superoxide radical and hydroxyl radical (Larson, 1988). The central role of GSH in the antioxidative defense is due to its ability to regenerate another water soluble antioxidant ascorbic acid, via the ascorbate glutathione cycle (Foyer and Halliwell, 1976; Noctor and Foyer, 1998).

2.4.2.3 Hydrogen donating non-enzymatic compound

Hydrogen donating antioxidants can donate hydrogen atoms to free radicals, can scavenge free radicals and prevent lipid oxidation. Chain breaking antioxidants donate hydrogen atoms to peroxyl radicals and convert them to more stable and nonradical products, thus they are very crucial in preventing lipid peroxidation (Decker, 1998). Antioxidant radicals formed from hydrogen donating antioxidants can react with alkyl, alkoxyl and peroxyl radicals of PUFA and generate a nonradical stable compound.

2.5 NATURAL ANTIOXIDANTS

It has been well conceived that plant derived dietary antioxidants play an important role in the maintenance of human health as our endogenous antioxidants fail to provide sufficient protection against the constant and unavoidable challenge of reactive oxygen species (Fridovich, 1998; Benzie, 2003). During the past decade, a great deal of attention has been focused on natural antioxidants such as vitamin E and C, flavonoids and polyphenols. In the early 1980s, Linus Pauling proposed that the antioxidant effect of high doses of vitamin C might help treat cancer. Therefore, consuming a diet rich in natural antioxidants has been associated with prevention and/or treatment of various kinds of diseases (Lugassi *et al.*, 2003; Mahajan and Tandon, 2004). In fact, there is clinical and epidemiological evidence correlating higher

consumption of food rich in antioxidants with a lower incidence of various human morbidities or mortalities. In recent years, prevention of cancer and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants (Ganguly, 2003; Chan *et al.*, 2005), suggesting that a higher intake of such compounds should lower the risk of mortality from these diseases. Antioxidants derived from fruits, vegetables, spices and cereals are very effective and have reduced interference with the body's ability to use free radicals constructively (Kahkonen *et al.*, 1999; Wolfe *et al.*, 2003). Many of these compounds possess anti-inflammatory, artherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades.

2.5.1 Sources of natural antioxidants

Aromatic and medicinal plants are in use since time immemorial, not only as a source of medicine but also, to serve innumerable human cause. Since natural antioxidants are present in plants, therefore the basic source of these compounds for humans is plant-derived products. However with the advent of synthetic antioxidants, this ageold practice of using plant derived formulations as an inherent part of folk medicine remedies received a sudden setback. Nevertheless, a majority of world population still widely depends on traditional herbal medicine.

First systematic study on antioxidant properties of herbs could however, be traced back to early fifties, when Chipault and coworkers performed a screening study of 72 different spices for their antioxidant activity (Chipault *et al.*, 1952, 1956). The result bought into focus two prominent herbs like Rosemary (*Rosmarinus officinalis*) and Sage (*Salvia officinalis*) as effective sources of antioxidants, which later also received considerable attention in past few decades. Further studies by various research groups have also proven the effectiveness of these antioxidative plants, which later resulted in their commercial exploitation. Many other spices commonly used in our diet have also been extensively studied with respect to their antioxidant property; turmeric, ginger, cinnamon and clove are few of them (Selvam *et al.*, 1995; Mathew and Abraham, 2006). Inspite of all controversies, spices remain one of the most promising sources of natural antioxidants till date. Active components present in these spices and responsible for their antioxidant properties are mainly phenolic acids, flavonoids,

natural pigments (curcumin, turmerin) and terpenes (e.g., rosmanol, carnosol) from rosemary and sage. Also, fruits, berries, vegetables, cereals, legumes and tea have been widely explored and their composition well established since they are commonly used in human diet (Chambers *et al.*, 1996; Miller *et al.*, 2000; Dasgupta and De, 2006). Majority of the active components accountable for their antioxidant potential belong to the group of polyphenols; flavonoids in vegetables, phenolic acids in cereals, anthocyanidins in fruits and berries, ascorbic acid in all variety of citrus fruits. However, in recent years tea extracts have also received much attention. Biologically active components of the tea extracts are mainly phenolic compounds like epigallocatechins, which possess strong radical scavenging activity *in vivo* (Henning *et al.*, 2004). Another rich source of antioxidants is red wine (Ghiselli *et al.*, 1998), that contains many valuable fruit peel-derived polyphenols (resveratrol), whose moderate consumption is also associated with a lower risk of coronary heart disease and other beneficial effects (Howard *et al.*, 2002; Rupasinghe and Clegg, 2007). Therefore, it is inevitable that richest source of antioxidants are fruits, vegetables, cereals and legumes, tea, coffee, wine, beer, and also herbs and spices (Sikora *et al.*, 2008).

2.5.2 Advantages of plant derived natural antioxidants over synthetic, microbial and animal origin antioxidants

Lipid soluble vitamins and selenium occur in animal derived food for example, milk and fish lipids, eggs, however their concentration is very less and their presence is also dependent on kind of feed consumed like carotenoids content in milk lipids, eggs. Thus products derived from animals are not significant sources of antioxidants in human diet (Sikora *et al.*, 2008). Many synthetic antioxidants such as propyl gallate (PG), butylated hydroxytoluene (BHT) and tertiary butylhydroxyquinone (TBHQ) were earlier recommended as synthetic antioxidants for use in health, food and cosmetic industry (Barlow, 1990; Abdalla *et al.*, 1999). However, some of their physical properties such as their high volatility and instability at elevated temperatures, toxicity, higher manufacturing cost, strict legislation on the use of synthetic food additives and consumer preferences aroused the need to find alternatives to synthetic antioxidants. There are also reports about serious side effects including the liver toxicity and cancer development (Hayashi *et al.*, 1993). Plants possess an inherent ability to synthesise an array of aromatic compounds which enable them to counteract reactive oxygen species or oxygenic stress as part of their defense strategy. Hence, they serve as potential

source of natural antioxidants. Also these are found to be safer, reliable and compatible with human diet without any side effects as often reported from use of synthetic antioxidants (Atolaiye *et al.,* 2009; Jennings and Akoh, 2009). Thus, there is an emergent and rampant need for the search of more potent safer natural antioxidants that can protect the human body from free radicals and retard the progress of many chronic diseases. The antioxidant activity of flavonoids can be attributed to their ability to reduce free radical formation and to scavenge free radicals (Pietta, 2000). The use of plants or herbs as antioxidants in processed foods is gaining remarkable importance in the food industry as an alternative to synthetic antioxidants.

2.5.3 Mechanism of action of natural antioxidants

The natural antioxidants like the flavonoids may prevent oxidative stress mediated tissue injury by direct scavenging of free radicals resulting in a more stable and less toxic radical (Pietta, 2000). Epicatechin and rutin are also powerful radical scavengers. Some specific flavonoids like quercetin are known to chelate iron, thereby removing a causal factor for the development of free radicals (Murota *et al.,* 2004). Also few of them may exert their antioxidant effect by inhibitory action of enzymes responsible for generation of free radicals. For example, the scavenging ability of rutin may be due to its inhibitory activity on the enzyme xanthine oxidase, a potential source of superoxide anion (Chang *et al.*, 1993). Antioxidant can modify the gene expression through the ARE (Antioxidant response element) and associated transcription factors Nrf-1 & Nrf-2 (Havsteen, 2002; Vries *et al.,* 2008). They may also act as regulators of redox state through interaction with transcription factors NFk, AP1 & p53. Dietary polyphenols can stimulate antioxidant transcription and detoxification defense systems through ARE.

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

The past few decades have witnessed a tremendous resurgence in interest and utility of medicinal plant products in various parts of the world. Therefore, in context of worldwide importance given to plant derived natural antioxidants and hepatoprotective agents, there is ample scope to explore Indian dietary and medicinal plants due to their widely diversified plant flora. Factors like geographical location, diverse climatic condition and topographical features can well explain the diverse nature of different classes of antioxidant phytochemicals. Thus, keeping Indian perspectives in view, these

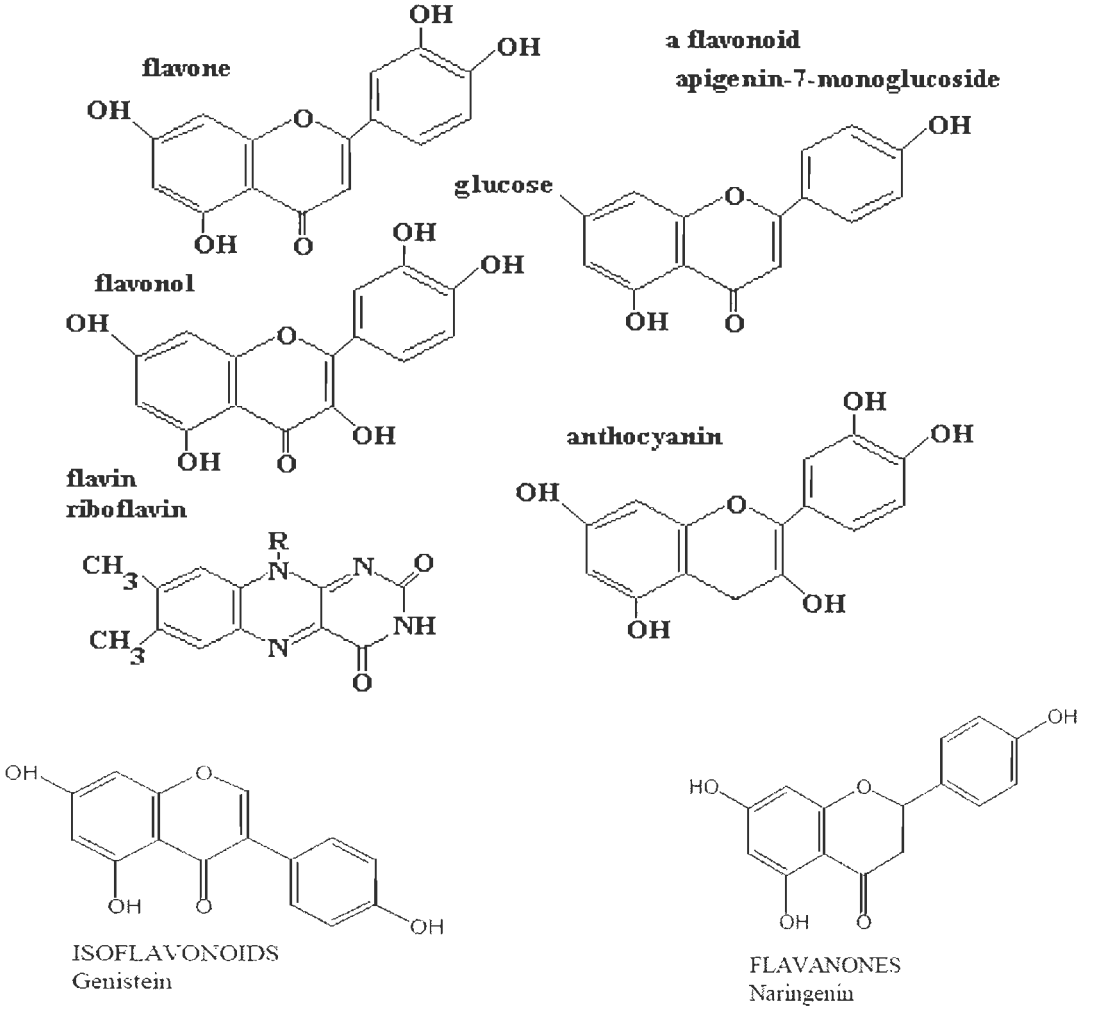
factors are quite compatible and enriching for our innumerable plant diversity. Results obtained from natural antioxidant therapy in various diseases have prompted several research groups in India to explore our vast reservoir of plant flora as potential source of therapeutic phytochemicals. During past few years a large number of medicinal plants have been extensively studied for their free radical scavenging activity and hepatoprotective potential (Gupta and Singh, 2007; Jyothi *et al.*, 2008; Mandal *et al.*, 2008). Rigorous efforts have been made in the recent past to explore the vast reservoir of natural antioxidants from the Indian medicinal plant perspective. In due course of scientific development, informative review articles have been summarized to give the scope of diverse nature of natural antioxidant classes present in Indian plant flora (Dahanukar *et al.*, 2000). The potential application of these therapeutic agents in the treatment of many human diseases including liver toxicity has been scientifically validated (Ali *et al.*, 2008; Samarth *et al.*, 2008; Gutierrez and Solis, 2009).

2.5.5 Classification of plant derived natural antioxidants

2.5.5.1 General introduction on Flavonoids and their structure-activity relationship

Flavonoids, constitute one of the most ubiquitous groups of plant phenolics derived from higher plants. Most of them occur as glycosylated derivatives in plants. These aromatic compounds are synthesized/ formed in plants from the aromatic amino acids, phenylalanine and tyrosine and malonate units. They are known to display a multitude of pharmacological and biochemical actions that includes, anti-inflammatory, antiallergic, antimicrobial, hepatoprotective, antiviral, antimutagenic/ anticarcinogenic and many more properties (Tapas *et al.*, 2008). The antioxidant function and enzyme modifying actions of flavonoids may attribute for many of their pharmacological activities. The compounds appear to possess variable mechanisms of action, which include radical scavenging and metal ion complexation. Numerous studies by different research groups have been carried out to determine the necessary structural features for flavonoids to be effective radical scavengers. The flavonoids consist of two benzene rings (ring A and B) linked by an oxygen-containing heterocycle (ring C). They can be categorized in six groups that include flavones, flavonols, flavanones, flavanonols, and anthocyanins, based on the common carbon structures (Harbone, 1980). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of

A and B rings. The flavones have a double bond between C2 and C3, flavanones have a saturated C2-C3. Flavononols have an additional hydroxyl group at the C3 position and flavanonols are saturated between C2 and C3 with a hydroxyl group at the C3 position. Anthocyanidins are water-soluble flavonoids that are aglycones of anthocyanins. The principle naturally working anthocyanidins are pelargonidin, cyanidin, paeonidin, delphinidin, petudin and malvidin. According to some investigations, leucoanthocyanins are present in wood, bark, nutshells, flowers, leaves and fruits. They are morphologically much more widespread than the normal anthocyanins.



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

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.*, 1996). Flavonoids have the most potent antioxidant activities because of the chemical structures with o-diphenolic group, a 2-3

double bond conjugated with the 4-oxo function, the hydroxyl groups in positions 3 and 5. Previous studies have shown that flavonoids having more hydroxyl groups, or hydroxyl groups ortho to one another, proved better antioxidants. The B ring of flavonoids is more electron rich than the A and C rings. This makes B ring more susceptible to attack by radicals. These properties are however, consistent with oxidation mechanisms of phenols; hydroxyl groups act as electron donating substituents, and ortho hydroxylation helps to stabilize the phenoxyl radical (Steele *et al.*, 2002). Antioxidant activities of flavonoids are also influenced by hydroxylation and the presence of sugar moiety. Hydroxyl substituents on the flavonoid ring increase the antioxidant activity, while substitution by methoxy groups is linked with diminished antioxidant activity (Acker *et al.*, 1996; Kemertelidze *et al.*, 2000; Silva *et al.*, 2002; Wolfe and Liu, 2008).

2.5.5.2 Importance of Flavonoids as antioxidants and hepatoprotective agents

From the earlier work, it has been determined that the major contributing constituents of plant products for their antioxidant effect is mainly due to phenolic compounds, such as flavonoids, phenolic acids, tannins and diterpenes etc (Chung *et al.*, 1998; Pietta, 2000). The flavonoids isolated from various dietary and medicinal plant sources have been suggested to play a preventive role in the development of cancer, heart disease and ageing related diseases related to excessive oxidative stress due to their antioxidant properties (McKay *et al.*, 2007; Das *et al.*, 2008; Song *et al.*, 2009). According to free radical theory, blocking or retarding the chain reaction of oxidation is one of the practicable strategies to prevent oxidative stress induced hepatotoxicity (Paya *et al.*, 1993; Dometrovic *et al.*, 2008). It is known that some flavonoids are able to reduce xenobiotic-induced hepatotoxicity in animals. The inhibitory activity of flavonoids on free radical production could be related to their hepatoprotective effects since exogenous antioxidants may counteract the damaging effects of oxidative stress, in coordination with natural systems like glutathione, tocopherol or protective enzymes. Some flavonoids, like quercetin and silibinin, can protect cells and tissues from toxic effects of reactive oxygen species and elicit their antioxidant activity by scavenging of free radicals and other oxidising intermediates, chelation of iron or copper ions, and from inhibition of oxidases (Pietta, 2000; Leopoldini *et al.*, 2006; Chang *et al.*, 2007; Panah *et al.*, 2009). It is well documented

in literature that flavonoids from *Silybum marianum* widely used in the treatment of liver disorders exert not only a positive effect on intact liver cells or cells not yet irreversibly damaged, but also stimulate their regenerative capacity after partial hepatectomy (Skottova *et al.*, 2004; Pradhan and Girish, 2006). Oxidative stress can enhance the progression of chronic inflammatory liver diseases, and therefore, in addition to therapies based on the etiological factors, the use of flavonoid antioxidants may also be justified in the treatment of chronic liver diseases (Yuan *et al.*, 2008; Kim *et al.*, 2009).

2.6 *IN VITRO* METHODS FOR EVALUATION OF ANTIOXIDANT POTENTIAL

It has been widely accepted that antioxidant potential of plant derived natural antioxidant depends on its inherent structure. Moreover, the reactivity of potent antioxidants varies depending on the 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. Since most phytochemicals are multifunctional, their activity and mechanism would largely depend on the composition and conditions of the test system. Therefore, a reliable antioxidant protocol requires the measurement of more than one property relevant to either food or biological systems (Frankel and Meyer, 2000; Moreno, 2002).

FRAP (Free Radical Absorbance Power) assay uses an oxidation/reduction reaction to measure the ability of a sample to reduce iron (III) to iron (II). In this assay, there is no pro-oxidant and regarded as direct test of total antioxidant power of any compound (Benzie and Strain, 1996).

Phosphomolybdenum Complex assay first developed by Prieto *et al.* (1999) is based on the change in absorbance after the reduction of molybdenum (VI) to molybdenum (V) in presence of a reducing species like an antioxidant.

Scavenging of the stable radical 2, 2' diphenyl-1-picryl hydrazyl (DPPH) assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH$^{\cdot}$ The free radical DPPH$^{\cdot}$ is reduced to the corresponding hydrazine when it reacts with hydrogen donors.

TEAC (Trolox Equivalent Antioxidant Capacity) assay was first reported by Miller *et al.* (1993). It is based on the inhibition of the absorbance of radical cation 2, 2-

azinobis-(3-ethylbenzothiazoline-6-sulphonate ($ABTS^{\cdot+}$) by antioxidants, which has a characteristic long wavelength absorption spectrum showing main absorption maxima at 415 nm, and secondary absorption maxima at 660, 734 and 820 nm. Hydroxyl radical scavenging is calculated using the deoxyribose assay: a mixture of ferric chloride ($FeCl_3$) and ethylenediamine tetraacetic acid (EDTA) in the presence of ascorbate reacts to form iron(II)-EDTA plus oxidized ascorbate, H_2O_2 then reacts with iron (II)-EDTA to generate iron (III)-EDTA plus $OH^{\cdot}$ in the so called Fenton reaction. Those radicals not scavenged by other components of the reaction mixture attack the 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 $OH^{\cdot}$ of a substance added to the reaction mixture is measured on the basis of inhibition of the degradation of deoxyribose (Halliwell, 1990; Aruoma *et al.*, 1993).

2.7 EXPERIMENTAL PLANT *FICUS BENGALENSIS*

2.7.1 About *Ficus bengalensis* and its use in traditional medicine

Ficus bengalensis L. is a tree belonging to the family Moraceae. This tree grows in almost all parts of India and is widely distributed in the Sub-Himalayan tract and Peninsular India. The main flowering season is from April to May. *Ficus bengalensis* L. is an ornamental plant named in Arabic Teen Benghaly. Stem-bark, root -bark, aerial roots, leaves, vegetative buds and milky exudates of the plant are used in medicine. It is useful in biliousness, ulcers, vomiting and vaginal complaint. The leaves are used in treatment of ulcers. The root is useful in gonorrhoea and syphilis. Stem bark is hypoglycemic, tonic, astringent, antidiarrhoeal and antidiabetic. Seed is tonic. The aerial roots are useful in obstinate vomiting and leucorrhoea and are used in osteomalacia of the limbs. The buds are useful in diarrhoea and dysentery. The latex is used to treat indigestion, rheumatism, and toothache (Warrier *et al.*, 1995).

2.7.2 Phytochemistry of *Ficus bengalensis L.*

A number of compounds have been isolated from *F. bengalensis* and chemically characterized (Lanhers *et al.*, 1987). These include n-nonadodecane, β-amyrin, β-sitosterol, lupene-3-one, lupeol acetate, 24-hydroxy β-sitosterol, palmitic acid and palmitoyl glycerol from the bark of *Ficus bengalensis* L were isolated by different

methods of chromatographic techniques. Several reports on phytochemistry of *F. bengalensis* showed the presence of flavonoids and triterpenoids (Elgindi, 2004) and sterols.

2.7.3 Ethnopharmacology of *F. bengalensis L.*

An ethnopharmacological study of *F. bengalensis L.* (Moraceae) was undertaken earlier to evaluate its pharmacological properties. Several pharmacological properties of *F. bengalensis L.* are reported such as; antimicrobial (Parekh and Chanda, 2006), antihyperglycemic (Edwin *et al.*, 2008), immunomodulatory (Gabhe *et al.*, 2006), antitumor, antioxidant (Shukla *et al.*, 2004), antidiarrhoeal (Mukherjee *et al.*, 1999), antirheumatic, skin disorders like sores (Warrier *et al.*, 1995), anthelmintic activity (Aswar *et al.*, 2008).

Chapter 3

Screening, Fractionation and Identification of Antioxidant Constituents from *F. bengalensis.*

3.1 INTRODUCTION

As part of our innate defense system, our body has evolved complex antioxidant mechanisms to combat toxic effects of deadly free radicals. The role of external supply of antioxidants has been widely believed to be one of the promising approaches in the prevention and treatment of many oxidative stress mediated human diseases including liver pathophysiological conditions (Shen *et al.*, 2007; Lengyel and Tulassay, 2008; Chakraborty *et al.*, 2009). However, natural antioxidants from dietary and medicinal plant sources are gaining edge over others from synthetic, microbial and animal origin (Fogden and Neuberger, 2003; Sikora *et al.*, 2008).

In recent years, lot of attention has been directed towards credentials of medicinal and dietary plants with antioxidant potential to be utilised for human cause, therefore research on medicinal plants has attracted a lot of attention globally (Govindrajan *et al.*, 2005; Mazumdar and Rehman, 2008; Madhuri and Pandey, 2009). Several research groups worldwide have made substantial efforts to screen and evaluate potential sources of natural antioxidants from plants (Buricova and Reblova, 2008; Souri *et al.*, 2008; Demiray *et al.*, 2009; Veeru *et al.*, 2009). Flavonoids and other phenolic compounds of plant origin widely suggested to be safe and bioactive, have also been reported as potent ROS scavenger, thus they are viewed as promising therapeutic drugs for free radical pathologies (Parshad *et al.*, 1998; Lee *et al.*, 2000).

Most of past investigations have revolved around the evaluation of antioxidant activity of crude extracts from plants and herbs; however with rapid advancement and development of sophisticated techniques for isolation/ purification and characterisation of bioactive fraction have led to discovery of novel compounds with therapeutic potential and establishment of structure-activity relationship (McRae *et al.*, 2007; Nair *et al.*, 2009). Also, the antioxidant activity of plant origin is dependent on the type and polarity of the extracting solvent as well as on the test system and the substrate to be protected by the antioxidant (Moure *et al.*, 2000; Kang and Lee, 2001). Therefore it has been emphasized to assess different dietary and medicinal plants for their antioxidant activity on the basis of reliable *in vitro* based assays (Moreno, 2002).

Indian subcontinent due to its compatible geographical location and climatic condition and widely diversified plant flora richly endowed with variety of potent phytochemicals, has been demonstrated to deliver preventive role in many traditional as well as therapeutic medicines (Dahanukar *et al.*, 2000). Moreover, from the huge

reservoir of aromatic, spicy or medicinal plants, only a few have found a niche for application as antioxidants in food products. Besides, scientific information on antioxidant properties of various plants that are less widely used as food or as medicine is still rather scarce and consequently rarely brought in use. Therefore, the assessment of such properties remains an interesting, useful and challenging task, particularly for finding new sources of natural antioxidants, functional foods and nutraceuticals.

Keeping this whole perspective in view, this present work is an attempt to screen and evaluate fifteen different medicinal and dietary plants for their antioxidant property by means of reliable *in vitro* based assays. The present study mainly focused on *F. bengalensis* based on the screening results from the present study. Although the use of *F. bengalensis* in numerous physiological disorders has been widely acknowledged in Indian traditional medicine (Cherian, 1992; Warrier *et al.*, 1995) and presence of a number of triterpenoids, flavonoids, sterols, glycosides have been reported (Subramanian and Nair, 1970; Elgindi, 2004). However, the antioxidant potential of *F. bengalensis* has not yet been extensively studied. Therefore, the present work provides the detailed scientific evidence regarding antioxidant potential of *F. bengalensis*. Under the present work bioactivity guided extraction, fractionation and identification of active fraction from *F. bengalensis* have been performed. The results are presented and discussed.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals and plant materials

All common chemicals and solvents used in the present study were of analytical grade with highest purity and purchased from SRL, S.D Fine, Hi-Media 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) were purchased from Sigma Chemical Co. (St. Louis MO, USA) and MERCK Co. (Darmstadt, Germany) unless otherwise mentioned.

Plant materials: The fifteen different Indian dietary and medicinal plants were selected based on their therapeutic and other application in traditional medicine and screened for their antioxidant activity and major phytochemical content. These selected plant samples (*Cyperus rotundus, Boerhaavia diffusa, Hemidesmus indicus, Cassia occidentalis, Hibiscus abelmoschus, Vitis vinifera, Asparagus racemosus, Ferula*

asafoetida, Ficus carica, Hedychium spicatum, Celastrus paniculatus) were obtained from Raj Rajeshwari nursery, Jwalapur, Haridwar, and local area of Roorkee (*Beta vulgaris, Punica granatum, Ficus bengalensis*), Uttarakhand, India and were identified by botanical expert at Shantikunj, Haridwar and in the Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India. A specimen copy of these plants is kept in the departmental herbarium facility.

3.2.2 Extraction, preparation and screening for antioxidant activity

The fresh plant parts from fifteen different dietary and medicinal plants as given in Table 3.1 were washed thoroughly under running water and were allowed to dry in shade. The dried plant parts were frozen into liquid nitrogen and grinded to fine powder with the help of mortar and pestle. The aqueous extract of different part of each plant was prepared by soaking 10 g of fine powder in phosphate buffer (0.1 M; pH 7.4) (w/v 1:10) for 3 hours in dark. Similarly, the methanolic extract was prepared by soaking same amount of fine powder in methanol (w/v 1:10) for 24 hours at 40°C. The extracts were filtered through three layer muslin cloth and filtrates were centrifuged at 4000 x rpm for 15 min and clear supernatants were collected. The supernatants thus obtained were dried in rotary evaporator in case of methanolic extracts and lyophilized in case of phosphate buffer, respectively. The dried residue were stored at -80°C and used further for evaluation of antioxidant activity and phytochemical determination.

3.2.3 Evaluation of antioxidant activity of extracts

The preliminary evaluation of the aqueous and methanolic crude extracts of the above mentioned plants used in the present study were performed using DPPH free radical scavenging assay (Brand-Williams *et al.*, 1995) and phosphomolybdenum reduction assay (Prieto *et al.*, 1999) as described in detail later (chapter 4, section 4.2.2.5 and 4.2.2.7 respectively).

3.2.4 Quantitative estimation of total phenolic and sugar content

The total phenolic content of the aqueous and methanolic crude extract of different plants were determined by the Folin-Ciocalteu method (Singleton *et al.*, 1977). One mL of respective extract 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 reaction mixture was incubated at 30°C for 1 hour. Absorbance was measured at

the end of incubation time at 765 nm. A calibration curve was prepared using gallic acid as a standard phenolic with a concentration range of (0.01–0.1 mg per mL). The linear equation y = 0.0112x + 0.0061 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.

Quantitative determination of total sugar content of the different extract was performed as per method described by *Dubois et al.* (1956). Briefly, 50 µL (10%) solution of different extract samples was made upto 100 µL final volume with distilled water. Further 50 µL phenol solution (80% phenol by weight) was added to it and vortexed and finally sulphuric acid (2 mL) was added in a streak and the mixture was allowed to stand for 10 min at 25° C. Absorbance was measured at 490 nm. A calibration curve was prepared using glucose as a standard with a concentration range of (0.1– 1 mg per mL). The amount of total sugar was calculated with the help of glucose calibration curve linear equation y = 0.0015x - 0.009. The total sugar content was expressed in terms of Glucose Equivalent (GlcEq) per 0.1g sample.

3.2.5 Extraction and fractionation of antioxidant constituents from stem bark of *F. bengalensis*

Stem bark of *F. bengalensis* was washed thoroughly under running water and was allowed to dry in shade for several days. Dried plant part was weighed and frozen into liquid nitrogen and then powdered with the help of mortar and pestle. The powdered bark sample (250 g) was extracted with methanol (w/v 1:10) by maceration at room temperature for 3 days. The extracts were filtered with Whatmann no #1 filter paper and supernatant was collected. The clear supernatant was evaporated and dried powder was called crude extract of *Ficus bengalensis* stem bark (CFBS) and further used for bioactivity guided fractionation. The CFBS was redissolved in 400 mL methanol/water (40:60 v/v) and was further successively liquid-liquid partitioned with increasing polarity to remove fatty substances using hexane, dichloromethane and ethylacetate. Crude methanolic extract was partitioned with hexane in a separating funnel. Hexane partition was removed in vacuum. The aqueous–methanol mother liquor was evaporated in vacuum to remove methanol and the remaining aqueous extract was successively partitioned with dichloromethane (DCM) and ethylacetate.

Partitioned organic solvent fractions were evaporated at 50°C with the help of rotary evaporator to get fraction extract powder. The remaining aqueous mother liquor was lyophilized to obtain an aqueous partition. Both organic and aqueous phases were evaluated for their phenolic, flavonoid and glycoside content and antioxidant activity. Dried ethylacetate fraction (3g) was re-suspended in 5 mL of methanol, fractionated on silica gel column using Benzene: Ethylacetate as elution medium (2:3 to 0:1 v/v) and eluted at flow rate of 1 mL per min. 10 mL fractions were collected and each elution fraction was monitored for its flavonoid content and antioxidant activity using DPPH free radical scavenging assay. Eluates from 13-16 were pooled on basis of similar antioxidant activity and maximum flavonoid content. This pooled fraction was named as Flavonoid Enriched Fraction of *F. bengalensis* (FFB).

3.2.6 Qualitative and quantitative determination of antioxidant constituents in FFB

3.2.6.1 Spectrophotometric determination of glycosides in FFB constituents

Quantitative determination of glycoside content in FFB was performed as per method described (Antan *et al.*, 1995). Briefly, 1 mL (1%) solution of FFB was mixed with 1 mL of 8% solution of vanillin containing 10 mL of 72% sulfuric acid. The reaction mixture was mixed thoroughly, heated at 60°C for 10 min and then cooled on ice to stop the reaction. The absorption 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 of sample.

3.2.6.2 Spectrophotometric estimation of phenolic and flavonoid in FFB constituents

Total phenolic content was determined using the standard method as described (Singleton *et al.*, 1977) and detailed method is described previously (section 3.2.4). Total flavonoid content was estimated using the protocol described by Chang *et al.* (2002) with slight modifications. Briefly, 0.5 mL of extract (1g per 10 mL) 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. The mixture was incubated at 25°C for 30 min. The absorbance of the reaction mixture was measured at 415 nm using UV/Visible spectrophotometer (CARY 100 Bio, Varian, Netherland). The calibration curve was

prepared using rutin solutions at concentrations 12.5 to 100 µg per mL in methanol. The total flavonoid content of FFB was expressed in terms of mg Rutin equivalent per g sample.

3.2.6.3 Thin layer chromatography identification of active constituents in FFB

TLC method was employed for the qualitative observation of antioxidant constituents with the help of suitable developing reagents and solvent system. TLC analysis of FFB antioxidant constituents were performed on silica gel plates 60 G_{254} (Merck, Germany), preactivated in an oven at 110-120°C for an hour. TLC chamber was saturated with the mobile phase atleast an hour before analysis. A 20 mg per mL solution of extract was prepared in methanol and 5 µL aliquots were applied in narrow streak to the plates with a micropipette. Ethylacetate: Chloroform: formic acid (3:3:1) was used as solvent system.

3.2.6.4 UV visible spectroscopy of FFB antioxidant constituents

UV-VIS Spectrophotometer (CARY 100 Bio, Varian and Netherland) was used to determine the type of constituents in the active fraction of FFB. The active fraction FFB was dissolved in methanol and its UV-VISIBLE spectra was collected in the range of 200-600 nm.

3.2.6.5 FTIR characterization of antioxidant constituents from FFB

Lyophilized powder of FFB 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 (Thermo Nicollet Nexus FTIR, USA).

3.2.6.6 Direct infusion Electro spray ionization-mass spectrometry (ESI-MS) characterization of antioxidant constituents from FFB

Electrospray mass spectrometric analyses was performed on a Brucker LC-MS-NMR instrument equipped with a Brucker mass electrospray (Esquire 2000 series, Agilent, Zurich, Switzerland). 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 mL per min in both positive and negative scan mode. The FFB constituents were analyzed by direct

infusion in ESI-MS at a flow-rate of 10 mL per min. The antioxidant fraction was dissolved in HPLC grade methanol (50:50) and filtered with 0.25 micron membrane before injection in mass spectrometer. Mass spectra were acquired in negative scan mode detection and ESI-MS conditions from 200-1200 m/z.

3.2.6.7 NMR spectroscopy of antioxidant constituents from FFB

Sample (5 mg) of FFB fraction from *F. bengalensis* were cleaned using various solvent systems starting with non polar solvents e.g., hexane and then introducing, methanol, ethylacetate, chloroform and acetone. The cleaned samples were weighed and dissolved in maximum 2 mL deuterated DMSO-d6. The samples were then pipetted into NMR tubes. The ^{1}H NMR spectra were recorded by using Bruker DRX 500 at 500 MHZ.

3.2.7 Statistical analysis

All qualitative and quantitative experiments in separation and fractionation of antioxidants constituents from *F. bengalensis* 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 fifteen Indian medicinal plants for their antioxidant activity

In the present study, an attempt has been made to do systematic evaluation of radical scavenging activity and total antioxidant potential of fifteen different dietary and medicinal plants. The list of these fifteen plants and their respective plant parts used in the study are presented in Table 3.1. Very scarce data is available on radical scavenging and/or antioxidant properties of these plants since majority of them have not been thoroughly investigated before in this regard. Therefore, isolating and identifying the radical scavenging compounds in these plants and determining their individual activity in comparison to reference compound is a crucial and challenging

task. 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). Most of herbs / plant extracts were traditionally prepared in water; however recent studies (Parekh *et al.*, 2005) have shown that plant extracts prepared in organic solvent like methanol display more consistent activity compared to their aqueous counterpart. These activities might depend on the compounds being extracted by each solvent, the polarity of the solvents, and their intrinsic bioactivity. However, it may be acknowledged that water soluble components such as carbohydrates and glycosides are important biomolecules of considerable therapeutic potential (Kren and Martinkova, 2001; Newman *et al.*, 2008) that are sparingly soluble in dry methanol and less polar solvents.

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 methanolic extract were used for evaluating antioxidant potential. The antioxidant potential of a preparation can not be determined by a single assay. However, it has been widely suggested that a battery of assays based on different mechanistic principles could reveal the true antioxidant potential of diverse class of phytochemical constituents. Therefore in the present study, screening for the antioxidant potential of selected plants was performed by DPPH and Phosphomolybdenum assays which work on two different principles. Since the $DPPH^{\bullet}$ stable free radical assay can accommodate a large number of samples in a short period of time and is sensitive enough to detect natural compounds at low concentrations, it was used in the present study for the primary screening of both methanolic and aqueous crude extracts of different plant parts for their free radical scavenging activity. Thus, it is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extract. These two *in vitro* methods selected to screen out plants have also been used by other scientific groups and have recommended the reliability of these assays to validate the antioxidant activity *in vitro* condition (Arouma, 2006).

The results of antioxidant activity are summarized in Table. 3.2. As evident from the results that a great variation occurred in antioxidant activity of the different plants in both aqueous as well as methanolic preparation. Among the fifteen different plants tested (*Hibiscus abelmoschus*, *Ferula asafoetida, Beta vulgaris*, *Ficus carica, Punica granatum*, *Achyranthes aspera* and *Hedychium spicatum*) showed poor

antioxidant and radical scavenging activity. On the other hand methanolic extract of *Ficus bengalensis* showed highest free radical scavenging activity (IC_{50} = 0.0335 mg per mL) and total antioxidant activity (194.8 µmol AAE per g extract). Thus *Ficus bengalensis* was found to have strongest antioxidant activity. The observed differences in the antioxidant activity of various plants aqueous as well as methanolic extracts in the present study may be due to variations in the active constituents and difference in phytochemical constituents which is logical and expected. It has been suggested from previous studies that a number of phytochemical constituents may attribute for antioxidant activity (Benzie, 2003). Since various phenolics (Slusarczyk *et al.,* 2009; Abdel Hameed, 2009; Souza *et al.,* 2008), flavonoids (Chicaro *et al.,* 2004) and glycosides (Rahman and Moon, 2007) have been reported to account for the antioxidant potential of plants. Therefore in order to get an indication about the nature of active constituents present in the preparation, the qualitative estimation of both the aqueous and methanolic extracts of all plants were determined and attempt was made to see the correlation with antioxidant activity. The preliminary phytochemical analysis of aqueous and methanol extract from selected plants is presented in Table 3.3 and 3.4, respectively. The results were consistent with aforementioned view since phytochemical screening of both methanolic and aqueous extract of the tested plants revealed some differences in their phytochemical constituents. It was observed that nature of phytochemical constituents varied among different plants in both aqueous as well as methanolic extracts. However, only *Ficus bengalensis* methanolic extract demonstrated maximum quenching ability towards DPPH free radical followed by *Celastrus paniculatus* stem extract and correlate well with its high phenolic and flavonoid content. Several other plant extracts included in our study also tested positive for the presence of either phenolics or flavonoids or both of them, however, they did not show significant radical scavenging abilities, probably because of very low phenolics or flavonoid content.

Both aqueous and methanolic preparation from *F. bengalensis* showed high content of phenolic, flavonoids and glycosidic nature of compounds. Though previous studies have suggested that presence of phenolics, flavonoids and glycosides may contribute for antioxidant activity in various plant extracts (Hseu *et al.,* 2008; Yang *et al.*, 2008). It is likely that the higher antioxidant activity observed in *Ficus bengalensis* could be due to their high flavonoid content which is further confirmed from

quantitative analysis of different phytochemicals. Althoug it is well established from earlier studies that flavonoids may attribute for antioxidant activity in many plants, however it is not necessary that higher concentration of flavonoids in *F. bengalensis* are the soul contributor for their antioxidant activity. Since, some compounds though present in low quantity could contribute greatly for the antioxidant potential of particular plants. Therefore, the observed higher antioxidants activity in *F. bengalensis* is indeed due to their high flavonoid content need to be further investigated.

The preliminary phytochemical analysis of the selected plant indicated that in most of the plants mainly phenolic, flavonoids, glycosides, saponin and terpenoids were present in good amount. It is widely considered that plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators; therefore it was logical to determine their total amount in the selected plant extracts. On the basis of this preliminary study, total phenolic and sugar content of these selected fifteen 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 sugar content was measured spectrophotometrically and expressed in terms of mg GluEq per g extract. On the basis of phytochemical analysis *Ficus bengalensis* showed highest yield of total phenolic content (12.13 mg GAE per 0.1 g extract) in methanolic extract in comparison to the aqueous extract (1.02 mg GAE per 0.1 g extract) among all selected fifteen plants. In a number of earlier phytochemical studies, polar nature of compounds is reported from aqueous/methanolic extraction and our phytochemical study conforms to the same observations. In phytochemical study of several plant sources, there is assumption that most of polar extractable constituents may be found in water/methanol extraction (Harbonne, 1998).

3.3.2 Evaluation of antioxidant activity and phytochemical content from different parts of *F. bengalensis*.

Since the preliminary screening studies showed that *F. bengalensis* have strong antioxidant activity, further study was focused on this plant only. In order to select most effective plant part, the antioxidant activities of different parts of *F. bengalensis* were further investigated. The methanolic extracts of different part of *F. bengalensis* (leaf, aerial root, stem bark) were prepared as described earlier in material method section (3.2.2). Antioxidant activities of methanolic preparation of these parts were

determined using DPPH free radical scavenging assay described before and reducing assay. The antioxidant activity of different plant parts extract is summarized in Fig. 3.1 (a) and (b). The result clearly indicated that methanolic extracts of stem bark of *F. bengalensis* demonstrated maximum free radical scavenging activity (IC_{50}= 0.0335 mg per mL) in comparison to low scavenging activity of leaf (IC_{50}= 0.139 mg per mL) and aerial root (IC_{50}= 0.0467 mg per mL). The reducing potential of stem bark was also higher (1.41 ± 0.01) compared to leaf and root part at any given concentration. The findings from the present study support earlier traditional use of these plants for their bark derived medicine. The results also support the fact that during methanolic extraction, major phytochemical constituents suggested being polar in nature like flavonoids (Pietta, 2000).

Since flavonoids constitute one of the most diverse and widespread group among plant phenolics and possess a broad spectrum of chemical and biological activities including radical scavenging properties, therefore determination of their concentration in plant extract is also mandatory. Therefore, total flavonoid content along with phenolic and glycosides content of methanolic extracts of different parts of the plant were also determined. The result illustrated in Table 3.6 revealed that much variation occurred in phenolics, flavonoids and glycoside content of aerial root, stem bark and leaves of *Ficus bengalensis*. The stem bark of the plant found to have high flavonoid (143.68 ± 6.21) content compared with other parts of plants. As suggested previously that the high antioxidant activity of stem bark of *F. bengalensis* may be due to its high content of flavonoid. But that need to be confirmed from further experimental analysis. Flavonoids from several plants have been attributed for their antioxidant activity (Sharififar *et al.*, 2009; Mohanty *et al.*, 2009; Lazaro, 2009; Najda *et al.*, 2008) and it is likely that it may be responsible for antioxidant activity of *F. bengalensis* which need to be confirmed from further studies described later.

3.3.3 Total phenol, flavonoid and glycoside content and antioxidant activity of liquid liquid partitioned organic solvent fraction from *F. bengalensis*.

Since the bark was found to be the best source for antioxidant activity as compared to other parts. The bark extracts of the plant have been used for purification and analyzing the active constituents and evaluation of the antioxidant potential. Solvent extraction is frequently used for isolation of the antioxidants and both extraction yield and antioxidant activity of the extracts are strongly dependent on the

solvent, due to the different antioxidant potential of compounds with different polarity. For these reasons, comparative studies for selecting the optimal solvent providing maximum antioxidant activity are required for each substrate. After solvent fractionation as mentioned in material and method section, both aqueous and organic fractions were evaluated for their antioxidant activities. The schematic representation of liquid-liquid partitioning of methanolic extract of *F. bengalensis* is shown in Fig 3.2. The liquid liquid partitioning of 20 g of *F. bengalensis* methanolic extract resulted in following yield of partitioned fractions: hexane (2.42 g), dichloromethane (1.90 g), Ethylacetate (3.6 g) and aqueous fraction (11.9 g). Table 3.7 illustrates the total phenolic, flavonoid and glycosides content of different liquid-liquid partitioned fraction n-hexane, dichloromethane and ethylacetate fraction. Ethylacetate phase of partitioned fraction showed highest phytochemical constituents in comparison to other partitioned fractions. Ethylacetate phase from partitioned fraction of *F. bengalensis* extract demonstrated highest amount of flavonoid content (mg Rutin Eq per g extract, 142.68 ±7.32) followed by phenolic content (mg GAE per g extract, 115.92 ± 6.58) and glycoside content GEq per g extract (86.76 ± 4.78). Therefore, the phytochemical yield from *F. bengalensis* partitioned fractions clearly supports the earlier view that polar compounds are usually extracted in polar solvents (Rice and Evans, 2000).

The antioxidant potential of the various partitioned fractions were also determined by FRAP assay. Fig 3.2 depicts Ferric reducing antioxidant power (FRAP) activity of different partitioned fractions of *F. bengalensis* extract in terms of µmol Fe^{2+}/L. Ethylacetate fraction of *F. bengalensis* extract demonstrated highest reducing power in terms of µmol Fe^{2+}/L. Earlier studies pertaining to liquid- liquid partitioning experiments demonstrated that polar phase like ethylacetate fraction contained mostly polar water soluble phytochemicals like phenolics, flavonoids and glycosides (Senevirathne *et al.*, 2006; Tung *et al.*, 2007). Our results further support the earlier view.

3.3.4 Bioactivity guided fractionation of active constituents of Ethyl acetate fraction of *F. bengalensis* extract

Majority of the active antioxidant constituents were retained in the ethylacetate fraction from *F. bengalensis*. Since this fraction also showed highest flavonoid content

and the flavonoid compounds are reported to be contributing factor for the antioxidant activity in many plants (Abraham *et al.*, 2008; Wu *et al.*, 2008; Ahmad *et al.*, 2009). Therefore, an attempt was made to purify the active constituents, which are responsible for the antioxidant activity in *F. bengalensis* by bioactivity guided fractionation method. The ethylacetate fraction obtained from *F. bengalensis* was further fractionated on silica gel column chromatography as already described in material and methods. The result of fractionation is depicted in Fig 3.4. It was observed that most of the antioxidant activity was retained in the fraction 13-16th which also possessed maximum amount of flavonoid content. This clearly indicates the possibility of an existing correlation between antioxidant activity and flavonoid content. This fraction was called flavonoid enriched fraction of *F. bengalensis* (FFB). Our results are in agreement with earlier reports which have demonstrated the role of flavonoids as major contributors of antioxidant activity (Souza *et al.*, 2007; Kaviarasan *et al.*, 2008). Thus the flavonoids are the main constituents contributing for antioxidant activity in *F. bengalensis*. The antioxidant activity is described in terms of percentage DPPH free radical scavenging activity with 10 µg per mL fraction. Total flavonoid content were expressed in terms of µg Rutin Eq per 0.1 mg fraction amount. Results showed that pooled active fraction from 13-16th indicated maximum free radical scavenging activity expressed in terms of 62.04 % activity at concentration 10 µg per mL fraction. The total flavonoid content was found to be highest showing 78.72 µg Rutin Eq per 0.1 mg fraction. Our results are in consonance with earlier reports showing strong antioxidant activity of several plants with flavonoid compounds as major phytochemical constituents. In this study, the bioactivity guided fractionation of active constituents 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 flavonoid compounds, stationary phases of polyamide, cellulose, silica gel, and Sephadex LH-20 were mostly used by several phytochemical fractionation study (Vuckis *et al.*, 2008; Hostettman, 2004).

3.3.5 Analysis and identification of active constituents of FFB from *F. bengalensis*

The active fraction (FFB) obtained from *F. bengalensis* from silica gel column chromatography were analyzed for its constituents using various analytical techniques such as TLC (Thin Layer Chromatography), UV-VIS Spectrophotometry, FTIR (Fourier Transformation Infra Red), ESI-MS (Electo Spray Ionization–Mass Spectrometry) and NMR 1-D.

3.3.5.1 TLC analysis of FFB constituents

In order to identify the active components, TLC separation of FFB fraction from *F. bengalensis* was performed on silica gel 60 G_{254} plate using ethyl acetate: chloroform: formic acid (3:3:1) solvent system. The TLC result is shown in Fig. 3.5 TLC analysis resulted in their major constituents with Rf value 0.63, which have been identified as flavonoid compound (leucopelargonidin derivative) due to similar Rf values (Paris and Etchepare, 1967). Thus, it is clear that the antioxidant constituents of FFB from *F. bengalensis* are flavonoid components including leucopelargonidin derivative.

3.3.5.2 UV-VISIBLE Spectroscopy analysis of FFB

The UV-VIS spectrum of active fraction (FFB) is illustrated in Fig. 3.6 (a) and indicated the major peaks at 201 and 280 nm. The peak at 201 nm indicates the presence of glycosidic nature of compound on the basis of earlier study of glycoside constituents from plant sources, since it has been reported that major absorption for standard glycosides occurs in far UV-VIS range 200-220 nm (Devon *et al.*, 2001). The other peak observed at 280 nm in the Fig.3.6 (a) indicates the presence of flavonoid compound (leucopelargonidin). Our results are supported by matching the standard spectra of flavonoids and spectral report of other plant sources (Joslyn *et al.*, 1968). This is further confirmed from the fact that on hydrolysis of methanolic solution of sample with conc HCl, a broad peak at 531 nm was observed which corresponds to those of anthocyanins (Takizawa *et al.*, 1979).

3.3.5.3 FTIR characterization of FFB constituents

The FFB fraction was also analyzed by FTIR analytical technique. An infrared spectrum mainly shows the type of bonds and functional groups constituting the compounds. The FTIR spectrum of FFB constituents of *F. bengalensis* is shown in

Fig. 3.7. The major peak at 3376 cm-^{1} has been indicated and suggested to account for the hydroxyl (-OH-) group. In earlier reports showing FTIR characterization of phytochemical constituents, broad peak in the range of 3300-3400 cm^{-1} indicated the presence of hydroxyl groups (Takizawa *et al.*, 1979). A wavelength of 2949-2919 cm-^{1} and 1460-1326 cm-^{1} were also recorded for the FFB constituents which confirmed C-H bonds with methyl groups (-CH_3-) or methylene groups (-CH_2-) (Fasoyiro *et al.*, 2006). The peaks at 1611.72 cm-^{1} and 1519.54 cm-^{1} were consistent with the presence of carbonyl and aromatic ring respectively (Tachakittirungrod *et al.*, 2007). Since ortho and para substituted benzenes show one band each at 770-735 cm-^{1} and at 840-800 cm-^{1} respectively. Therefore, the two corresponding peaks at 819.92 cm-^{1} and 767.76 cm-^{1} are characteristic peak for substituted benzene rings (Huang *et al.*, 2004).

3.3.5.4 Direct infusion ESI-MS characterization of FFB constituents from F. bengalensis

The analysis of the FFB fraction from *F. bengalensis* was also done using ESI-MS technique. Fig. 3.8 depicts the direct infusion ESI-MS spectra of FFB fraction in negative ion mode and the scanning range was 200-1200 m/z. On the basis of fragmentation ion spectra analysis, mass ion at 255.15, 283.20, 341.02 and 451.03 m/z was observed. The spectral analysis on comparison with standard flavonoid compounds indicated the presence of chrysin, luteolin and aglycone and glycosylated forms of leucopelargonidin derivative, as major components in FFB fraction from *F. bengalensis*. 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.

3.3.5.5 Proton nuclear magnetic resonance (H^1 NMR)

The proton NMR spectra of FFB fraction from *F. bengalensis* is presented in Fig. 3.9. It showed different peaks at 2 ppm, 2.1 ppm and 2.3 ppm showing the presence of methyl group attached to aromatic structure. The presence of an aromatic ring structure implies the presence of a benzene ring structure (Fasoyiro *et al.*, 2006). Harborne (1984) reported the range of 6.6 - 8 ppm for structure of aromatic compounds with benzene ring attached to hydrogen atom (Ar-H). The proton resonances due to the rhamnose moiety appeared in the region 3.18- 5.30 ppm. The anomeric proton of the

rhamnose moiety was observed as a singlet at 5.30 ppm. This spectral data value is in consonance with previous report by Abou-Zaid and Nozzollilo (1999).

CONCLUSION

Among the fifteen plants used in the present study the methanolic extract of *F. bengalensis* stem bark demonstrated highest antioxidant activity. A flavonoid enriched fraction accounting for the antioxidant activity was obtained from methanolic extract of stem bark of *F. bengalensis* using liquid-liquid partitioning and column chromatography. Further analysis using various analytical tools revealed the presence of chrysin, luteolin and aglycone and glycosylated forms of leucopelargonidin derivative as major flavonoid constituents. This is the first report showing antioxidant potential of *F. bengalensis* and identification of active constituents.

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

S No	Local Name	Scientific Name	Family	Plant parts used
1	Nagar motha	*Cyperus rotundus L.*	Cyperaceae	Rhizome
2	Punarnava	*Boerhaavia diffusa Linn.*	Nyctaginaceae	leaf
3	Sariva	*Hemidesmus indicus L.*	Asclepiadaceae	Root
4	Kasondi	*Cassia occidentalis L.*	Fabaceae	Leaf, seed
5	Lata Kasturi	*Hibiscus abelmoschus L.*	Malvaceae	Seed
6	Kali draksha	*Vitis vinifera L.*	Vitaceae	Seed
7	Shatavari	*Asparagus racemosus L.*	Liliaceae	Root
8	Heeng	*Ferula asafoetida L.*	Umbelliferae	Leaf
9	Anjeer	*Ficus carica L.*	Moraceae	Fruit
10	Kapur kachrii	*Hedychium spicatum L.*	Zingiberaceae	Rhizome
11	Jyotismati	*Celastrus paniculatus L.*	Celastraceae	Leaf , stem, root
12	Vata	*Ficus bengalensis L.*	Moraceae	Leaf, stem bark, root
13	Pomegranate	*Punica granatum L.*	Saxifragaceae	Whole fruits
14	Apamarg	*Achyranthes aspera Linn.*	Amaranthaceae	Leaf
15	Beet	*Beta vulgaris L.*	Carecaceae	Whole fruits

Table 3.2 Total antioxidant activity of methanolic and aqueous extract of fifteen Indian dietary and medicinal plants.

Scientific Name	Methanol extract		Aqueous extract	
	DPPH IC_{50} (mg per mL)	PM (µmol AAE per g extract)	DPPH IC_{50} (mg per mL)	PM (µmol AAE per g extract)
Cyperus rotundus L.	0.051 ± 0.01	168.85± 8.1	0.054 ± 0.94	25.66 ± 0.79
Boerhaavia diffusa Linn.	0.047 ± 0.04	78.9 ± 3.45	0.490 ± 0.07	37.41 ± 0.81
Hemidesmus indicus L.	0.052 ± 0.02	136.12 ± 6.24	0.153 ± 0.03	124.03 ± 9.29
Cassia occidentalis L.	0.064 ± 0.03	182.18 ± 15.53	0.438 ± 0.06	138.61 ± 7.97
Hibiscus abelmoschus L.	0.027 ± 0.03	34.49 ± 2.43	0.263 ± 0.03	117.71 ± 11.03
Vitis vinifera L.	>2	135.38 ± 12.11	>2	193.29 ± 12.42
Asparagus racemosus L.	0.051 ± 0.11	112.97 ± 8.62	0.059 ± 0.03	80.63 ± 5.8
Ferula asafoetida L.	0.548 ± 0.06	52.3 ± 3.85	0.632 ± 0.04	34.51 ± 2.57
Ficus carica L.	>2	56.27 ± 1.07	> 2	13.96 ± 0.77
Hedychium spicatum L.	>2	180.85 ± 5.77	> 2	8.6 ± 0.71
Celastrus paniculatus L.	0.036 ± 0.12	135.1 ± 5.41	0.960 ± 0.06	17.8 ± 0.39
Ficus bengalensis L.	**0.0335 ± 0.01**	**194.8 ± 14.96**	> 2	181.26 ± 9.06
Punica granatum L.	0.267 ± 0.02	38.26 ± 0.97	> 2	21.71 ± 2.33
Acyranthes aspera Linn.	0.820 ± 0.06	24.61 ± 0.79	0.636 ± 0.03	29.06 ± 0.39
Beta vulgaris L.	>2	28.58 ± 1.04	> 2	27.5 ± 1.08

Results are mean of triplicate measurements ± SD.

Table 3.3 Preliminary phytochemical evaluation in aqueous extract of fifteen Indian dietary and medicinal plants.

Scientific Name	Phenolic test	Flavonoid test	Glycosides/ saponin test	Steroid/ terpenoids test	Alkaloid test
Cyperus rotundus L.	++	++	++	++ +	-
Boerhaavia diffusa Linn.	++	++	++	++	++
Hemidesmus indicus L.	++	++	+++	+++	-
Cassia occidentalis L.	++	+++	+++	++	-
Hibiscus abelmoschus L.	-	++	+++	-	-
Vitis vinifera L.	++	++	+++	-	-
Asparagus racemosus L.	++	-	-	-	++
Ferula asafoetida L.	++	-	-	+++	-
Ficus carica L.	++	+ + +	++	++	++
Hedychium spicatum L.	-	-	++	++	+++
Celastrus paniculatus L.	++	-	-	+++	+++
Ficus bengalensis L.	++	++ +	+++	++	-
Punica granatum L.	+++	-	++	-	-
Acyranthes aspera Linn.	-	-	++	++	++
Beta vulgaris L.	-	++	-	++	-

- Absent ++ Average +++ Good

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

Scientific Name	Phenolic test	Flavonoid test	Glycosides/ saponin test	Steroid/ terpenoids test	Alkaloid test
Cyperus rotundus L.	+++	++	++	+++	-
Boerhaavia diffusa Linn.	+++	++	++	++	++
Hemidesmus indicus L.	++	++	+++	+++	-
Cassia occidentalis L.	+++	+++	-	++	-
Hibiscus abelmoschus L.	-	++	-	-	-
Vitis vinifera L.	++	++	-	-	-
Asparagus racemosus L.	++	-	++	++	++
Ferula asafoetida L.	-	-	-	+++	-
Ficus carica L.	++	+ + +	++	++	++
Hedychium spicatum L.	-	-	++	++	+++
Celastrus paniculatus L.	++	++	-	+++	+++
Ficus benghalensis L.	+++	++ +	++	++	-
Punica granatum L.	++	-	++	-	-
Acyranthes aspera Linn.	-	-	++	++	++
Beta vulgaris L.	-	++	-	++	-

- Absent ++ Average +++ Good

Table 3.5 Total phenol and sugar content determination in aqueous and methanol extract of various plants.

Scientific Name	Aqueous extract		Methanol extract	
	Phenolic content (mg GAE per 0.1g extract)	Sugar (mg GluEq per 0.1g extract)	Phenolic content (mg GAE per 0.1g extract)	Sugar (mg GluEq per 0.1g extract)
Cyperus rotundus L.	1.67 ± 0.21	1.66 ± 0.12	5.19 ± 0.19	0.64 ± 0.01
Boerhaavia diffusa Linn.	1.59 ± 0.23	1.14 ± 0.08	4.63 ± 0.11	0.74 ± 0.02
Hemidesmus indicus L.	2.06 ± 0.22	3.19 ± 0.05	3.35 ± 0.07	0.31 ± 0.01
Cassia occidentalis L.	3.45 ± 0.16	3.71 ± 0.12	5.16 ± 0.34	0.46 ± 0.46
Hibiscus abelmoschus l.	2.09 ± 0.15	3.71 ± 0.24	9.45 ± 0.09	0.44 ± 0.03
Vitis vinifera L.	2.55 ± 0.25	3.69 ± 0.12	1.98 ± 0.17	0.63 ± 0.04
Asparagus racemosus L.	3.56 ± 0.15	0.45 ± 0.03	2.95 ± 0.19	0.93 ± 0.02
Ferula asafoetida L.	3.27 ± 0.17	0.49 ± 0.04	0.92 ± 0.09	0.70 ± 0.02
Ficus carica L.	2.56 ± 0.18	0.44 ± 0.03	2.24 ± 0.15	0.89 ± 0.04
Hedychium spicatum L.	1.45 ± 0.03	0.34 ± 0.05	4.65 ± 0.09	2.44 ± 0.28
Celastrus paniculatus L.	2.65 ± 0.16	0.45 ± 0.01	8.92 ± 0.16	0.66 ± 0.03
Ficus bengalensis L.	1.02 ± 0.04	1.33 ± 0.08	12.13 ± 1.90	1.65 ± 0.03
Punica granatum L.	5.89 ± 0.27	0.72 ± 0.03	6.34 ± 0.10	0.63 ± 0.02
Acyranthes aspera Linn.	0.12 ± 0.03	1.28 ± 0.08	0.79 ± 0.06	0.28 ± 0.03
Beta vulgaris L.	1.45 ± 0.14	0.29 ± 0.03	1.84 ± 0.09	0.34 ± 0.02

Results are mean of triplicate measurements ± SD.

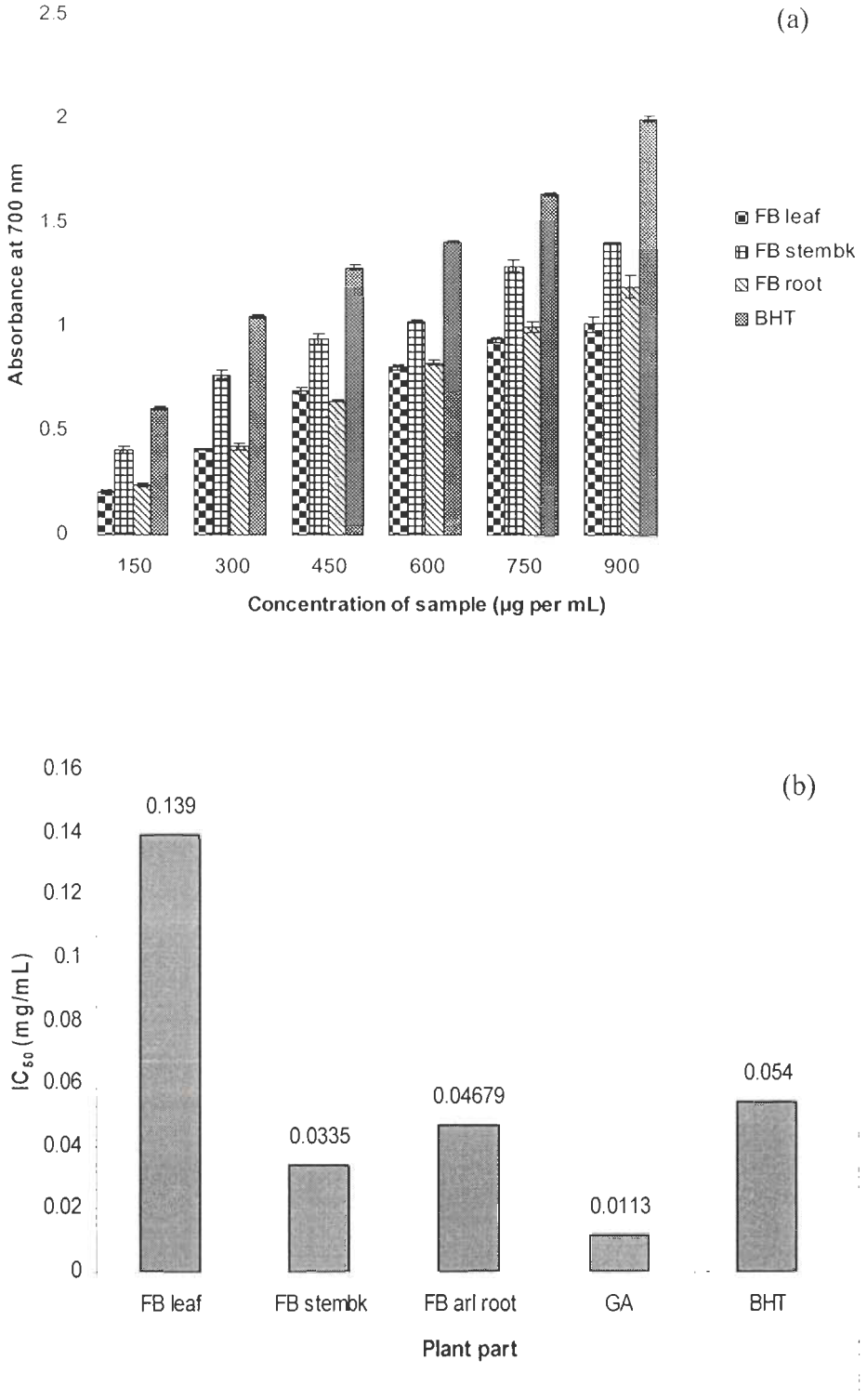


Fig. 3.1 Antioxidant activity of different parts of *F. bengalensis* (a) Reducing power (b) Free radical scavenging (DPPH scavenging) activity of different parts of *Ficus bengalensis*.

Table 3.6 Total phenol, total flavonoids and total glycosides content of different plant parts extract from *F. bengalensis*

Sample	**Methanolic extracts**		
	Phenolic (mg GAE per g)	Flavonoids (mg Rutin Eq per g extract	Glycosides mg GEq per g extract
	Ficus bengalensis plant parts		
Root	51.12 ± 1.93	36.76 ± 1.32	56.87 ± 2.65
***Bark	121.16 ± 5.28	**143.68 ± 6.21**	94.38 ± 3.60
Leaves	116.43 ± 6.38	56.36 ± 2.27	76.42 ± 3.84

Results are mean of triplicate measurements ± SD.

Table 3.7 Total phenol, flavonoids and glycosides content of different organic solvent fraction from *F. bengalensis* crude methanolic extract.

Name of sample	Different organic solvent fraction from CFBS extract.		
	Hexane	Dichloromethane	Ethyl acetate
Total phenol (mg GAE per g extract)	33.12 ± 1.57	31.58 ± 1.21	115.92 ± 6.58
Total flavonoids (mg Rutin Eq per g extract)	25.38 ± 0.95	16.29 ± 0.57	142.68 ± 7.32
Total glycosides (mg GEq per g extract)	4.79 ± 2.75	15.38 ± 0.19	86.76 ± 4.78

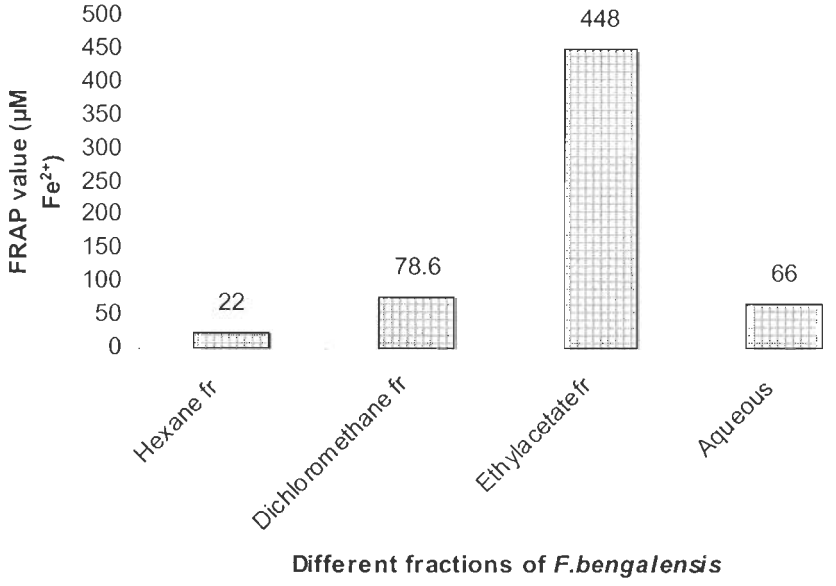


Fig. 3.2 Ferric reducing antioxidant power (FRAP) of liquid-liquid partitioned different fractions from *F. bengalensis extracts* at 20 µg/mL. The FRAP value is expressed in terms of µM Fe^{2+}.

Stem bark powder (250 g) from *F. bengalensis* was extracted with methanol (2.5 Liters) by maceration at room temperature for three days.

⇩

The crude methanolic extract vaccum dried powder (20 g) was redissolved in 400ml methanol/ water mixture (40:60) and partitioned into different fraction for further fractionation.

⇩

Liquid-liquid partitioning for fractionation into different organic solvents with their increasing polarity with (v/v 1:1) ratio

⇩

Partitioned organic solvents fractions were evaporated at 50°C with the help of rotary evaporator to get fraction extract powder

⇩

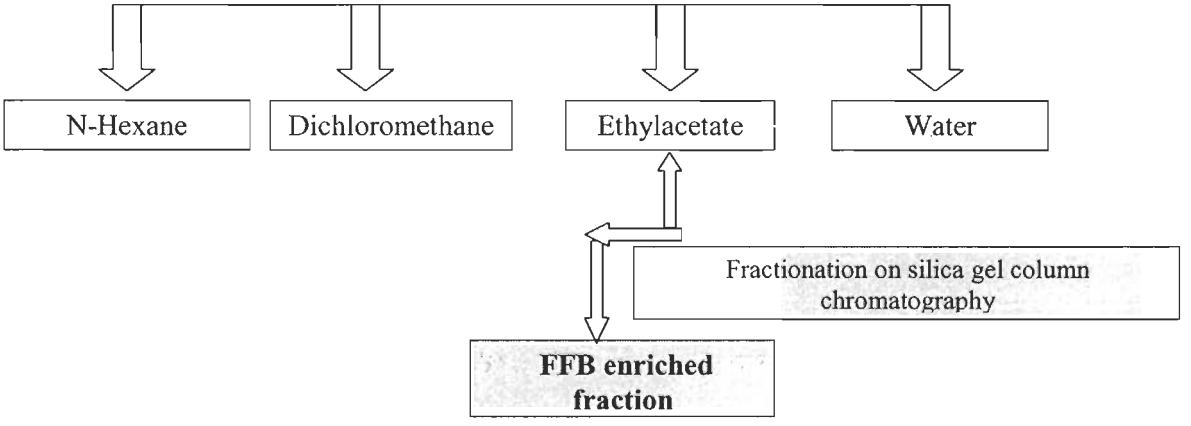


Fig 3.3 Schematic flow diagram for bioactivity guided liquid-liquid partitioning and fractionation of enriched fraction from *F. bengalensis* stem bark.

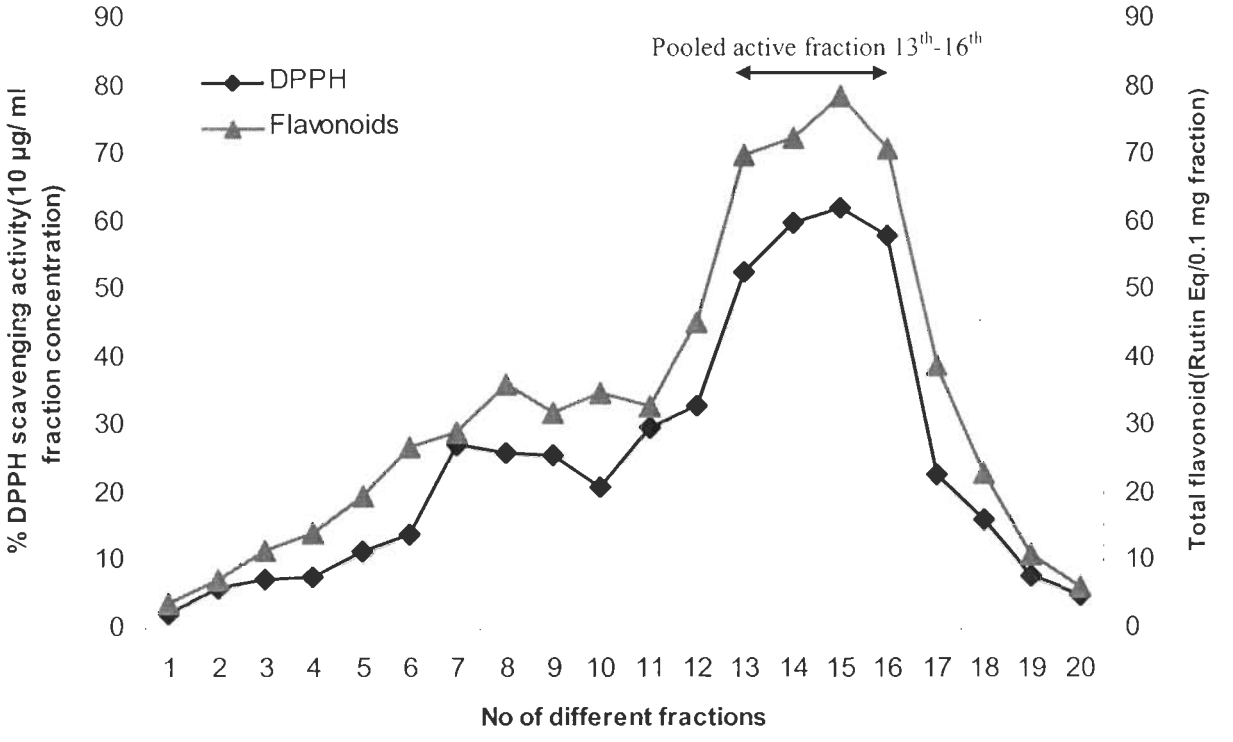


Fig. 3.4 Illustrates the bioactivity guided fractionation of flavonoid enriched fraction from stem bark of *F. bengalensis*

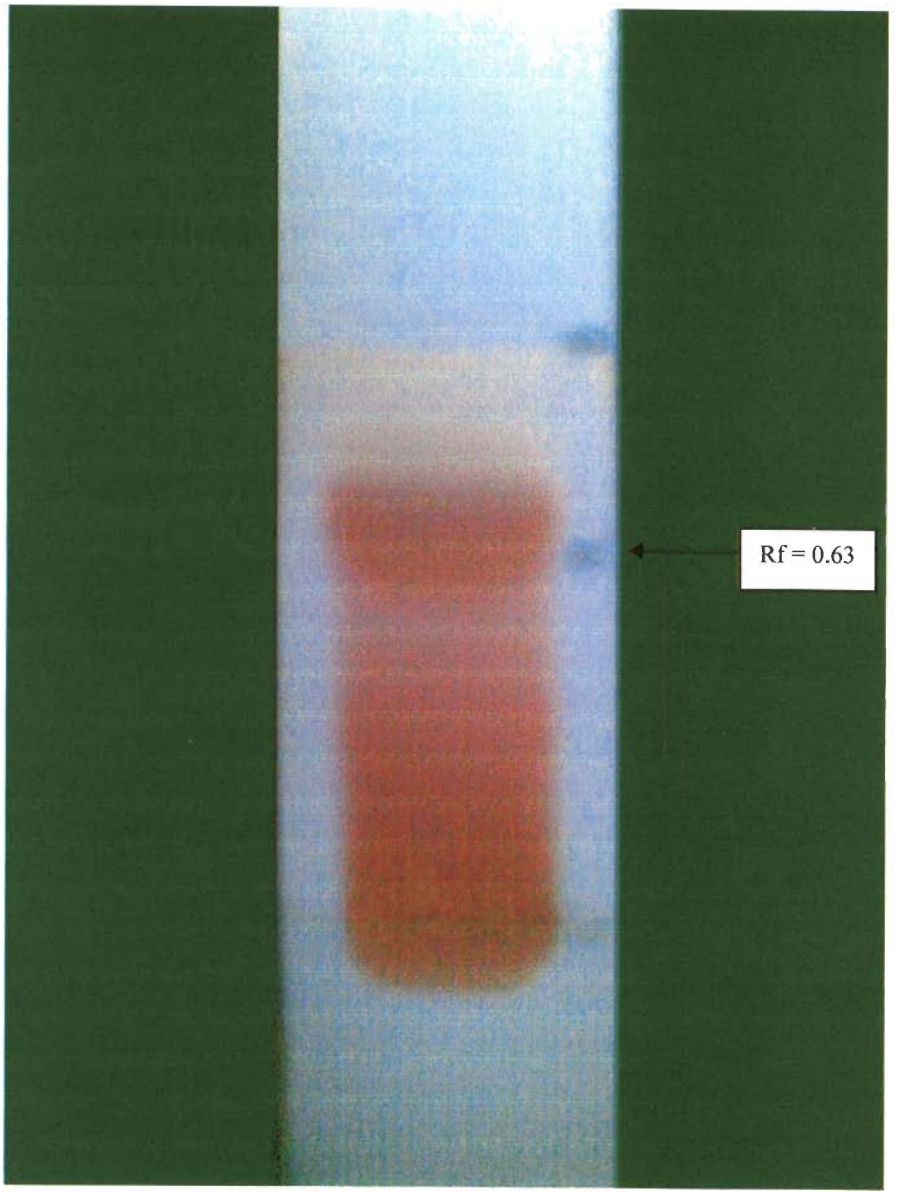


Fig 3.5 TLC profile of FFB fraction from *F. bengalensis.* Developing solvent: Ethyl acetate-$CHCl_3$-HCOOH (3 : 3 : 1)

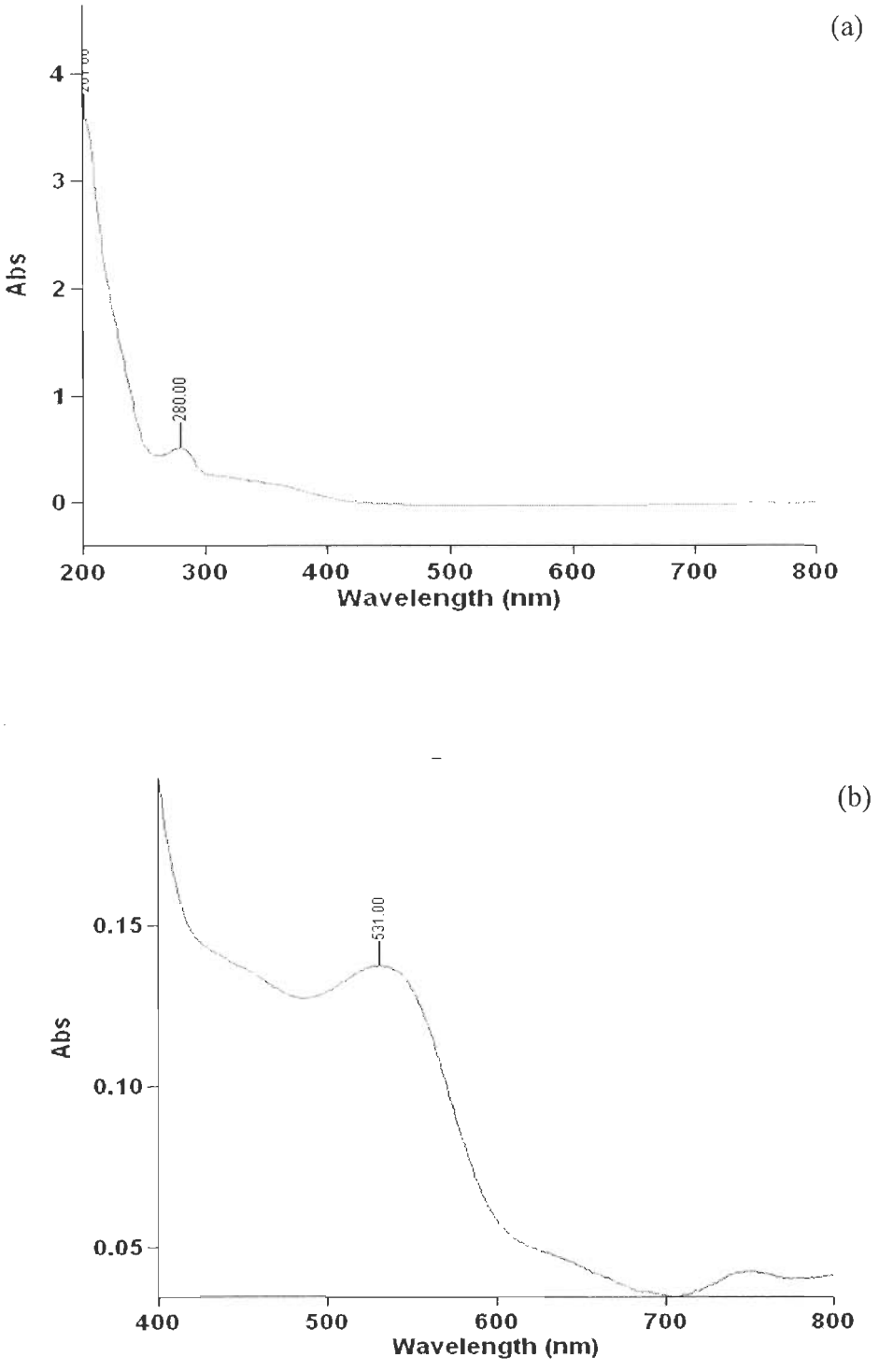


Fig. 3.6 (a) and (b) UV-VISIBLE spectra of FFB from *F. bengalensis.*

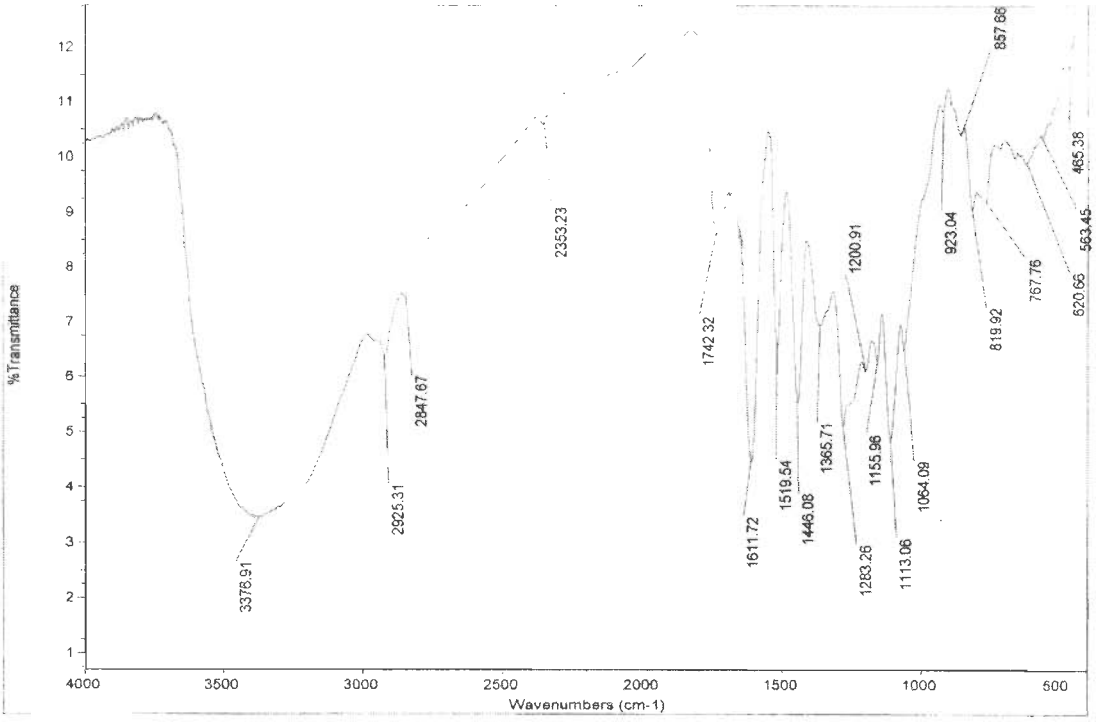


Fig 3.7 Shows the FTIR spectra of FFB constituents from *F. bengalensis.*

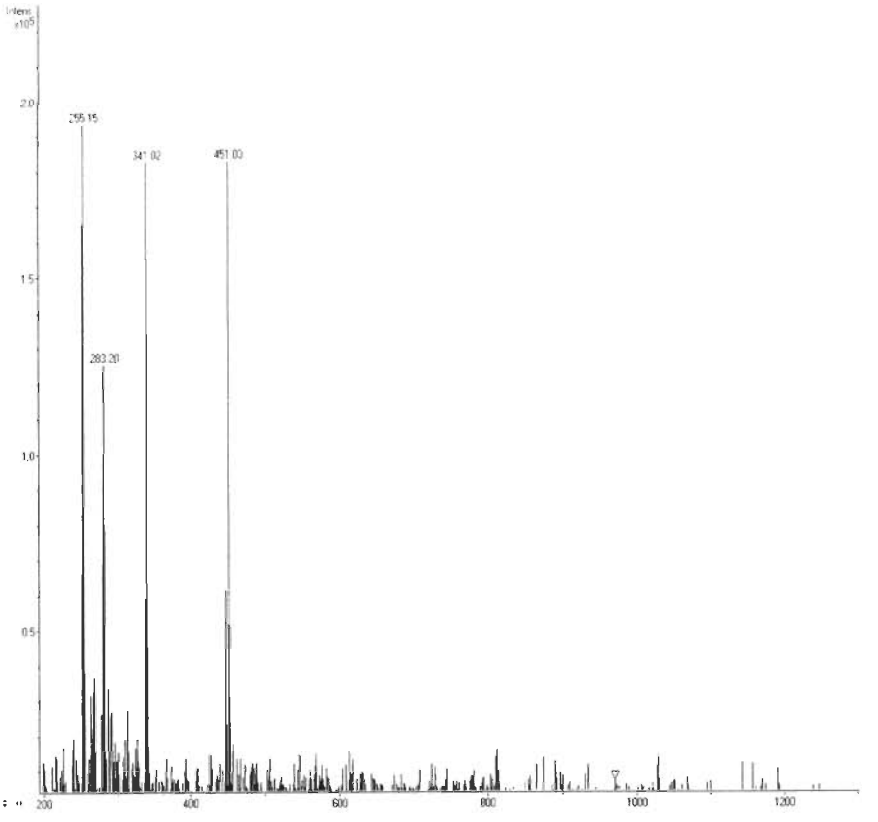


Fig. 3.8 Direct infusion ESI-MS spectra of FFB constituents in negative ion mode and the scanning range was 200-1200 m/z. [M-H]

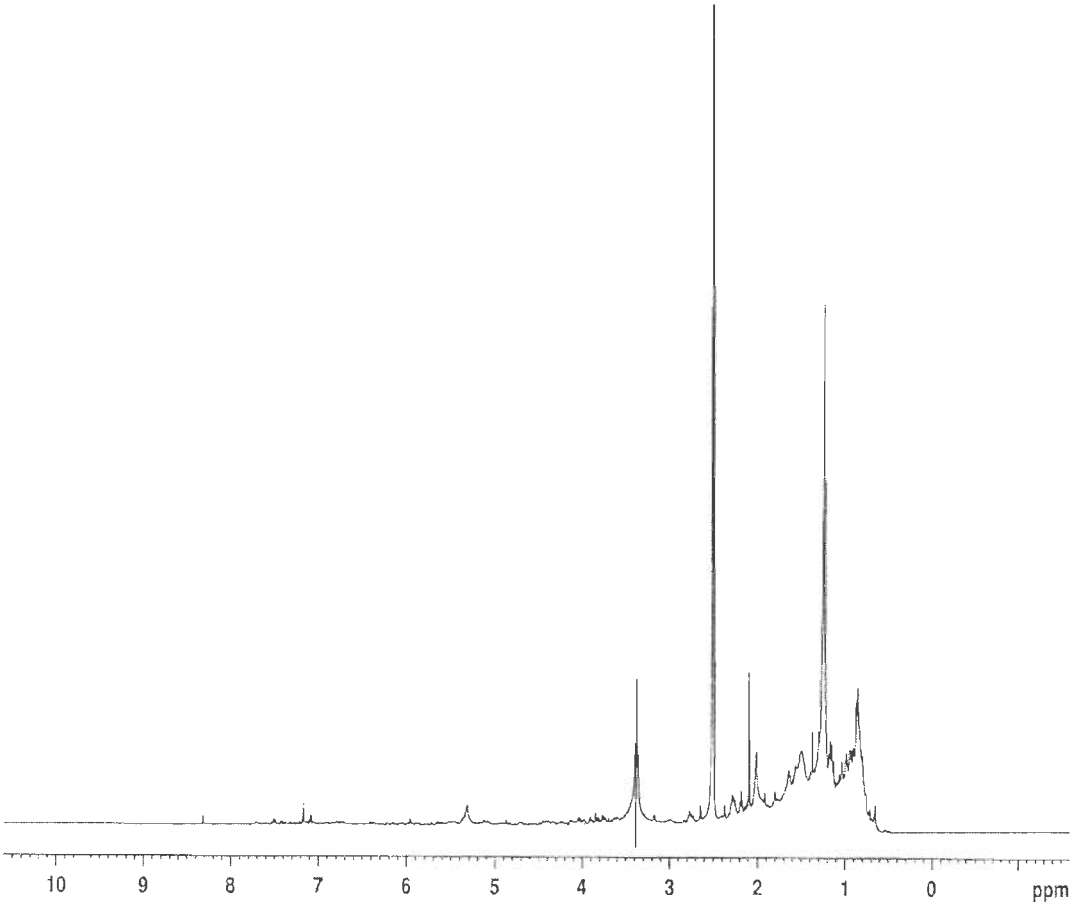


Fig 3.9 Proton Nuclear Magnetic Resonance (H^1 NMR) spectra of FFB constituents from *F. bengalensis*

Chapter 4

Evaluation of antioxidant potential and free radical scavenging activity of crude (CFBS) and flavonoid enriched fraction (FFB) from *F. bengalensis*.

4.1 INTRODUCTION

Reactive oxygen species (ROS) mediated oxidative stress plays a significant role in the pathogenesis of numerous diseases like cancer, diabetes, atherosclerosis, aging, rheumatoid arthritis etc (Pham-Huy *et al.,* 2008; Moussa, 2008). ROS vary widely in nature and act differently to mediate immediate damage to target biomolecules like nucleic acids, lipids and proteins (Bandyopadhyay *et al.,* 1999; Halliwell, 2007). It is evident that the supplementation of dietary antioxidants may be useful in preventing the deleterious consequences of oxidative stress (Lugasi *et al.,* 2003; Frei, 2004; Meydani, 2006), due to inefficiency of our own endogenous defense systems and the existence of some physio-pathological situations (cigarette smoke, air pollutants, UV radiation, high polyunsaturated fatty acid diet, inflammation, ischemia/reperfusion, etc.) resulting in unregulated production of ROS.

Owing to the diverse nature of antioxidants and their varying mechanism of action towards free radicals, the evaluation of antioxidant properties may not be an easy task. The potential antioxidants have been suggested to counteract differently with a particular nature of compounds. Therefore, no single assay will accurately reflect all antioxidants in a mixed or complex system. Therefore, it is emphasized to perform several antioxidant capacity assays to fully elucidate a full profile of antioxidant capacity since multiple reaction characteristics and mechanisms are usually involved in them (Benzie, 2003; Li *et al.*, 2008). A number of sensitive *in vitro* systems have been developed which are easier, faster and more cost effective compared to traditional *in vivo* bioassays (Mello *et al.,* 2006; Apak *et al.,* 2007). Therefore, direct testing of antioxidant activity *in vitro* is more logical because a substance with low activity *in vitro* conditions will probably not be effective *in vivo* (Frei, 1999). Besides, demonstrating antioxidant efficacy *in vivo* requires use of valid *in vivo* models. Moreover, results from the *in vitro* methods can enable assessment of potential *in vivo* efficacy. In due course of scientific development, several reliable *in vitro* antioxidant methods for measuring antioxidant activity of food, beverages and biological samples have been developed on the basis of their diversity, functionality, nature and potential to counteract different free radicals (Pellegrini *et al.,* 2003; Bompadre *et al.,* 2004). Thus in order to establish the antioxidant efficacy of a group of similar nature of compounds, we need to evaluate its oxidant potential using a battery of *in vitro* antioxidant assays involving different mechanisms including hydrogen donation ability,

chain breaking property, metal chelation and modulators of innate antioxidant defense system (Sanchej-Marino, 2002). The preliminary study regarding the antioxidant potential of CFBS and FFB constituents of *F. bengalensis* stem bark methanolic extract was performed earlier (Chapter-3) using a limited assay system. In present study the evaluation of antioxidant potential of CFBS and FFB constituents have been done using a battery of antioxidant assay system in order to elucidate their full profile of antioxidant capacities.

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. 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) were purchased from Sigma Chemical Co. (St. Louis MO, USA) and MERCK Co. (Darmstadt, Germany) unless otherwise mentioned.

4.2.2 Evaluation of *in vitro* antioxidant activity of (CFBS and FFB) from *F. bengalensis*

The *in vitro* antioxidant potential of CFBS and FFB from *F. bengalensis* were evaluated using a battery of *in vitro* based assays and is described below separately.

4.2.2.1 Ferric Reducing Antioxidant Power Assay (FRAP assay) determination of (CFBS and FFB) from F. bengalensis

The FRAP assay was carried out according to the procedure of Benzie and Strain (1996) with slight modifications. The FRAP reagent was prepared by mixing acetate buffer, 300 mmol/L (pH 3.6), 10 mmol /L TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mmol/L HCl and 20 mmol/L ferric chloride solution in proportion of 10:1:1 (v/v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37°C for an hour in a water bath prior to use. 50 µL of antioxidant sample (CFBS and FFB) with varied concentration (0.02, 0.04, 0.06, 0.08 0.1 mg per mL) were added to 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was then measured at 593 nm after 5 min against a reagent blank. The calibration curve was

prepared using iron (II) sulphate solution with concentration range (200μM-1400μM). The FRAP value of antioxidant samples was expressed in terms of μmol Fe (II)/ L.

4.2.2.2 Reducing capacity assessment of (CFBS and FFB) from F. bengalensis

The reducing capacity of the extract was assessed by method of Oyaizu (1986). Different concentration of both CFBS and FFB (150-900 μg) in 1 mL of distilled water was mixed with phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 10 g per L). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 mL, 100 g per L) was added to the mixture, which was then centrifuged at 3000 x rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1 g/L). The absorbance was measured at 700 nm. Butylated hydroxytoluene and ascorbic acid were used as standards.

4.2.2.3 Hydroxyl radical scavenging potential of CFBS and FFB from F. bengalensis

The hydroxyl radical scavenging activity of CFBS and FFB was measured by deoxyribose method. 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 100 mM KH_2PO_4-KOH, pH 7.4, 10 mM $FeCl_3$, 10 mM H_2O_2, 1mM ascorbic acid, 1 mM EDTA and 10 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 an hour pre-incubation at 37°C with varied concentration of antioxidant samples: FFB (25, 50, 75 and 100 μg per mL) and CFBS (50, 100, 150, 200 and 250 μg per mL) in final volume of reaction mixture. Mixture was heated at 80°C for 30 min with 1 mL of 2-TBA (0.5% 2-TBA in 0.025 M NaOH) and 1 mL of 10% trichloroacetic acid (TCA) in a water bath for 45 min. After cooling, absorbance of the mixture was measured at 532 nm. Along with antioxidant constituents, butylated hydroxyl toluene was used as positive standard.

4.2.2.4 Metal chelating potential of CFBS and FFB from F. bengalensis

The metal chelating effect of CFBS and FFB was determined by the ferrous ion chelating assay as per the modified method of Decker and Welch (1990). In brief, the reaction mixture contained 1 mL of antioxidant sample with varied concentration (20, 40, 60, 80, 100 and 120 μg per mL), 3.70 mL of deionized water, 0.1 mL of 2 mM

$FeCl_2$. After 30 seconds, 200 µL of 5 mM ferrozine was added to activate the reaction mixture. The reaction mixture was incubated at 25°C for 10 min, and its chelating activity was measured at 562 nm. Ethylenediaminetetracetic acid (EDTA) and Butylated hydroxyanisole (BHA) were used as positive standard. The metal chelating activity was calculated by using the following equation:

Chelating rate % = (1- Absorbance of sample at 562 nm/ Absorbance of control at 562 nm) x 100.

4.2.2.5 Free radical scavenging activity of CFBS and FFB from F. bengalensis

The free radical $ABTS^{*+}$ (2, 2'-azinobis 3-ethyl benzothiazoline-6-sulfonic acid) scavenging assay was performed according to standard protocol (Re *et al.*, 1999) with slight modifications. The free radical 2, 2'-azinobis 3-ethyl benzothiazoline-6-sulfonic acid ($ABTS^{*+}$) was produced by reaction of ABTS solution (7 mM) with potassium persulphate (2.45 mM) and mixture was allowed to stand in dark for 12-16 hours before use. For aqueous extract ABTS solution was diluted with PBS (pH 7.4) to an absorbance of 0.700 ± 0.002 at 734 nm and for methanolic extract diluted with ethanol. Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid), a vitamin E analogue was used as standard for the preparation of calibration curve. The free radical scavenging activity was expressed in terms of mmol TEAC (Trolox equivalent antioxidant capacity). Free radical scavenging activity was also performed by DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay according to method described by (Brand-Williams, 1995). 100 µL aliquots of both CFBS and FFB of varied concentration were mixed with 3.9 mL methanolic/ethanolic DPPH solution (6×10^{-5} M). Absorbance was measured at 517 nm. The standard gallic acid was used as positive control against DPPH free radical scavenging assay. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect \%} = (A_0 - A_1/A_0) \times 100$$

Symbol A_0 indicate the absorbance of the control reaction and A_1 is the absorbance in the presence of antioxidant samples.

4.2.2.6 Superoxide radical scavenging potential determination of CFBS and FFB from F. bengalensis.

The super oxide radical scavenging activity was determined by the method described by Nishimiki (1972) with slight modification. Solutions containing 1 mL of 156 µM NBT (Nitro blue tetrazolium) dissolved in 100 mM phosphate buffer, pH 7.4, 1 mL of 468 µM NADH in 100 mM phosphate buffer (pH 7.4) and various concentration (20, 40, 60, 80 and 100 µg per mL) of antioxidant samples (CFBS and FFB) were mixed and the reaction was started by adding 100 µL of 60 µM PMS (Phenazine methosulfate) solution. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was measured against control samples (without NADH). Butylated hydroxytoluene and quercetin were used as positive control in this assay.
The superoxide scavenging activity (%) was calculated as follows:

Scavenging activity = [1-(absorbance of sample at 560 nm) / (absorbance of control at 560 nm)] × 100.

4.2.2.7 Evaluation of total antioxidant capacity of CFBS and FFB from F. bengalensis by phosphomolybdenum assay

Total antioxidant power of the extracts was assessed with the phosphomolybdenum reduction assay, as per method (Prieto *et al.,* 1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate / Mo (V) complex at acidic pH. Three mL reagent solution containing ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (600 mM) was mixed with 0.3 mL of both CFBS and FFB constituents diluted in methanol at varied concentration (25, 50, 100, 250 and 500 µg per mL) respectively. The samples were incubated for 60 min at 37°C and 90°C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. For reference, the appropriate solutions of ascorbic acid have been used.

4.2.3 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

In the present study, an attempt have been made to validate the antioxidant potential of CFBS and FFB from *F. bengalensis* stem bark by employing a battery of *in vitro* based biochemical assays towards the different nature of free radicals generated *in vitro* and *in vivo* system. The earlier reports support the logic to involve different antioxidant assays to validate the reaction properties of varied class of phytochemicals available from plant sources (Rice-Evans, 2000).

4.3.1 Ferric reducing antioxidant power (FRAP) determination of (CFBS, FFB) from *F. bengalensis*

Ferric reducing antioxidant power of antioxidant constituents of CFBS and FFB from *F. bengalensis* is shown in Fig. 4.1(a). The FRAP value is expressed in terms of µmol Fe II / L, which is calculated with the help of ferrous ammonium sulphate calibration curve depicted in Fig.4.1(b). The results suggest that fractionated FFB possessed almost two fold increased reducing power with respect to their crude extract CFBS. From these results, it can be concluded that both constituents have enough antioxidant potential in terms of reducing potential. The FFB was found to be better than the standard antioxidant ascorbic acid.

4.3.2 Test for ferric ion reducing capacity of CFBS, FFB from *F. bengalensis*

For the measurement of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in presence of CFBS and FFB from *F. bengalensis* stem bark. The results are shown in Fig 4.2. An increase in the reducing power of CFBS, FFB and the reference compounds ascorbic acid and butylated hydroxytoluene was observed with increasing concentration. The reducing power increased trend was in the following order CFBS < BHT < Ascorbic acid < FFB. At 0.9 mg/ mL concentration, the absorbance values of CFBS, FFB, butylated hydroxyl toluene and ascorbic acid at 700 nm, were 1.29 ± 0.012, 2.9 ± 0.01, 2 ± 0.013 and 2.7 ± 0.058, respectively. It is clear

from the result that the reductive capability of the FFB was comparable to ascorbic acid and was better than butylated hydroxytoluene.

4.3.3 Free radical scavenging activity of CFBS, FFB from *F. bengalensis* using DPPH and ABTS free radical scavenging assay

The free radical scavenging activities of CFBS and FFB from *F. bengalensis* were evaluated using DPPH and ABTS assays and the result is illustrated in Table 4.1. The calibration curve for standard antioxidant gallic acid against DPPH free radical and TEAC standard curve for ABTS assay are depicted in Fig. 4.3 (a) and Fig. 4.3 (b), respectively. The free radical scavenging activity against DPPH free radical was expressed in terms of IC_{50} concentration (µg per mL). The results shown in Table 4.1 clearly indicate that FFB has strong free radical scavenging activity with IC_{50} value summarized in decreasing order CFBS (2.73 ±0.143) < FFB (1.69±0.131) < Gallic acid (1.19 ± 0.10). The result clearly indicate that fractionated antioxidant constituent FFB possessed almost two fold strong free radical scavenging activity against DPPH free radical in comparison to CFBS. The IC_{50} value of FFB constituents was found to be comparable with standard gallic acid compound.

The free radical scavenging activity against ABTS free radical is expressed in terms of TEAC value (the TEAC value represents the concentration of a Trolox solution that has the same antioxidant capacity as the extract). The TEAC values for various samples were found to be in following increasing order CFBS (0.625±0.06) < FFB (1.80±0.10) < Gallic acid (2.23±0.19) (Table 4.1). The higher TEAC value for the FFB compared to CFBS clearly indicates that its constituents attributes for the antioxidant activity.

4.3.4 Hydroxyl radical scavenging potential of CFBS and FFB from *F. bengalensis*

The hydroxyl radicals generated from the Fe^{3+}–ascorbate–H_2O_2 system were detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA at low pH form a pink chromogen. The hydroxyl radical scavenging activity of CFBS and FFB from *F. bengalensis* is illustrated in Table 4.2. 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) indicate higher efficiency to scavenge hydroxyl radicals. The inhibitory concentration values in decreasing order for antioxidant constituents were CFBS (158.54 ± 4.29) < FFB (46.29 ± 2.821) < Butylated hydroxyl toluene (26 ± 1.13).

4.3.5 Metal chelating potential of CFBS and FFB from *F. bengalensis*

The ferrous ion metal chelating potential of CFBS and FFB from *F. bengalensis* were evaluated by ferrozine assay that involves spectrophotometric determination of ferrozine-metal complex formation with absorbance maxima at 512 nm. The results are shown in Fig 4.4. The metal chelating efficiency was converted to percentage metal chelating potential. EDTA and BHA were used as reference metal chelators in this experiment to compare with the metal chelating efficiency of the antioxidant constituents. As shown in Fig. 4.4, the formation of the Fe^{2+}-ferrozine complex was not completed and reduction in the absorbance occurs in the presence of CFBS and FFB constituents along with reference standard, indicating that the extracts have chelating activity and capture ferrous ion. The metal chelating efficiency in terms of inhibitory concentration IC_{50} was calculated from the analysis of this concentration dependent metal chelation graph. The metal chelating effect decreased in the order of EDTA (IC_{50}) > FFB (IC_{50}) > BHA (IC_{50}) > CFBS (IC_{50}).

4.3.6 Superoxide radical scavenging activity of CFBS and FFB from *F. bengalensis*

The superoxide radical scavenging potential of CFBS and FFB from *F. bengalensis* is depicted in Fig. 4.5(a). A concentration dependent increase in superoxide scavenging activity was observed for all the samples tested. At 100 µg per mL, FFB exhibited the strongest superoxide scavenging activity with value of 71.28 ± 0.882 %, which is comparable to that of Quercetin which exhibited 72.9 ± 0.79 % scavenging activity at the same concentration. The superoxide scavenging activity of FFB was much better than the crude preparation CFBS. The superoxide radical scavenging potential is also presented in terms of IC_{50} values (Fig 4.5b) and results are shown in decreasing order CFBS (90.12 ± 1.837) < FFB (31.39 ± 1.328) < Quercetin (14.46 ± 0.562) < Butylated hydroxyl toluene (10.12 ± 1.34) µg per mL.

4.3.7 Total antioxidant activity determination of CFBS and FFB from *F. bengalensis*

Total antioxidant capacity of CFBS and FFB constituents was evaluated using phosphomolybdenum assay and data is presented in Fig. 4.6 (a) and 4.6 (b), respectively. Ascorbic acid was used as reference compound. The spectrophotometer absorbance was recorded at 695 nm. The data analysis showed that concentration dependent increase (25-500 µg per mL) in absorbance was observed. All samples (FFB, CFBS and Ascorbic acid) exhibited dose dependent antioxidant activity at 37°C and 90°C. The activity as measured by the intensity of the absorbance at 250 and 500 µg per mL concentrations was maximum for FFB (0.7623 ± 0.008 and 1.5248 ± 0.03, respectively) at 90°C incubation temperature compared to ascorbic acid (0.7018 ± 0.03 and 1.3237 ± 0.028, respectively). However for all other tested concentrations ascorbic acid showed maximum activity at both incubation temperatures followed by FFB and CFBS respectively.

4.4 DISCUSSION

The previous chapter described and discussed about the antioxidant potential of *F. bengalensis* bark methanolic extract. The active fraction from this plant was purified by liquid-liquid partitioning and column chromatography. The crude and the active fraction obtained after bioactivity guided partitioning were named as CFBS and FFB, respectively. The major constituents of the fraction were also identified as flavonoids (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; Rajasekaran *et al.,* 2008; Yang *et al.,* 2008). Although, the medicinal potential and traditional uses of *F. bengalensis* has been reported (Mukherjee *et al.,* 1998; Aswar *et al.,* 2008) but their antioxidant potential have not been reported so far. Therefore, antioxidant efficacy of the CFBS (crude) and FFB (flavonoid enriched fraction) from *F. bengalensis* have been evaluated in the present study using various established and commonly used *in vitro* assay methods.

The antioxidant potential of CFBS and FFB fraction in the present study was determined by their reducing potential using FRAP assay. FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+} TPTZ)

complex and producing a blue coloured ferrous tripyridyltriazine (Fe^{2+}–TPTZ) with an absorption maximum at 593 nm (Benzie and Strain, 1996, 1999). Because the ferric-to-ferrous iron reduction occurs rapidly with all reductants with half reaction reduction potential 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 has been suggested to be more reliable and effective (Benzie and Strain, 1999, Blomhoff *et al.*, 2006).

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 (Pin-Der-Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990; Salah *et al.*, 1995; Duh *et al.*, 1999). 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).

In the present study the reducing power of FFB was found to be more potent than the standard synthetic antioxidants like butylated hydroxyl toluene and ascorbic acid. Our data on the reducing power of FFB suggest that it is likely to contribute significantly towards the observed antioxidant effect. The reducing capacity of CFBS and FFB might be due to presence of compounds with di and monohydroxy substitutions in the aromatic ring which is evident from the identification of the active

constituents, which possess potent hydrogen donating abilities as suggested in earlier studies (Shimada *et al.*,1992; Pin-Der Duh, 1998).

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 (Siddhuraju, 2006; Elmastas *et al.*, 2006). Our data are in well agreement with earlier observation as FFB showed strong free radical scavenging activity and high reducing power as well. The activity is attributed to their flavonoid constituents. The flavonoid hydroxyl groups that serve as plant antioxidants have lower redox potential which enable them to act as a reducing agent and a hydrogen donor in the given assay (Pietta, 2000). The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity. Reducing power assay has also been used to evaluate the ability of natural antioxidants to donate electrons. This observation is in agreement with 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. In number of plants the high reducing power observed are linked to their phenolics (Hajimahmoodi *et al.*, 2008; Nayaka *et al.*, 2008), flavonoids (Moein *et al.*, 2008) and glycosidic nature of compounds (Hsue *et al.*, 2008).

The antioxidant potential of CFBS and FFB was also evaluated by their free radicals scavenging ability. There are several mechanisms by which antioxidants can act; one of them is by scavenging of reactive oxygen and nitrogen free radicals. The $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals are the two most widely used and stable chromogen compounds to measure the antioxidant activity of biological material. The finding indicated that fractionated FFB constituents demonstrated strong antioxidant activity by scavenging both the $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ free radical.

The ability of FFB constituents to donate hydrogen atom or reduce free radicals as reflected from the results can be well interpreted based on remarkable antioxidant activity as referenced from earlier studies (Brand-Williams *et al.*, 1995; Sanchej Merino *et al.*, 2000).

In order to explain the antioxidant activity of extracts further, the total free radical scavenging potential was estimated by determining their efficiency to scavenge DPPH free radical. The DPPH is stable free radical at room temperature, which produces a purple colour solution in methanol. It is reduced in the presence of an

antioxidant molecule, giving rise to a stable diamagnetic molecule (yellow-coloured diphenyl picrylhydrazine) that shows strong absorption maxima at 517 nm. Any molecule that can transfer electrons or hydrogen atoms to DPPH can react with it and reduce the absorbance at 517 nm (Fogliano *et al.*, 1999). The degree of discoloration indicated the scavenging potential of the antioxidant compounds or extracts in the term of hydrogen donating ability (Mosquera *et al.*, 2007). It is suggested that antioxidative activity of flavonoids are related to their conjugated rings and hydroxyl groups (Pietta, 2000), so it is not surprising that CFBS and FFB constituents demonstrated strong free radical scavenging activity in the present study.

The TEAC assay was reported by Re *et al.* (1999) based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\cdot+}$) compared with a reference antioxidant standard (Trolox). High TEAC value indicates that the mechanism of antioxidant action of extracts was as a hydrogen donor and it could terminate the oxidation process by converting free radicals to the stable forms. The measurement of radical scavenging activity of any antioxidant is commonly associated with the use of DPPH method because it is quick, reliable and reproducible method to search the *in vitro* general antioxidant potential of pure compounds as well as plant extracts (Koleva *et al.*, 2002; Mosquera *et al.*, 2007). Wang *et al.*, 1998 found that some compounds which have $ABTS^{\cdot+}$ scavenging activity did not show DPPH scavenging activity. Therefore, a correlation between these two models may not be obvious, among biological samples containing a variety of antioxidants. However, in present study FFB showed high free radical scavenging activity in both assays. Thus it has constituents which have ability to scavenge both free radicals which also indicates its strong antioxidant potential.

Hydroxyl radical is biologically relevant and most reactive oxygen species among all ROS, owing to its strong ability to react with variety of biomolecules, such as polyunsaturated fatty acids, proteins, carbohydrates and DNA (Halliwell *et al.*, 1992). Hydroxyl radicals react with these biologically active molecules by hydrogen withdrawal, double bond addition, electron transfer and radical formation, and initiates auto-oxidation, polymerization and fragmentation (Liu and Ng, 2000). Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from phospholipid membranes, and thus bring about peroxidic reactions of lipids. Therefore, the higher hydroxyl scavenging activity shown by FFB, can be used to minimize the adverse effects from the hydroxyl radical (Lopes *et al.*, 1999). This view is further supported by

the fact that both CFBS and FFB are potential iron chelators. The iron (II) chelating properties of the antioxidant extract may be attributed to their endogenous chelating agents, like the flavonoids (Acker *et al.*, 1998). The results of the present study, together with previous work support the same findings. This study shows that FFB has a marked capacity for iron binding, suggesting the presence of flavonoids that has potent iron chelating capacity i.e, Fe^{2+} ions. Therefore, it is likely that the chelating effect of CFBS and FFB on metal ions maybe responsible for the inhibition of deoxyribose oxidation. The scavenging activities of phenolic substances like flavonoids might be due to the active hydrogen donating ability of hydroxyl substitutions. Since flavonoids present in the extract are good electron donors, they may accelerate the conversion of H_2O_2 into H_2O.

The findings from above experiment can be correlated with promising role of constituents in the *in vivo* and *in vitro* protection against oxidative damage reaction mediated by iron and ascorbate. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, however, the complex formation is disrupted, resulting in a decrease in the magenta colour of the complex. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelator (Yamaguchi *et al.*, 2000). It was reported that the chelating agents which form σ bond with a metal, are effective as secondary antioxidants, because they reduce the redox potential thereby stabilising the oxidised form of the metal ion (Gordon, 1990). Bivalent transition metal ions particularly Fe^{2+} ion has the ability to move single electrons by virtue of which it can catalyse the formation of the first few radicals to initiate the propagation step of radical chain reaction during lipid peroxidation. Therefore, metal chelating capacity plays a significant role in antioxidant mechanism (Geckil *et al.*, 2005), since it reduces the concentration of the catalysing transition metal in lipid peroxidation (Duh *et al.*, 1999). Chelating agents may inhibit lipid oxidation by stabilizing transition metals. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. The iron (II) chelating properties of the antioxidant extract may be attributed to their endogenous chelating agents, like the flavonoids (Acker *et al.*, 1998). The results of the present study, together with previous works support the same findings. This study shows that FFB has a marked capacity for iron binding, suggesting the presence of flavonoids that has potent iron chelating capacity i.e., Fe^{2+} ion.

The superoxide radical is considered one of those few typical free radicals which is involved in the generation of other deadly free radicals like H_2O_2, $^{\cdot}OH$, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury and thus directly or indirectly play a major role in the oxidative damage to biomolecules (Fridowich, 2001). Superoxide has also been observed to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals. The ability to scavenge superoxide radicals may be of one of the approaches adopted by antioxidant constituents towards free radicals as suggested (Kostyuk *et al.*, 1996; Yoshida *et al*, 2002). In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen by a PMS/NADH coupling reaction reduces the yellow dye NBT to produce the blue formazon, which is measured spectrophotometrically at 560 nm (Nishimiki *et al.*, 1972). Antioxidants elicit their action by inhibiting the formation of blue coloured NBT complex. The decrease of absorbance at 560 nm in presence of antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Removal of superoxide in a concentration dependent manner by antioxidant constituents of FFB from *F. bengalensis* may be attributed to the direct reaction of its constituents with these radicals. However, it may be due to direct reaction or by inhibition of the enzymes involved in its formation or both that need to be further investigated as we have only measured the direct reaction in present study. The higher inhibitory effects of the stem bark extracts FFB of *F. bengalensis* on superoxide anion formation noted herein possibly renders them as promising antioxidants.

Total antioxidant potential was also evaluated by phosphomolybdenum reduction assay. This assay is based on the ability of potential antioxidants to donate electron and reduce the phosphomolybdate complex giving coloured reaction. Though the formation of the green phosphomolybdenum complex could proceed at room temperature, however this reaction showed a positive dependence on temperature, so that a significant yield increase was observed at higher temperature (Matkowski and Piotrowska, 2006). Both CFBS and FFB showed concentration and temperature dependent increase in antioxidant activity but FFB has highest activity at 90° C and found to be even better than ascorbic acid. This further confirms the strong antioxidant potential of FFB of *F. bengalensis*.

CONCLUSION

It is clear from the present study that antioxidant potential of FFB from *F. bengalensis* are due to its high scavenging potential of various reactive oxygen species/free radicals and metal chelating abilities as evident from *in vitro* assays. The antioxidant efficacy was almost similar to well known antioxidant agents like quercetin, BHA and ascorbic acid and found to be more effective and potent than few other antioxidants from other plants reported earlier. The broad range of antioxidant activity of the extracts indicates the potential of FFB from *F. bengalensis* as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

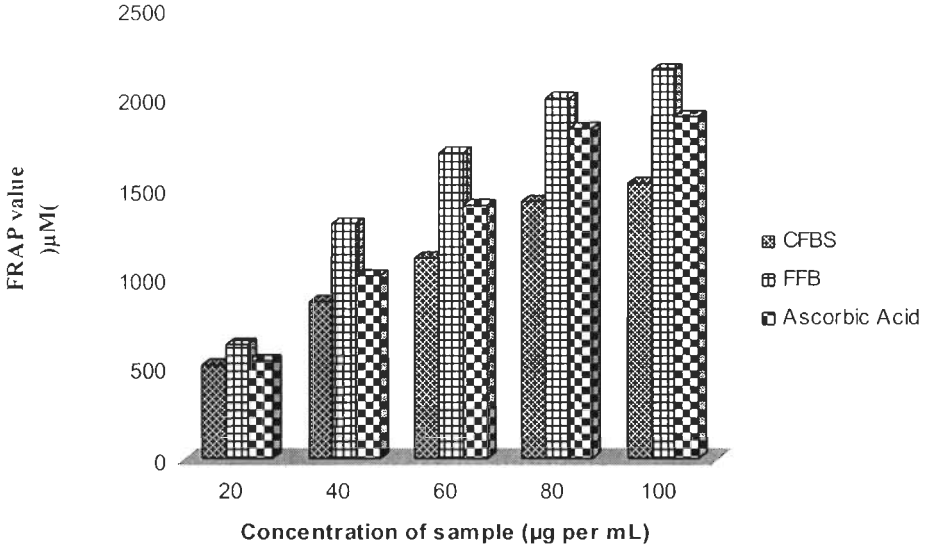


Fig 4.1 (a) The ferric reducing antioxidant power of antioxidant constituents from *F. bengalensis*. FRAP value (µM) for CFBS, FFB from *F. bengalensis* and Ascorbic acid at different concentrations.

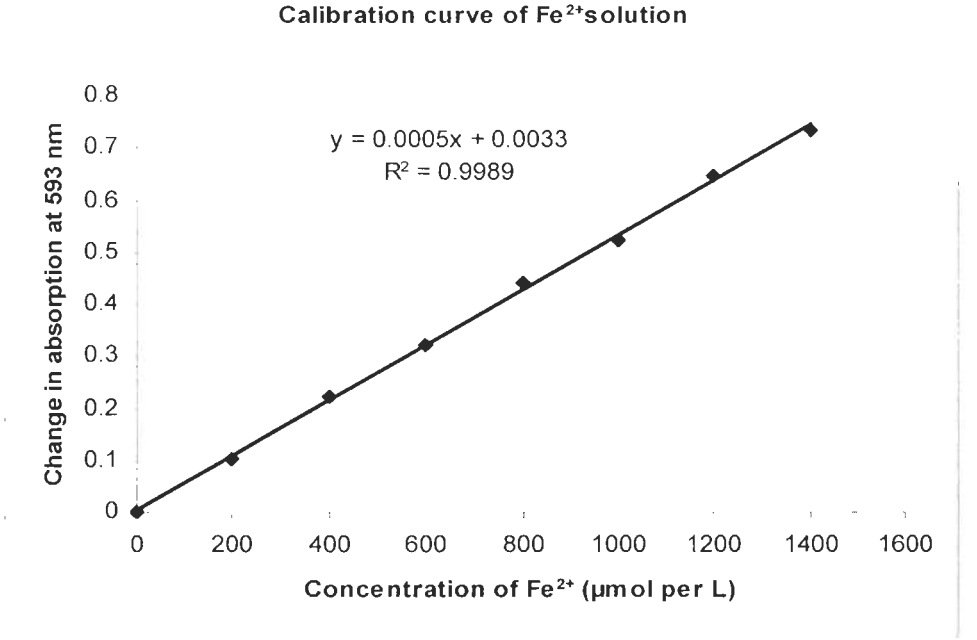


Fig 4.1 (b) The calibration curve for standard Ferrous ammonium sulphate for FRAP.

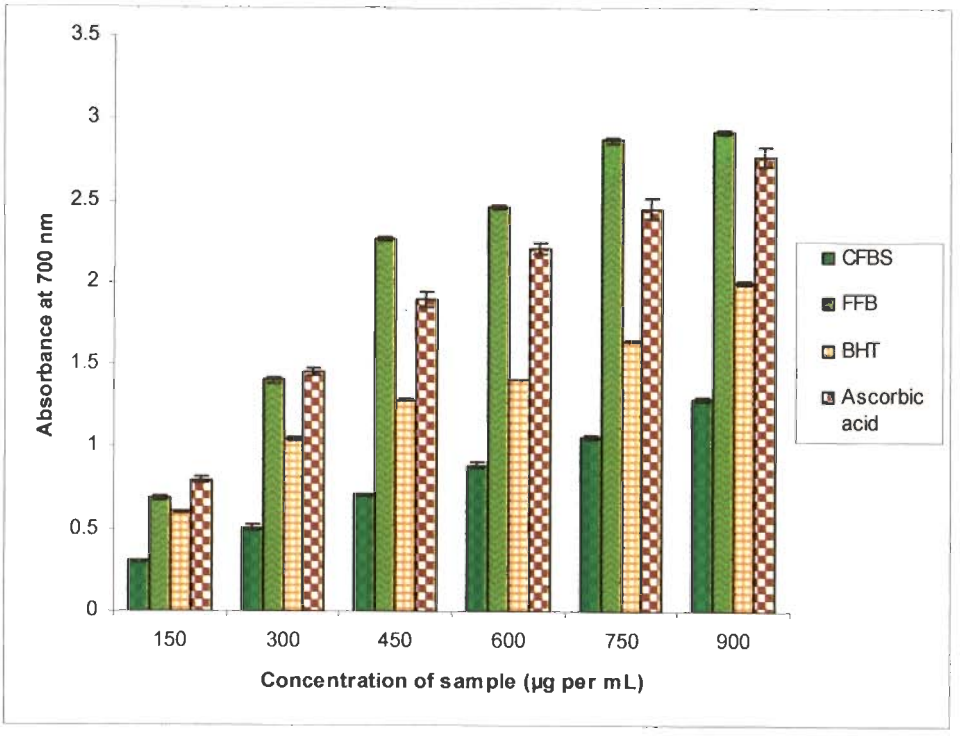


Fig. 4.2 Reducing power of CFBS, FFB, Butylated hydroxyl toluene and Ascorbic acid. Results are mean of triplicate measurements ± SD. (P value ≤ 0.01 when compared with control).

Table 4.1 Free radical scavenging activity of CFBS and FFB using ABTS and DPPH free radical scavenging assay

Name of Sample	DPPH assay (IC_{50} µg per ml)	ABTS assay (TEAC value)
GA(Gallic acid)	1.19 ± 0.10	2.23±0.19
CFBS	2.73 ±0.143	0.625±0.06
FFB	1.69±0.131	1.80±0.10

Results are mean of triplicate measurements ± SD. (P value ≤ 0.05 when compared with control).

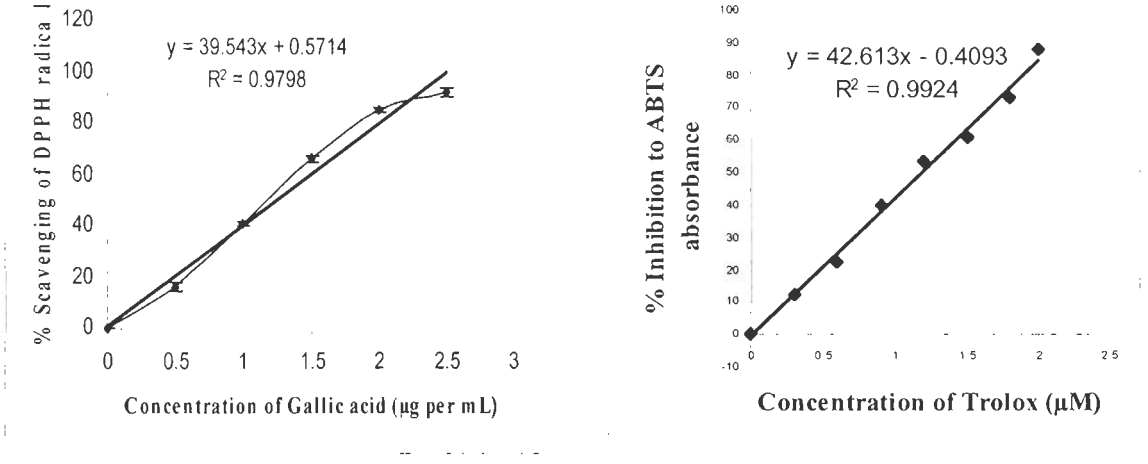


Fig 4.3 (a) The calibration curve of Gallic acid against DPPH free radical scavenging assay and Fig 4.3 (b) TEAC calibration curve for ABTS assay.

Table 4.2 Hydroxyl radical scavenging activity of CFBS and FFB constituents from *F. bengalensis*

Sample (µg per mL)	**Absorbance (532 nm) with ascorbic acid**	**% Inhibition**
Oxidizing mixture only	0.8920	
CFBS 50	0.6900	22.64 ± 0.73
CFBS 100	0.5424	39.20 ±0.87
CFBS 150	0.4577	48.69 ± 1.18
CFBS 200	0.2909	67.39 ±0.56
CFBS 250	0.2564	71.26 ±1.58
FFB 25	0.5591	37.32 ± 1.92
FFB 50	0.4262	52.21 ± 1.71
FFB 75	0.3577	59.89 ± 2.38
FFB 100	0.2794	68.67 ± 2.98

(IC_{50} CFBS = 158.54 ± 4.29 µg per mL)

(IC_{50} FFB = 46.29 ± 2.82 µg per mL)

(IC_{50} BHT = 26 ± 1.128 µg per mL)

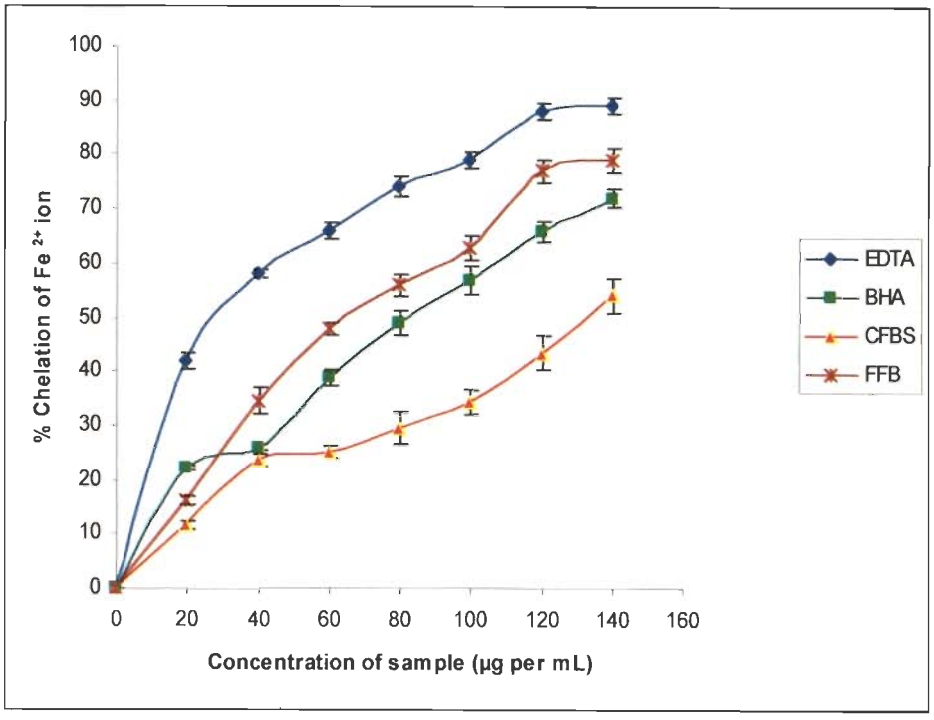


Fig. 4.4 Metal chelating activity of different concentrations of (CFBS and FFB from *F. bengalensis*). Each value is expressed as mean of triplicate measurements ± SD. (P value ≤ 0.05 when compared with the control).

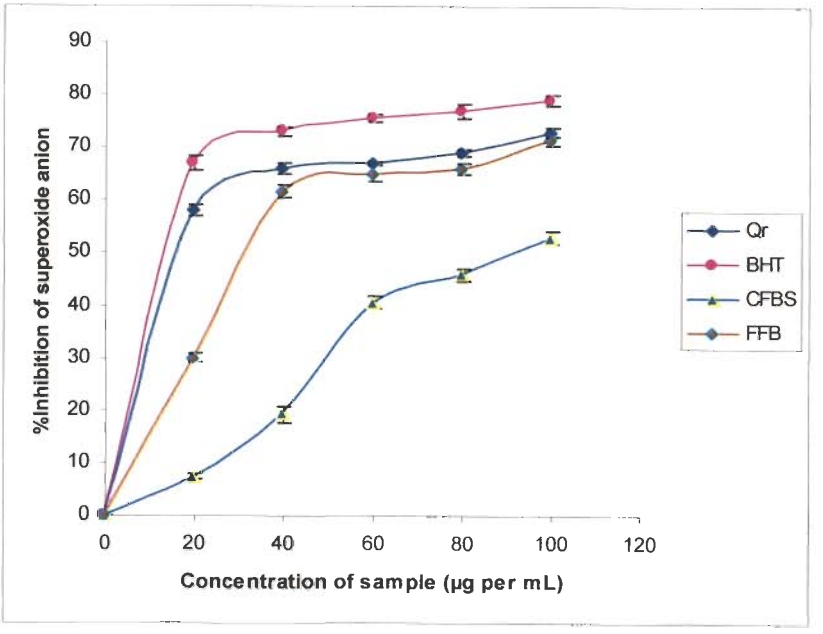


Fig. 4.5 (a) Superoxide radical scavenging potential of CFBS, FFB, Quercetin and Butylated hydroxyl toluene constituents. Results are mean of triplicate measurements ± SD. (P value ≤ 0.05 when compared with the control).

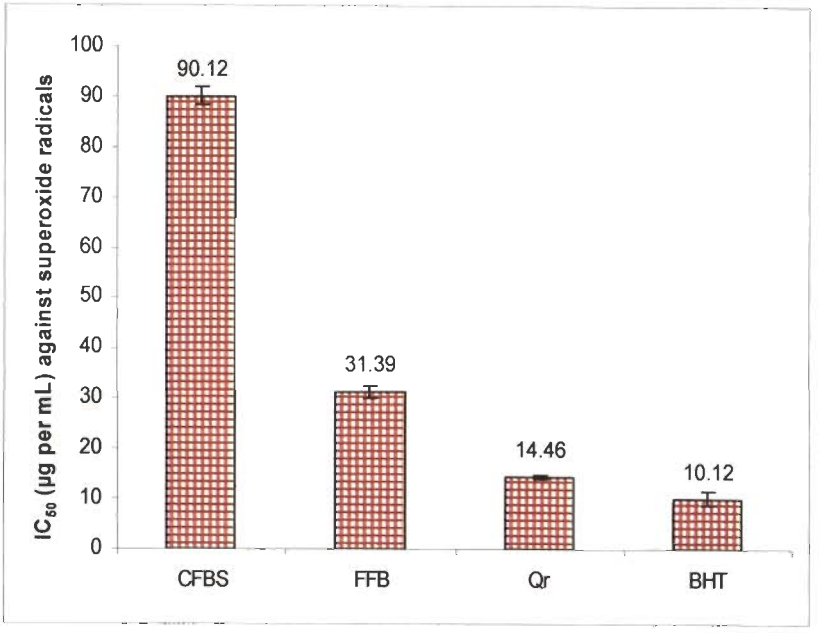


Fig. 4.5 (b) IC_{50} (µg per mL) values of CFBS, FFB, standard compounds Qr and BHT for superoxide anion scavenging activity. Lower IC_{50} values indicate higher antioxidant activity. (Values are mean of triplicate measurements ± SD).
CFBS- Crude methanolic extract of *F. bengalensis* stem bark; FFB-Flavonoid enriched fraction of *F. bengalensis* stem bark; Qr- Quercetin; BHT-Butylated hydroxy toluene

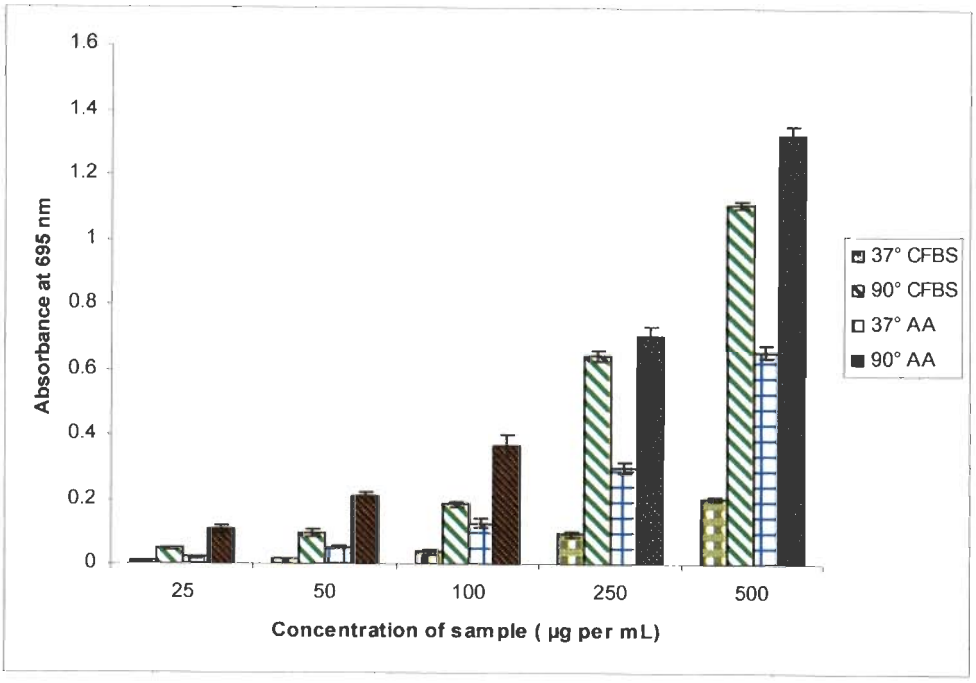


Fig 4.6 (a) Comparison of phosphomolybdenum formation of CFBS with standard compound ascorbic acid at different incubation temperature 37°C and 90°C. Results are mean of triplicate measurements ± SD. (P value ≤ 0.05 when compared with the control).

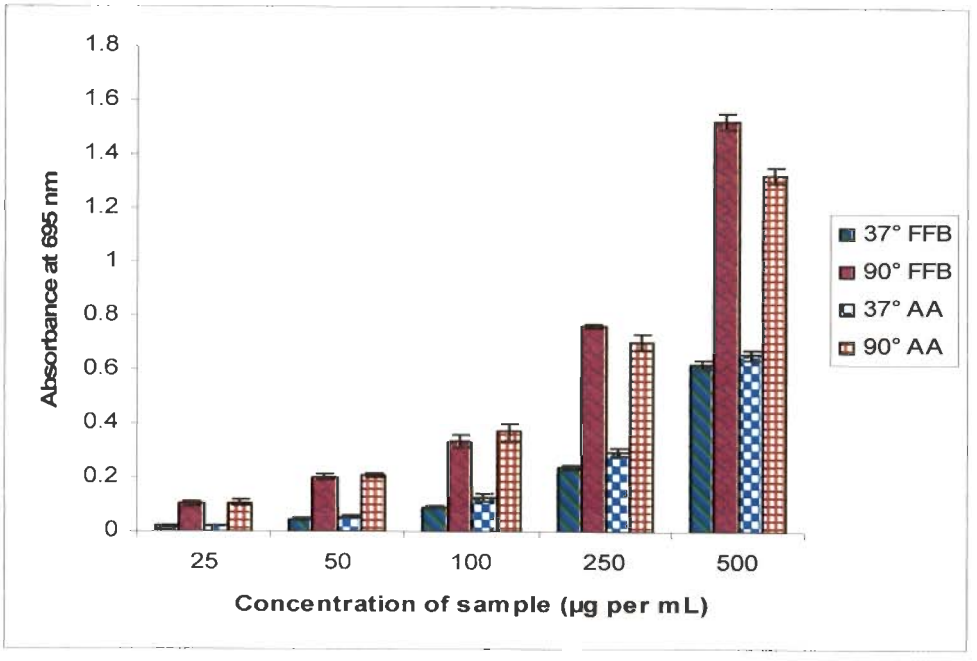


Fig 4.6 (b) Comparison of phosphomolybdenum formation of FFB with standard compound ascorbic acid at different incubation temperature 37°C and 90°C. Results are mean of triplicate measurements ± SD. (P value ≤ 0.05 when compared with the control).

Chapter 5

Protection against Oxidative Damage to Biomolecules by Flavonoid Enriched Fraction (FFB) From *F. bengalensis.*

5.1 INTRODUCTION

ROS in the form of superoxide ($O_2^{\cdot-}$), Hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) mostly attack biological molecules such as lipids, proteins, enzymes, DNA and RNA and thus pose a continuous and formidable challenge to all living systems (Valko *et al.*, 2007; Halliwell *et al.*, 2007). Although the organism possesses several defense mechanisms 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 which if remained unchecked, can cause irreversible and irreparable damage to biomolecules (Arouma, 1998; Djordjevic, 2004; Valko *et al.*, 2004). The extent of damage caused due to particular biological molecules depends on the type of ROS species and their concentration. 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 oxidation induced cell death and disease development (Sies, 1997; Valko *et al.*, 2007). There are several reports emphasizing the generation of different marker oxidation products during oxidative damage to biomolecules (Dalle-Donne *et al.*, 2006). However, with recent advancement and development of several analytical methods for the measurement of such marker *in vitro* as well as *in vivo* experiments (Aruoma, 1998), it has become possible to evaluate the level of protection by antioxidant agents of interest against oxidative damage to biomolecules (Frei, 2004). It has been investigated 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). 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 sulfhydral groups and amino acid modification (Stadman, 2001; Levine and Stadman, 2003). All these modifications can be used as markers of protein damage by free radicals *in vivo* and *in vitro* study. Oxidative stress induced peroxidation of membrane lipids can be very damaging since it leads to alterations in the membrane fluidity and inactivation of membrane-bound receptors or enzymes but also generate a large number of potentially damaging oxidation products. A number of marker products like malondialdehyde and isoprostanes are formed during lipid peroxidation and are used as markers in assessment of lipid peroxidation level (Wiswedel *et al.*, 2005; Dalle-Donne

et al., 2006). Oxidative DNA damage can be correlated with an increased risk of cancer development (Thompson, 2006). Both part of DNA including sugar as well as nucleotide bases are important molecular targets for free radical mediated oxidative damage. Therefore, DNA subjected to attack by hydroxyl radical generates a wide variety of base and sugar modification products (Halliwell and Aruoma, 1991). Free radicals cause extensive base modification as well as single-strand breaks in DNA (Marnett, 2000). Therefore, there is ardent need for sensitive and reliable tools to monitor the biomarkers produced due to oxidative damage to biomolecules. In the recent past, there has been much advancement in developing more reliable analytical methods for the measurement of such markers *in vitro* as well as *in vivo* experiments to evaluate the level of protection by antioxidant of interest against oxidative damage to biomolecules (Aruoma, 1998; Yu *et al.*, 2007; Yin, 2008).

Role of antioxidants in protecting our body against damage caused by ROS and as therapeutic agents in diseases is well established (Indap *et al.*, 2006; Pham-Huy *et al.*, 2008; Ali *et al.*, 2008). In this context the role of plant derived natural antioxidants have been appreciated and encouraged to prevent such implications. Therefore, attempts have been made by various groups worldwide to study the antioxidant potential of various plant preparation or extracts or their purified constituents (Choi and Lee, 2009; Atmani *et al.*, 2009; Borneo *et al.*, 2009; Orhan *et al.*, 2009) and their protective role against oxidative damage to biomolecules likes DNA (Jeong *et al.*, 2009; Wei *et al.*, 2008), proteins (Bahramikia *et al.*, 2009; Wang *et al.*, 2006) and lipids (Wang *et al.*, 2009; Ho *et al.*, 2008). It is well established that plant derived natural antioxidants belongs to several classes of phytochemicals mainly phenolic (Zhou *et al.*, 2009), glycosides (Abraham *et al.*, 2008), terpenoids (Sharififar *et al.*, 2007) and saponins (Dini *et al.*, 2009) etc., which are reported to show different extent of antioxidant activity and mechanism towards free radicals. 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 (Shahidi, 2008).

Keeping the above logic and facts in mind, the aim of the present study was to assess the antioxidant efficacy of flavonoid enriched fraction (FFB) from *F. bengalensis* (described previously in Chapter 3) and evaluate its protective potential

against oxidative damage to DNA, protein and lipid employing spectrophotometric, electrophoresis and molecular techniques.

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. Calf thymus (Ct) DNA and pUC18 plasmid were purchased from Bangalore Genei Pvt. Ltd. India.

5.2.2 Protective potential of FFB constituents against oxidative damage to DNA

Different analytical methods were carried out to evaluate the protective potential of FFB constituents against oxidative damage to DNA. Among different methods available to determine protective role against DNA damage, the methods including spectrophotometric MDA determination and agarose gel electrophoresis techniques are widely used.

5.2.2.1 Electrophoretic study on oxidative damage to Calf thymus DNA induced by metal catalyzed oxidation system (MCO) system

The reaction mixture contained 4 µL of calf thymus DNA (0.5 µg), oxidation system MCO [Fe^{2+} (4 µL, 1 mM) and H_2O_2 (6 µL, 1 mM)]. The oxidation reaction was carried in the presence or absence of varied concentrations of FFB (10, 20, 40 and 60 µg per mL) and positive standard Quercetin (50 µg per mL). Incubation was allowed for 15 min at 37°C. 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)

5.2.2.2 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 [Fe^{2+} (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 out in the presence or absence of varied concentration of FFB (10, 20, 40 and 60 µg per mL) and positive standard Quercetin (50 µg per mL). After 30 min, reaction was stopped by addition of 2 µL of loading dye. The plasmid samples were

analyzed by electrophoresis on 1% agarose horizontal slab gel submerged in TAE buffer (1X) as described above for calf thymus DNA oxidation. After staining with ethidium bromide, the plasmid bands were visualized, photographed and analyzed with the Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA). The level of plasmid nicking was expressed as % DNA in relaxed form of plasmid (Form II).

5.2.2.3 Spectrophotometric determination of calf thymus DNA oxidation using TBARS method

Spectrophotometric study to evaluate protective potential against oxidative damage to Calf thymus DNA was studied using the oxidation reaction mixture containing Ct DNA (0.1 mg per mL) and oxidation system MCO [Fe^{2+} (200 μM) and H_2O_2 (500 μM)] in final concentration. The oxidation reaction was carried in the presence or absence of different concentrations of FFB (25, 50, 100, 150 and 200 μg per mL) and positive standard quercetin (75 μg per mL). After the oxidation reaction was over, the TBARS production was determined employing TBA-MDA estimation by method described (Okhawa *et al.,* 1979) with slight modification.

5.2.3 Protective potential of FFB against oxidative damage to protein

The protective potential of FFB was evaluated using spectrophotometric and SDS-PAGE electrophoresis.

5.2.3.1 Oxidative damage reaction methods to Bovine serum albumin (BSA) protein

Oxidation reaction mixture contained oxidation system MCO (Halliwell *et al.*, 1987) [Ascorbic acid (0.1 mM) + EDTA (0.05 mM) + $(NH_4)_2Fe(SO_4)_2$ (0.05 mM) + H_2O_2 (1 mM)] in 50 mM phosphate buffer (pH 7.4) and BSA (1 mg per mL). The assay was performed at 25°C for 30 min in the presence or absence of varied concentration of FFB (50-250 μg per mL). Glutathione was used as a positive control. In another set of oxidation reaction, HOCl was used instead of H_2O_2 based MCO system. Oxidative damage to BSA protein (1 mg per mL) was induced with 0.75 mM HOCl reagent (concentration was calculated $\square_{290}$=350 M^{-1} cm^{-1}). The reaction mixture was incubated at 25°C for 20 min in presence or absence of different concentrations of FFB and 2.5 mM of ascorbic acid was added to stop the reaction (Wang *et al.,* 2006).

5.2.3.2 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 Levin *et al,* (1995) with some modifications. The oxidized BSA pellets obtained above were resuspended in 500 µL of 50 mM phosphate buffer (pH 7.4) and derivatized with 500 µL of 10 mM dinitrophenyl hydrazine (DNPH) dissolved in 2M HCl. Tubes were incubated at 25°C for an hour. Samples were vortexed every 10-15 min. In order to precipitate proteins, 500 µL of (20% w/w) trichloroacetic acid was added and centrifuged for 3 min at 11,000×g. Supernatant was discarded and pellets were washed thrice with one mL of ethanol/ethylacetate (1:1). After the final rinse, supernatant was discarded and the precipitated protein was redissolved in 600 µL of 6M guanidine in 20 mM KH_2PO_4, with final pH 2.3. The solution was vortexed thoroughly and incubated for 15 min at 37°C. Insoluble material was pelleted by centrifugation for 3 min at 10,000 x g. The absorbance of BSA-DNP conjugates were recorded at 370 nm. The final data were expressed as nmol of protein carbonyl per mg of protein (ε_{375} = 22,000 $mol^{-1}cm^{-1}$) for the DNPH derivates.

5.2.3.3 Analysis of oxidative damage to BSA and their inhibition by FFB using SDS PAGE technique

To determine protein damage by MCO system in presence or absence of FFB, reaction was further discontinued by addition of reaction inhibitor and oxidized samples were lyophilized and pellets were subjected to SDS-PAGE. Twenty micrograms of BSA (from re-suspended 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 samples were loaded in a 10% polyacrylamide gel and electrophoresed at 100 V. After running, gels were stained with 0.2% Coomassie brilliant blue for 1 hour, destained and scanned in BIO-RAD gel documentation system (Bio-Rad, Hercules, CA, USA) for densitometric analysis with 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).

5.2.4 Lipid peroxidation in rat brain homogenate and their inhibition by FFB antioxidant constituents

Lipid peroxidation in rat brain homogenate was induced by iron/ascorbate reaction system. The extent of Fe (II)/ascorbate induced lipid peroxidation was further determined by TBA-assay. Wistar albino rats (3-4 months) old and weighing around (120-150 g) was anaesthetized under mild ether anesthesia; brain was excised and washed with 0.95 M NaCl solution. The brain of rats were homogenized with a hand homogeniser in ice cold Tris-HCl buffer (25 mM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 20,000 x g for 20 min. 0.5 mL of supernatant was incubated with 0.3 mL of varied concentrations (25, 50, 100, 150 and 200 µg per mL) of FFB in the presence of 0.1 mL of 10 mM $FeSO_4$ and 0.1 mL of 0.1 mM ascorbic acid at 37°C for an hour. The reaction was stopped by addition of 0.75 mL of 28 % trichloroacetic acid (w/v 28 %) and 0.5 mL of 2-thiobarbituric acid (2-TBA, 1% w/v) was added to the reaction mixture followed by heating at 100°C for 45 min. After centrifugation, all precipitated proteins were removed and the color of the malondialdehyde (MDA)-TBA complex in the supernatant was detected at 532 nm. The decrease in absorbance was recorded at 532 nm for lipid peroxidation inhibition potential. The values of MDA were expressed as nmol per mg of protein. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = (A - A1) / A \times 100\ \%$$

where, A was the absorbance of the control, and Al was the absorbance of the test sample

5.2.5 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:

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

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

5.3 RESULTS

5.3.1 Protective effects of FFB constituents against oxidative damage to DNA

Protective role of FFB against MCO induced oxidative damaged to DNA was studied using spectrophotometric and agarose gel electrophoretic techniques.

5.3.1.1 Spectrophotometric evaluation of protective potential of FFB constituents against oxidative damage to DNA

Protection by different concentrations of FFB against oxidative DNA damage was determined in terms of the damage to its deoxyribose sugar moiety. The MDA production was determined during DNA oxidation in the presence of MCO agent in absence or presence of varied concentrations of FFB (25-200 µg per mL). The result is depicted in Fig 5.1 showing extent of reduction in MDA generation during DNA oxidation. The reduction in MDA content during DNA oxidation was observed and at 100 µg per mL concentration 54.23 % reduction was observed, which was equivalent to standard quercetin protective potential showing 47.45 % reduction in MDA content at 75 µg per mL. The presence of various concentrations (25-200 µg per mL) of FFB constituents prevented the free radical-mediated DNA-sugar damage in a dose dependent manner. Thus, it clearly indicates that FFB has protective effect against oxidative damage to DNA.

5.3.1.2 Agarose gel electrophoresis study on evaluation of protective effect against oxidative damage to DNA

Protective effect against oxidative damage to DNA was also studied by agarose gel profile showing oxidatively damaged calf thymus DNA in presence or absence of FFB and the results are illustrated in Fig. 5.2 (a) and (b). It is clear from the gel that DNA damage induced due to oxidant system has been ameliorated significantly in the presence of FFB in a dose dependent manner (Fig 5.2 (a) Lane 3-6). The extent of protection to DNA damage by FFB is comparable to that of standard Quercetin. The gel densitometry analysis data is given in the Fig 5.2 (b) and data analysis clearly indicated an increase in the parent band intensity of calf thymus DNA with the increase in the FFB concentration.

The protective effect of FFB was also studied by inhibition of Fenton reaction induced pUC 18 plasmid DNA damage. The result is presented in Fig. 5.3 (a) and (b). The agarose gel photograph clearly shows the protective effect of FFB (Lane 3-6).

Control pUC18 DNA showed two bands, one of open circular that was merely visible and the other of supercoiled. Data analysis clearly indicates that treatment with $(NH_4)_2.FeSO_4$ and H_2O_2 in the absence of FFB led to the formation of open circular DNA by the strand scission of supercoiled DNA (Lane 2), whereas the presence of FFB prevented this strand scission in concentration dependent manner to a considerable extent (Fig 5.3 a; Lane 3-6) causing drastic decrease in the form II (open circular DNA) band intensity of pUC18 plasmid DNA. At higher concentration (60 μg per mL) of FFB, protection was almost similar to quercetin (lane7). The densitometric analysis of the gel is depicted in Fig 5.3(b) which further confirmed the experimental data.

5.3.2 Protective effect of FFB against oxidative damage to protein

The protection of FFB against oxidative damage to protein was studied using DNPH based spectrophotometric technique and assessing the fragmentation profile of protein on SDS-PAGE using BSA.

5.3.2.1 Spectrophotometric study to evaluate protective effect of FFB against oxidation to protein

BSA samples were oxidized in absence or presence of varied concentrations of FFB constituents and the protein carbonyl index was measured with the help of DNPH based spectrophotometric assay. The result is presented in Fig. 5.4. The data analysis from results suggested that at concentration of 197.2 μg per mL, formation of protein carbonyl during oxidation to BSA was reduced to 50%. The standard glutathione was able to protect BSA oxidation at 200 μg per mL up to 51.64 %. Thus it is clear that efficiency of FFB to protect BSA oxidation was equivalent to glutathione in terms of their inhibitory concentration. Protein are important components of cell and tissue and are susceptible to oxidation by ROS and RNS e.g., $OH^{\cdot}$, HOCl and $ONOO^{-}$. It is clear from Fig 5.4 that FFB inhibited protein carbonyl derivatives production (due to protein oxidation induced by $OH^{\cdot}$).

5.3.2.2 SDS-PAGE fragmentation study to evaluate protective effect of FFB against oxidative damage to protein

The inhibition of MCO induced oxidative damage to protein by FFB was also studied by SDS-PAGE profile. The BSA was oxidized in absence or presence of various concentration of FFB. Glutathione was used as reference antioxidant for comparison. The Coomassie blue gel profile and its densitometry analysis was done

with the help of quantity one software and percentage band intensity was calculated and given in Fig. 5.5 (a) and 5.5 (b), respectively. Similarly, the protective effect of FFB was also studied against HOCl induced oxidation of BSA. The Coomassie blue gel profile and the densitometric analysis of BSA band was done with the help of quantity one software and percentage band intensity was calculated and given in Fig.5.5 (c) and 5.5 (d), respectively.

It is clear from both Fig 5.5 (a) and Fig 5.5 (c) that there is clear reduction in BSA oxidative damage. Lanes 3–7, clearly revealed that the samples treated with FFB, exhibited a concentration-dependent increase in band intensity compared to the control treated without FFB (lane 2). Similar observation was also made in case of HOCl induced oxidative damage. The efficacy of FFB was almost similar to the standard glutathione protection.

5.3.3 Study on lipid peroxidation inhibition potential of FFB constituents

Lipid peroxidation leads to rapid development of rancid and stale flavors and is considered as a primary mechanism of quality deterioration in lipid foods and oils (Nguyen *et al.,* 2008). Synthetic antioxidants, such as butylated hydroxyanisole (BHA) are added in food, during processing to suppress lipid peroxidation and consequently to improve food quality and stability. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver (Gutteridge, 1985). In our study *in vitro* lipid peroxidation was induced to rat brain homogenate by ferrous sulphate that occurs either through ferryl-perferryl complex or through $OH^{\cdot}$ radical by Fenton's reaction. Fe^{2+}/ascorbate model is a well-validated system for production of ROS. A combination of ascorbate and iron can trigger a Fenton's reaction with formation of highly reactive hydroxyl radicals which can cause chain-initiation reaction of lipid peroxidation.

The protective effect of FFB was studied against Fenton's reaction induced lipid peroxidation of brain homogenate. Inhibition of ascorbate/Fe^{2+} oxidation system induced lipid peroxidation in rat brain homogenate by FFB from *F. bengalensis* was determined by measuring the MDA production. The results are presented in Table 5.1. It is clear from the results that with increasing concentration of FFB there was reduction in MDA production. The IC_{50} for FFB found to be 56.96 ± 0.66 ug per mL which was much better than glutathione (112.50 ± 4.38 ug per mL). Thus FFB inhibited

$FeSO_4$/ascorbate induced lipid peroxidation in rat brain homogenate significantly and in a dose dependent manner.

5.4 DISCUSSION

Free radical mediated oxidative damage to biomolecules including DNA, protein and lipid is one of the noticeable pathways leading to oxidation induced cell death and disease 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 (Tripathi *et al.*, 2007; Srinivasan *et al.*, 2007). In the present study, the protective effect of FFB constituents from *F. bengalensis* against free radical mediated damage to DNA, protein and lipids have been evaluated.

Reactive oxygen species induced oxidative DNA damage produce a variety of modifications in DNA including base and sugar lesions, strand breaks, DNA-protein cross-link and base-free sites (Halliwell and Aruoma, 1991; Valko *et al.*, 2004). One of the most reactive radical species that induce lesions in DNA is the hydroxyl radical ($OH^{\cdot}$). 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).

To demonstrate the protective effect against oxidative damage to DNA, the calf thymus DNA and pUC18 plasmid were exposed to metal catalyzed Fenton reaction in absence or presence of varied concentrations of FFB constituents from *F. bengalensis*. A strong inhibition of oxidative damage to calf thymus DNA by FFB (20 ug per mL onwards) showed its protective ability against oxidative damage to DNA (Fig 5.2 a). The protective effect of FFB constituents from *F. bengalensis* also confirmed from the inhibition of oxidation induced strand breakage or nicking in SDCC pUC18 plasmid when exposed to Fenton's reaction. Conversion of supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage (Jung and Surh, 2001). The plasmid DNA was mainly of the supercoiled form in the absence of Fe^{2+} and H_2O_2 (Fig 5.3 a, Lane1). During the addition of Fe^{2+} and H_2O_2, the

supercoiled form of DNA decreased and converted into the relaxed circular form resulting from the cleavage of one of the phosphodiester chains of supercoiled DNA (scDNA) to produce a relaxed open circular form (ocDNA) indicating that OH• generated via Fenton's reaction produced DNA strand scission. However, in presence of FFB there is reduction in conversion of scDNA to ocDNA. These results indicated significant protection of plasmid DNA from oxidative damage by the FFB constituents, correlating well with its antioxidant property. Earlier, numerous workers (Kumar and Chattopadhyay, 2007; Park *et al.*, 2009) have employed this system to assess the biological activity of various plant derived antioxidants in protection against biomolecules. Similar observation regarding protection against oxidative damage to DNA have been reported in earlier studies (Moon *et al.*, 2006; Fabiani *et al.*, 2008) and our results are at par with the ones reported.

Oxidative DNA damage is generally regarded as carcinogenic and actively participates in many pathological processes, including cancer and aging (Izzotti, 2003). Generally, it is assumed that the hydroxyl radical scavenging and metal chelating ability of antioxidants account for their protective activity against oxidative damage to biomolecules (Ghanta *et al.*, 2007; Emen *et al.*, 2009). Since FFB constituents from *F. bengalensis* found to have strong free radicals scavenging activity (hydroxyl and super oxide) and metal chelating ability as described and discussed earlier (Chapter 4).

It is suggested that FFB 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 presence of flavonoids. It has been suggested that the importance of chelation of Fe^{2+} ions depends upon the activity of flavonoid antioxidants (Acker *et al.*, 1998). It has also been reported that antioxidant activities of flavonoid play a potential role in protection against oxidative damage to DNA (Hsieh *et al.*, 2005). The protective effect of flavonoids against DNA damage would be consistent with their number of hydroxyl group and also their position in the ring structure is relevant (Noroozi *et al.*, 1998). Flavonoid compounds extracted from several plant sources have been reported to have radical scavenging effect and Fe^{2+} ions chelating ability and provides protection against oxidative damage to DNA (Ghanta *et al.*, 2007; Singh *et al.*, 2009).

Proteins have many different and unique biological functions; therefore oxidative modification of protein can lead to diverse functional consequences.

Oxidative damage of proteins have also been implicated in the pathophysiology of human diseases and is currently a topic of considerable interest (Dalle-Donne *et al.*, 2006). Several mechanisms have been proposed to explain radical-mediated damage to proteins which may be initiated by electron leakage, metal ion dependent reactions and autoxidation of lipids and sugars. However, the most crucial is metal catalyzed oxidation system involvement in protein damage (Dean *et al.*, 1997; Stadtman and Levine, 1990).

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 (Dean *et al.*, 1986; Levine and Stadman, 2003). The principle of protein modification by ROS is 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 $OH^{\cdot}$ or by $OH^{\cdot}$ plus $O_2^{\cdot-}$, however, tryptophan, histidine and cysteine are the most vulnerable (Freeman and Crapo, 1982; Stadtman and Levine, 2003). 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 (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; Bahramikia *et al.*, 2009). The most common method for determination of protein oxidation is to evaluate the levels of carbonyl group, a stable product of protein oxidation. There are several reports suggesting that during oxidation of protein, there is sharp increase in protein carbonyl content (Banaclocha *et al.*, 1997; Boscia *et al.*,

2000). Protein carbonyls are induced *in vitro* and *in vivo* by a diverse range of agents, including metal-catalyzed oxidation, ozone, HOCl, singlet oxygen, and ionizing radiation. Increased protein carbonyls have also been detected in several disease states, such as rheumatoid arthritis, ischaemia-reperfusion injury to heart muscle, and skeletal muscle damage due to exhaustive exercise (Esme *et al.*, 2006; Lemarechal *et al.*, 2006). As a result, carbonyl formation is frequently used as an important biomarker for protein oxidation by most types of ROS (Dalle-Donne *et al.*, 2006). Thus, it has been recognized that oxidative modification is one of suitable markers to evaluate the oxidative stress condition.

In the present study, results are presented with the help of different methodologies available to study protein oxidative damage including DNPH based protein carbonyl determination and fragmentation study (Mayo *et al.*, 2003). FFB showed a concentration-dependent reduction of albumin oxidation, induced by the Fe^{3+}–ascorbate–H_2O_2 system, which resulted in formation of a carbonyl group. Several attempts have been made to find suitable antioxidant constituents to exert protective role during oxidative damage to protein (Dean *et al.*, 2001).

HOCl is not a free radical, but a potent chlorinating and oxidizing agent. During hypochlorite-mediated BSA oxidation, oxidation of SH (thiol) group of methionine and tryptophan residues occurs, leading to formation of protein carbonyls (Himmelfarb *et al.*, 2000; Hawkins *et al.*, 2003). Therefore, BSA carbonyl content can be used to test the ability of various compounds to scavenge HOCl. FFB treatment reduced the carbonyl formation thus it is a "good" HOCl scavenger.

Lipid peroxides are likely to be involved in numerous pathological events, including inflammation, metabolic disorders and cellular aging (Minoguchi *et al.*, 2006; Prashant *et al.*, 2007). This might be explained by the higher affinity of active antioxidant compounds toward the lipid and aqueous phases as well as the interface, which enter the liposomal bilayer more readily. The FFB inhibited the lipid peroxidation which was confirmed by reduction in MDA level in treated samples compared to control. These results were similar to those observed in the case of oxidative damage to protein and DNA and could be suggested that the inhibition could be caused by the absence of ferryl-perferryl complex or by scavenging the $^{\cdot}OH$ radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of

conversion of ferrous to ferric or by chelating the iron itself. Thus decrease in the MDA level in rat homogenate with an increase in the FFB concentration indicates its role as an antioxidant. This is attributed to its flavonoid constituents. The flavonoid constituents identified from several plant sources have been found to be good antioxidants against lipid peroxidation in rat brain homogenate (Wagner *et al.*, 2006; Nagulendran *et al.*, 2007).

CONCLUSION

The implication of oxidative stress in the etiology and progression of a wide variety of clinical disorders has led to further affirmation of the fact that antioxidants from multivarious sources may provide health benefits by acting as prophylactic agents. Oxidative damage to biomolecules may lead to severe impairment of their structural as well as functional aspects ultimately resulting in cell death. Our present study highlighted that FFB showed strong protective effect against oxidative damage to biomolecules like DNA, protein and lipids. This protective activity can be very well correlated with its metal chelation as well as radical scavenging abilities. Protective effect of FFB attributed by its strong antioxidant activity is due to presence of flavonoids.

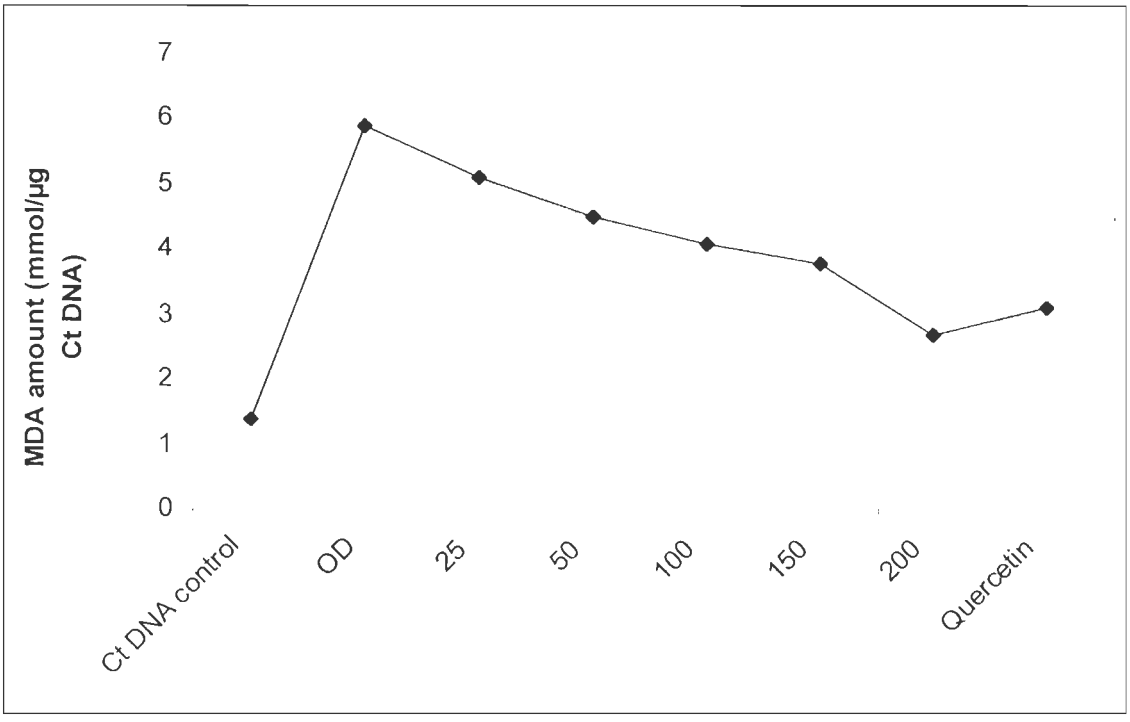


Fig. 5.1 Spectrophotometric determination of MDA generation in Ct DNA induced by MCO in the presence of FFB and positive standard quercetin.

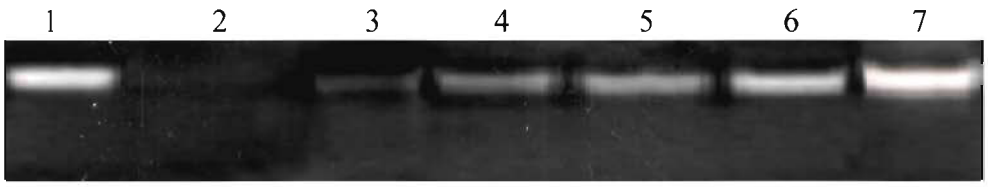


Fig 5.2 (a) Ethidium bromide stained agarose gel photograph showing calf thymus DNA (Ct DNA) damage by Metal catalyzed Fenton system (Fe^{2+} (1mM, 4µL) +H_2O_2 (1mM, 6µL) with or without FFB and standard quercetin. In brief, Lane 1.(Ct DNA only (0.5 µg), Lane 2.Reaction mixture (Ct DNA+MCO), Lane 3-6.(Reaction mixture + 10, 20, 40 and 60 µg per mL of FFB), Lane 7.(Reaction mixture + quercetin.50 µg per mL).

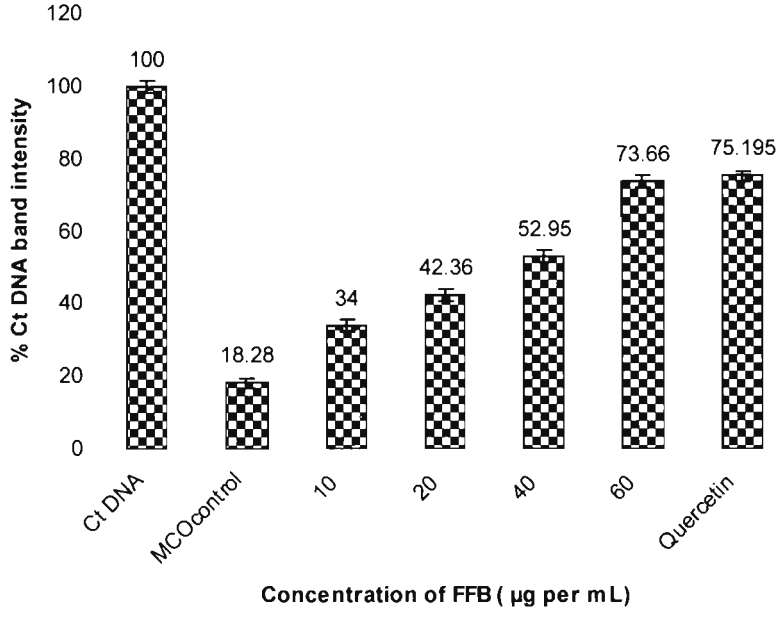


Fig 5.2 (b) Densitometric analysis of loss in calf thymus DNA band intensity in the presence of MCO and varied concentration of FFB fraction.

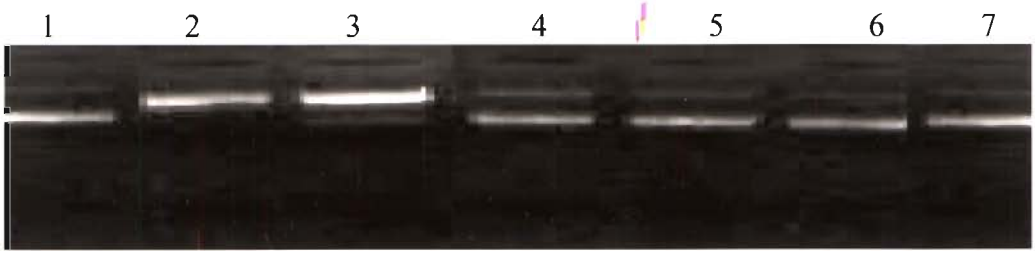


Fig 5.3 (a) Showing nicking of pUC18 induced by Metal catalyzed Fenton system (Fe^{2+} (1mM, 2µL) +H_2O_2 (1mM, 4µL). In brief, Lane 1.plasmid only (0.5 µg), Lane 2.Reaction mixture (plasmid+MCO), Lane 3-6.(Reaction mixture + 10, 20, 40 and 60 µg per mL of FFB), Lane 7.(Reaction mixture + quercetin. 50 µg per mL).

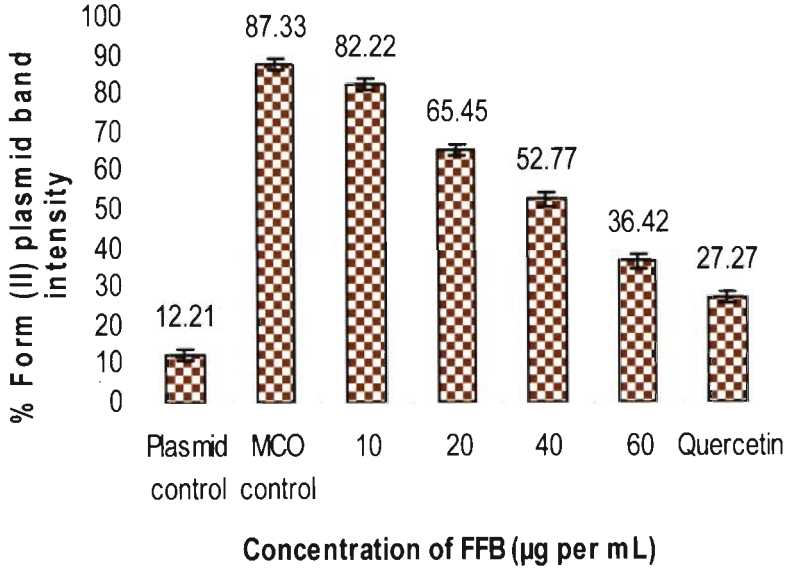


Fig 5.3 (b) Densitometric analysis of nicking of pUC18 plasmid in the presence of MCO and varied concentration of FFB fraction.

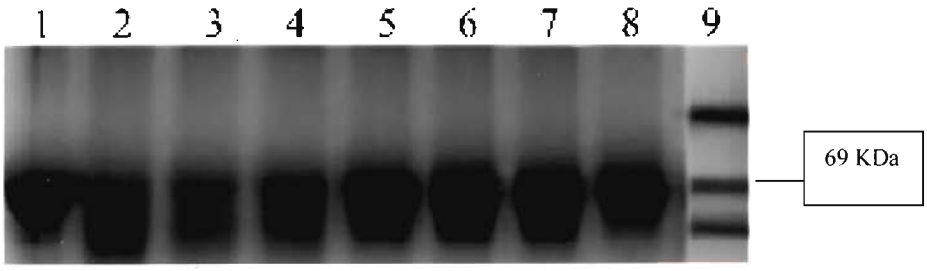


Fig 5.5 (a) Shows the SDS-PAGE results from BSA oxidation due to MCO system along with FFB and glutathione. Lane1. Contol BSA, Lane 2. BSA + MCO, Lane 3-7. BSA + MCO + FFB (50, 100, 150, 200, 250 μg per mL), Lane 8. BSA + MCO + glutathione (200 μg per mL), Lane 9. Molecular Marker.

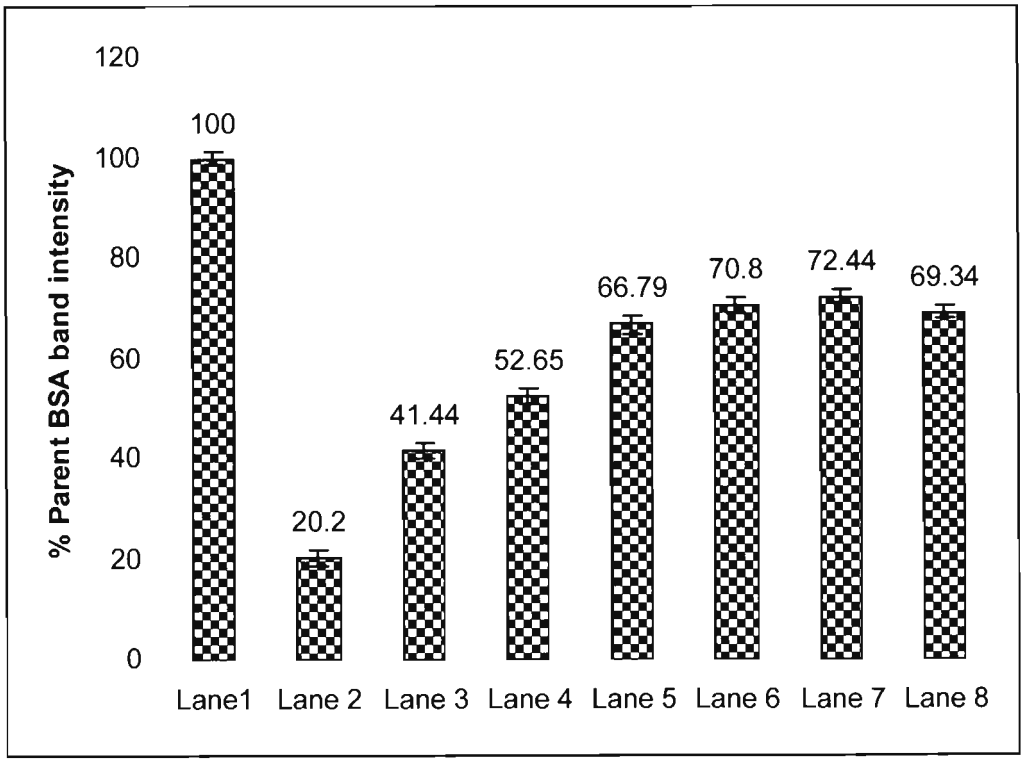


Fig 5.5 (b) Densitometric BSA band intensity analysis of control and oxidized BSA samples with or without FFB and glutathione compounds.

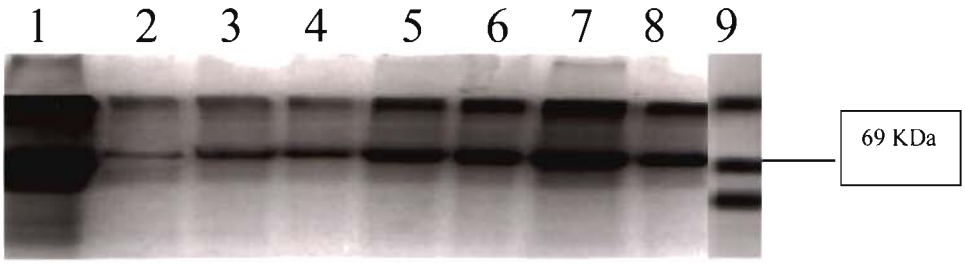


Fig 5.5 (c) Shows the SDS-PAGE results from BSA oxidation due to HOCl mediated oxidative system along with FFB and glutathione. Lane 1. Contol BSA, Lane 2. BSA + HOCl, Lane 3-7. BSA + HOCl + FFB (50, 100, 150, 200, 250 μg per mL), Lane 8. BSA + HOCl + glutathione (200 μg per mL), Lane 9: Molecular Marker.

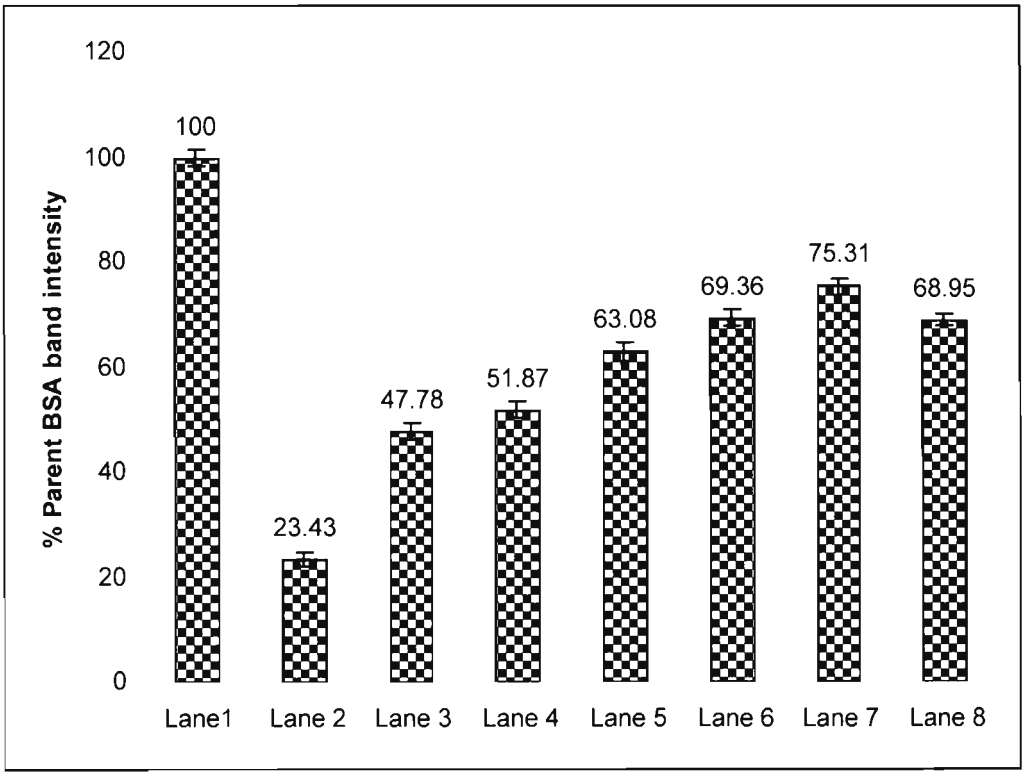


Fig 5.5 (d) Densitometric BSA band intensity analysis of control and oxidized BSA samples with or without FFB and glutathione compounds.

Table 5.1 Lipid peroxidation inhibition potential of FFB constituents from *F. bengalensis* against Ascorbic acid/Fe^{2+} oxidation system induced lipid peroxidation in rat brain homogenate.

Sample	**Fe^{2+} / Ascorbic acid induced Lipid peroxidation in rat brain homo**		
	MDA (nmol per mg protein)	I% a	IC_{50}
Control	3.59±0.29	-	FFB IC_{50}=56.96± 0.66 (µg per mL) GSH IC_{50}=126.14±4.38 (µg per mL)
FFB (25 µg per mL)	2.814±0.27**	21.74	
FFB (50 µg per mL)	1.90±0.15**	47.05	
FFB (100 µg per mL)	1.38±0.08**	61.49	
FFB (100 µg per mL)	1.23±0.24**	65.77	
FFB (200 µg per mL)	0.826±0.17**	77	

* The values are expressed as mean ± SD (n=3). TBARS were expressed by nmol of MDA produced in the presence of varied concentration of FFB 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.

Chapter 6

In vivo Hepatoprotective Effect of FFB from *F. bengalensis* against CCl_4 Induced Rat Liver Oxidative Damage

6.1 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). A dysfunctional liver cannot perform its tasks properly and consequently the body becomes subjected to toxicity resulting in an overall decline in metabolic function. 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). Thus, the drug induced hepatotoxicity has now become a significant cause of acute liver failure accounting for 50% cases. Imbalance in the pro-oxidant/antioxidant equilibrium in favor of pro-oxidant constitutes the oxidative stress condition which leads to heptotoxicity and associated with a number of pathophysiological events in the liver (Jaeschke *et al.,* 2002; Halliwell, 2007; Watkin and Seef, 2006; Tanikawa and Torimura, 2006). On the basis of above observations, liver protection has been a subject of considerable interest from biomedical perspective as it plays a crucial role in all aspects of metabolism and overall health (Seef *et al.*, 2001). Therefore, there is an ardent need to provide protection against toxicity and various liver disorders. Carbon tetrachloride (CCl_4)-induced liver damage has been extensively studied and widely used as a liver injury model to treat animals because CCl_4 induced hepatotoxicity is regarded as the analogue of liver damage caused by a variety of hepatotoxins in humans (Cesaratto *et al.,* 2004; Manibusan *et al.*, 2007). It is well established that a correlation exists between CCl_4 induced hepatotoxicity and oxidative stress. Therefore, CCl_4-induced liver damage is being used as a model for screening hepatoprotectors. Since the damaging effects of CCl_4 are oxidative stress-mediated, numerous investigations have been carried out on the hepatoprotective effects of antioxidants (Tirkey *et al.*, 2005; Lee and Jeong, 2007). Many previous studies have revealed that antioxidants prevent CCl_4 toxicity, particularly hepatotoxicity, by inhibiting lipid peroxidation, suppressing alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and increasing antioxidant enzyme activity (Kuriakose and Kurup, 2008; Balahoroglu *et al.*, 2008; Khan and Sultana, 2009). So, antioxidant compounds are currently being investigated as a therapeutic strategy in different liver pathologies. At present, in spite of an increasing need for agents to protect the liver from damage, modern medicine lacks a reliable liver

protective drug. Also, there is growing focus on the hepatoprotective substances from natural products because of severe side effects of synthetic agents (Barlow, 1990; Wang *et al.*, 2008; Dani *et al.*, 2008). Plants provide a formidable source of natural products (with pharmacological activity) since they accumulate these antioxidant phytochemicals as secondary metabolites through evolution as a natural means of surviving in a hostile environment. Therefore, a large number of scientific attempts have been made in the recent past to untap this vast reservoir of potentially useful phytochemicals from different plant sources and establish their protective role against oxidative stress induced liver damage (Stickel and Schuppan, 2007; Singh *et al.*, 2008; Park *et al.*, 2008; Guttierez and Solis, 2009). In the present research work, the active antioxidant constituents FFB from *Ficus bengalensis* was evaluated for its protective effects in CCl_4 induced oxidative damage to rat liver and their possible mechanism in different aspects of study at biochemical, histological and molecular level.

6.2 MATERIAL AND METHODS

6.2.1 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., Surat, India. The 2', 7'-dichlorofluorescein diacetate (DCFH-DA) was purchased from Sigma Chemical Co (St Louis MO, USA).

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

Thirty six healthy adult male Wistar strain albino rats weighing 200–280 g obtained from Indian Vetinary Research Institute (IVRI), Izatnagar, Bareilly, India were used in this study. All animals were housed in polypropylene cages (4 animals per cage), kept under standard 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 ad libitum. All rats were allowed to acclimatize for a week before commencement of experiment. All experimental procedures involving animals were reviewed and approved by the animal ethical committee of Department of Biotechnology, IIT Roorkee, Roorkee, India.

The animals were randomly divided into six groups with six rats in each and their initial body weights were taken and recorded. Group 1 served as control 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 (1 ml per kg. bw per week) for four weeks, which is well documented to induce hepatotoxicity. The first dose of CCl_4 was given in Group 3, 4 and 5 after one week of pretreatment with hepatoprotective agent FFB and silymarin. The FFB and silymarin were dissolved in physiological saline and then administered to Group 3 (FFB 50 mg per kg. bw, p.o), Group 4 (FFB 100 mg per kg.bw, p.o), Group 5 (Silymarin 75 mg per kg. bw, p.o), Group 6 (FFB 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 Group1 (ordinary control) and Group 6. The design and treatment performed *in vivo* experiment is summarized in Table 6.1.

6.2.3 Determination of total body weight, absolute liver weight and relative liver weight of rat under CCl_4 toxicity

The changes in total body weight, liver weight and relative liver weight were determining gravimetrically in different treatment groups.

6.2.4 Effects of FFB pretreatment on oxidative stress markers and antioxidant status level in blood serum and liver tissue

6.2.4.1 Preparation of blood serum

At the end of experiment, each rat was weighed and then anaesthesized in a chloroform saturated chamber. The thoracic region was opened to expose the heart. Blood samples were collected by direct cardiac puncture by means of a 5 mL hypodermic syringe and needle and placed in ice-cold 5 mL sample tubes. The blood samples were allowed to stand at 25°C for 30 min to allow clotting. Samples were centrifuged at 3000 x rpm for 10 min at 4°C. The collected blood serum was stored at −20°C for further analysis.

6.2.4.2 Preparation of rat liver homogenate

Entire liver tissues were quickly removed after animal sacrifice, rinsed with ice-cold physiological saline, blotted dry and weighed. After that small pieces of liver were frozen in liquid nitrogen and stored at −80°C until further biochemical analysis. To

prepare rat liver homogenate, 200 mg of liver tissue samples were homogenized with 2 ml (w/v 1:10) of 0.1 M phosphate buffer (containing 1 mM EDTA) pH 7.4; in a Potter–Elvehjem homogenizer. The rat liver homogenate samples were centrifuged at 12,000 X g for 30 min at 4°C. The supernatant of rat liver homogenate was collected and stored at −80°C for further analysis.

6.2.4.3 Evaluation of oxidative stress marker enzyme level in blood serum of experimental rats

Biochemical estimation of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) (Reitman and Frankel, 1957) and serum alkaline phosphatase (ALP) (King and King, 1954); lactate dehydrogenase (LDH) (Bergmeyer, 1965) were done as per methods described. Total Bilirubin, total triglycerides, total cholesterol were performed using biological kits (Span Diagnostics Pvt. Ltd., Surat, India) as per manufacturer's instruction. Total protein estimation in blood serum was done as per standard protocol (Bradford, 1976).

6.2.4.4 Evaluation of antioxidant status in rat liver tissue

The rat liver homogenate from different treatment groups were evaluated for their superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971), catalase (CAT) (Aebi, 1984) and reduced glutathione content (GSH) (Ellman, 1959) as levels of endogenous antioxidants system. Total protein concentration in liver tissue homogenate was determined using Bradford method (Bradford, 1976). Bovine serum albumin was used as standard. For CAT enzyme assay, 500 µL liver tissue homogenate was incubated with 5 µL ethanol for 30 min on ice. Mixture was treated with 5 µL of Triton X-100 (to a final concentration of 1%) and vortexed. 10 µL of the above sample was diluted with buffer and 5 µL of diluted sample was mixed with 495 µL of buffer, 60 µL H_2O_2 (10 mM) and 440 µL of distilled water. The decrease in absorbance was monitored at 240 nm for 5 min at 25°C. One unit of CAT activity is that which reduces 1 µmol of hydrogen peroxide per min. The CAT 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 superoxide radical (Beauchamp and Fridovich, 1971). 500 µL of supernatant, 1 mL of 50 mM of sodium carbonate , 400 µL of 25 µM NBT and 200 µL of 0.1 mM EDTA. Reaction was initiated by hydroxylaminehydrochloride. Change in absorbance was

measured at 560 nm. Control contains all ingredients except sample. Glutathione (GSH) content in the liver homogenates was determined according to the method described by Ellman (1959). Equal volume of tissue homogenate (double diluted) was mixed with 20% TCA (containing 1 mM EDTA) and mixture was allowed to stand for 5 min on ice. Mixture was centrifuged at 200 rpm for 10 min. 200 µL of supernatant was mixed with 1.8 mL of Ellman's reagent (0.1 mM) and volume made upto 2 mL. After completion of reaction absorbance was measured at 412 nm against blank.

6.2.5 Morphological and histopathological study on protective effects of FFB 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 (Hematoxylin and Eosin) stained light microscopy and scanning electron microscopy was conducted to evaluate the histopathological changes during CCl_4 induced toxicity.

6.2.5.1 Histopathological study using H&E staining light microscopy

Histopathological observation of different rat liver tissue was processed for determining the extent of hepatic damage and H&E staining was performed as per the method with slight modification (Krajain, 1963). Liver sections were evaluated for steatosis, inflammation, necrosis, and apoptosis. Fresh liver tissue from adult Wistar rats, cut to approximately 3–5 mm thick pieces and immediately submerged in 10% (v/v) neutral buffered formalin solution for fixation and embedding in paraffin. Formalin fixation of tissue specimens was performed over night (20–24 hours) at 25°C, unless otherwise mentioned. Formalin fixed tissues were dehydrated in 70%, 90%, and 100% ethanol, followed by xylene for 2 hour twice. After dehydration, the tissue was embedded in paraffin Paraplast Plus (Sherwood Medical Co., St. Louis) and infiltration was allowed to proceed for 2–3 hour at 60°C. Paraffin infiltrated tissues were embedded in a mold with liquid paraffin to form a block to facilitate better handling during microtome sectioning. For histopathology, 4-µM-thick paraffin-embedded liver sections were cut and stained with H&E for light microscopy.

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 second 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 400 X magnifications.

6.2.5.2 Protective effects of FFB pretreatment on surface morphology study of rat liver under toxicity by scanning electron microscopy

Liver tissue samples for scanning electron microscopy (SEM) were prepared according to standard protocols (Wisse, 1985). Briefly, liver tissues were fixed in 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. SEM-samples were examined under a scanning electron microscope at 30 kV (Leo 435 VP, England). Imaging and morphometric analysis were performed on randomly acquired digitized SEM images at magnifications of 2000X.

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

The extent of ROS formation in the liver homogenate was detected using the fluorescence intensity of oxidant sensitive probe like 2', 7'-dichlorofluorescein diacetate (DCFH-DA), a compound whose fluorescence sharply increases in the presence of oxidizing agents. DCFH-DA diffuses through the liver cell membrane and is enzymatically hydrolyzed by intracellular esterases to non fluorescent DCF-H, which is then rapidly oxidized to the highly fluorescent DCF in presence of ROS. The DCF is estimated by fluorescent spectrometer. Ten microlitre of liver tissue homogenate was incubated with 10 µL DCFH-DA (5 mM, 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 (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 fluorescence intensity (UF) per g liver tissue.

6.2.7 Protective effects of FFB 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 (Burton, 1968) and DNA laddering experiment.

6.2.7.1 Quantitative DNA fragmentation assay

Quantitative estimation of DNA fragmentation was determined by calorimetric diphenylamine assay as described (Burton, 1968). Liver samples from different groups were homogenized in chilled lysis buffer (10 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates (1 mL) were then centrifuged at 27,000 x g for 20 min to separate intact DNA in the pellet from the 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 and then centrifuged at 1500 x g for 10 min to remove proteins. Resulting supernatants, whether containing whole or fragmented DNA, were left to react with diphenylamine (0.088M) for 16-20 hours (25°C ± 0.02). Absorbance was measured at 600 nm. DNA fragmentation was expressed as a percentage of total to fragmented DNA. The protective effects of FFB were expressed as percentage of DNA fragmentation observed in comparison to CCl_4 treated control group.

6.2.7.2 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 (3g) 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 1000 x g for 10 min. The supernatant was removed to waste and the pellet re-suspended 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, 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 1000 x *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/mL = 1.0 absorbance unit at 260 nm. The concentration of DNA isolated from liver samples (3 g) was 1.9 ± 0.82 mg/g liver. The purity of DNA was determined from absorbance ratios $A_{230/260}$ and $A_{260/280}$.

6.2.8 Protective effect of FFB on lipid peroxidation level in rat liver intoxicated with CCl_4

The importance of lipid peroxidation in liver tissue induced by CCl_4 is extensively studied. To demonstrate the protective effect of FFB 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 the hepatic content of MDA and *in vivo* lipid peroxidation detection using Schiff's staining. The hepatic content of MDA is taken as a reliable biochemical index of lipid peroxidation in liver tissue.

6.2.8.1 Spectrophotometric determination of total MDA in rat liver tissue

Peroxidation was measured as the production of malondialdehyde (MDA), which in combination with thiobarbituric acid (TBA) forms a pink chromogen compound whose absorbance at 532 nm was recorded. 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 containing (1 mg protein) was mixed with 1 mL 20% TCA and 2 mL 0.67% TBA and heated for an hour at 100°C. After cooling, precipitate was removed by

centrifugation at 1000 x g for 10 min. The absorbance of the supernatant was measured at 535 nm against a blank that contains all the reagents except the tissue homogenate. The total MDA content was calculated with the help of calibration curve obtained from the reaction between varying MDA (1, 1 Tetraethoxy propane) concentrations and TBA given in 5.7 b and expressed in terms of nmol MDA per mg protein.

6.2.8.2 Histochemical detection of lipid peroxidation

Histological study on lipid peroxidation was performed as described by Pompella *et al.* (1987). In brief, tissue sections were stained with freshly prepared Schiff's reagent for an hour at 25°C, which detects aldehydes that originate from lipid peroxides. After the reaction with Schiff's reagent, tissue sections were rinsed in two changes of bisulfite solution (0.5% [w/v] $K_2S_2O_5$ in 0.05 M HCl) for 2-3 min each. After brief rinsing in distilled water, they were dehydrated in alcohol series 40%, 95% and 100% for approximately 1 min each, cleared in xylene and mounted in mounting medium. Tissue sections were visualized under light microscope to observe the extent of tissue staining.

6.2.9 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:

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

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

6.3 RESULTS

Evidences developed over the last few years have suggested that oxidative stress plays a central role in the development of acute liver toxicity by CCl_4 mediated free radical generation. Since free radical mediated oxidative stress condition plays a pivotal role in liver disease pathogenesis and progression, use of 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 antioxidant and free radical scavenging properties have been demonstrated to prevent and treat hepatopathies induced by CCl_4 mediated oxidative stress in liver tissue (Wu *et al.*, 2006; Bhaduria *et al.*, 2008).

6.3.1 Effects of FFB pretreatment on CCl_4 induced toxicity in relation to body weight and liver weight

The result of FFB pretreatment on CCl_4 intoxicated rat body weight and liver weight is summarized in Table 6.2. The mean body weight (241.66 ±14.2 g) of carbon tetrachloride treated control group animals was significantly ($P < 0.05$) lower than the untreated control (286.66 ± 16.12 g). Significantly higher absolute (5.96 ± 0.48 g) and relative liver weights (2.46 ± 0.16 g%) were observed in the carbon tetrachloride control rats compared with those in the normal control (4.01 ± 0.21g and 1.39 ± 0.03g %, respectively ($P < 0.05$). The pretreatment with FFB (50 and 100 mg per kg. bw per day) along with CCl_4 administration significantly inhibited the liver weight increase in dose dependent manner up to (31.00% and 46%) compared to CCl_4 intoxicated control group. The standard hepatoprotective agent silymarin showed 55.00 % percent reduction in the increased relative liver weight under CCl_4 induced toxicity. The animals fed with FFB (200 mg per Kg.bw) extract alone had a similar mean body weight and relative liver weight to that of normal control group.

6.3.2 Effect of FFB constituents on serum oxidative stress marker level in CCl_4 intoxicated experimental rat

The protective potential of FFB from *F. bengalensis* against CCl_4 induced liver toxicity was determined by measuring the serum oxidative stress markers like ALT, AST, ALP, LDH and bilirubin level in comparison to the CCl_4 intoxicated group. The results are summarized in Table 6.3. The data interpretation revealed that the rats treated with CCl_4 showed a significant hepatic damage as elicited by increase in levels of all these hepatospecific serum markers. However, a concentration-dependent decrease in their amount in serum was observed in rats pretreated with FFB constituents prior to administration of CCl_4 compared with CCl_4 treated rats alone ($P < 0.05$). Similar trend was also observed with bilirubin level.

6.3.3 Effect of FFB constituents on antioxidant status in liver from different CCl_4 toxicity groups

The status of total antioxidant enzyme status and antioxidant capacity of CCl_4 induced liver toxicity group and FFB treated animal groups were also determined and data are presented in Table 6.4. SOD activity in liver homogenate of CCl_4 treated control group was found to be 49.85 ± 3.86 U/ mg protein lower than 92.78 ± 4.38 U / mg protein in normal control group. However, a dose dependent increase in SOD activity was observed in rats pretreated with FFB prior to CCl_4 administration and was found to be (63.93 ± 4.37 U per mg protein at 50 mg per Kg b.w) and (76.65 ± 3.47 U per mg protein at 100 mg per Kg b.w); respectively.

Catalase activity in liver homogenate of CCl_4 control groups (85.82 ± 6.97 U per mg protein) was found to be conspicuously lower than in normal control group (220.27 ± 13.31 U per mg protein). Catalase activity in FFB pretreated groups was found to be (185.86 ±8.50 U per mg protein at 50 mg per Kg b.w) and (205.57 ± 7.57 U per mg protein at 100 mg per Kg b.w), respectively. CCl_4 treatment caused significant decrease in GSH level in liver tissue homogenates (1.45 ± 0.075 µmol per g of liver tissue) compared to (5.44 ± 0.135 µmol per g of liver tissue) in normal control group. Pretreatment with FFB in rats prior to CCl_4 administration resulted in pronounced increase in GSH levels with values (2.65 ± 0.23 µmol per g of liver tissue at 50 mg per Kg b.w) and (4.103 ± 0.102 µmol per g at 100 mg per kg b.w). Thus FFB has a positive effect on antioxidant enzyme status and antioxidant capacity.

6.3.4 Effect of FFB pretreatment on histopathological changes in rat liver under CCl_4 toxicity

Hepatoprotective effect of FFB was also investigated by monitoring liver histological parameters. Light microscope photomicrograph of H and E stained rat liver tissue showing protective effects of FFB on CCl_4 intoxicated rat is illustrated in Fig 6.1. No histological abnormalities were observed in control rats (Fig 6.1 a and A). Histology of the liver sections of normal control animals (Group I) showed normal hepatic architecture with well brought out central vein, well preserved cytoplasm and prominent nucleus and nucleolus (Fig. 6.1 a and A). Group II (CCl_4 control) showed loss of the normal liver architecture. Liver tissue histology exhibited intense centrilobular necrosis (N), vacuolization and macrovesicular fatty changes (F), ballooning degeneration and inflammatory infiltration (Fig. 6.1, b and B). Silymarin treated animals showed a closely normal hepatic architecture (Fig. 6.1, c and C). In the present study, we observed that administration of FFB (50 and 100 mg per kg.bw per

day) constituents as a pretreatment to CCl_4 treated groups, demonstrated revival of structural integrity of hepatocytes and less incidence of cellular necrosis, swelling and fatty degeneration in the histopathological study (Fig. 6.1, d and D and e and E, respectively).

Scanning electron microscope (SEM) photograph of rat liver surface is presented in Fig. 6.2 under different CCl_4 toxicity model experiment at 2000X magnification. High level of hepatocellular disorganization and necrosis was observed in CCl_4 treated groups (Fig. 6.2 b) and it was significantly reduced to normal (Fig. 6.2 a) in pretreated animal groups (Fig. 6.2 c and d) and was comparable to Silymarin treated animals (Fig. 6.2 e).

6.3.5 Protective potential of FFB in ROS production during CCl_4 toxicity

The ROS production in CCl_4 treated control and FFB pretreated animal groups was determined using DCFH-DA fluorescent probe. The DCFH-DA fluorescent probe study of *in vivo* CCl_4 intoxicated rat liver is depicted in Fig. 6.3 and the protective effect of FFB is clearly evident. The reactive oxygen species (ROS), such as superoxide anions and H_2O_2, are produced throughout cells during normal aerobic metabolism. A significant reduction in ROS was observed in FFB pretreated animal groups liver homogenate which was (826.47 UF per mg protein at 50 mg per Kg.bw) and (480.16 UF per mg protein at 100 mg per Kg.bw) compared to CCl_4 treated group (1311.65 UF per mg protein).

6.3.6 Protective role of FFB against oxidative damage to DNA during CCl_4 induced toxicity

The agarose gel electrophoresis showing the extent of DNA fragmentation pattern on rat liver genomic DNA under CCl_4 toxicity is depicted in Fig. 6.4 (a). FFB showed a strong inhibition of the DNA fragmentation and the ability of FFB to protect oxidative damage to DNA induced due to CCl_4 toxicity was more or less equivalent to standard silymarin (Fig. 6.4 a, lane 5 and 6, respectively).

The quantitative determination of DNA fragmentation induced by CCl_4 in rat hepatic tissue was also determined spectrophotometrically and result is illustrated in Fig. 6.4 (b). The comparative protective abilities of sylimarin and FFB are also presented. Results showed that CCl_4 administration induced an increase in DNA fragmentation in the rat hepatic tissues reaching approximately 140% of control values.

However concomitant administration of FFB (50 mg per Kg .bw and 100 mg per Kg.bw) significantly decreased hepatic DNA fragmentation whose value reached (124% for 50 mg per Kg.bw) and (116.5 % for 100 mg per Kg.bw) and their results were comparable to that of silymarin treated group.

6.3.7 Effects of FFB pretreatment on lipid peroxidation inhibition during CCl_4 induced toxicity

Fig 6.5 (a), (b) results present the total MDA generation as an index of extent of lipid peroxidation under CCl_4 induced toxicity and its inhibition on pretreatment with FFB from *F. bengalensis*. Rapid enhancement in total MDA levels (F= 4.38 nmol per mg protein, $P < 0.05$), were observed in CCl_4 intoxicated rats. However, pretreatment with 50 and 100 mg per kg.bw. FFB and Silymarin 75 mg per kg.bw. orally for twenty eight days, caused a significant reduction in MDA levels compared with rats treated with CCl_4 alone. The inhibition of lipid peroxidation in FFB treated animals groups was also evident from histological studies and result is presented in Fig. 6.6. The Schiffs stained liver tissue section result is presented in Fig. 6.6. showing extent of lipid peroxidation on rat liver tissue section under CCl_4 intoxication with or without pretreatment with hepatoprotective agent.

6.4 DISCUSSION

In present study the hepatoprotective potential of the antioxidant fraction FFB purified from *F. bengalensis* has been evaluated in CCl_4 induced hepatotoxicity model. Carbon tetrachloride is widely used as a hepatotoxic compound for screening the hepatoprotective activity of several natural antioxidants in experimental model systems (Cessarato *et al.*, 2004; Manibusan *et al.*, 2007). CCl_4 toxicity results from the bioactivation of the CCl_4 molecule to the trichloromethyl free radical by cytochrome P450 isozymes (P450s) in the endoplasmic reticulum (Recknagel and Glende, 1973; Slater, 1984; Recknagel *et al.*, 1989). Once the trichloromethyl radical is formed, it reacts with molecular oxygen to form the highly toxic trichloromethyl peroxy radical (Packer *et al.*, 1978; Slater, 1984). The free radicals then attack polyunsaturated 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 disruption of cell energy processes and protein synthesis (Recknagel *et al.*, 1989). Therefore, rapid and extensive lipid peroxidation of the membrane lipids has been proposed as the basis of CCl_4 hepatocellular toxicity (Basu, 2003).

A number of hepatospecific biomarkers or products have been identified, whose level alters significantly during liver injury (Poli *et al.*, 2008). These include abnormally high level of serum specific markers like ALT, ALP, AST, LDH etc, significant reduction in activities of antioxidant enzymes like SOD and CAT and depletion of important liver protectant like glutathione in liver homogenate and elevation in MDA level, end product of lipid peroxidation in liver tissue. These appear as significant indicators during generation of oxidative stress condition in CCl_4 intoxicated rats. Thus, hepatoprotective potential of any agent or compound may be assessed by measuring their protective effect on these serum and liver parameters.

The body weight decrease in rats after carbon tetrachloride administration was considered to be the result of the direct toxicity of carbon tetrachloride and the indirect toxicity related to liver damage. The inhibition of the body weight decrease by the pre-treatment of FFB was considered as evidence of their efficacy in preventing carbon tetrachloride induced subacute hepatic damage. Direct change in organ weight or organ/body weight ratio has been used as an index of CCl_4 toxicity (Uemitsu *et al.*, 1986). There was slight increase in relative liver weight of CCl_4 alone treated animal group (Table 6.2) compared to normal animal group. The changes in liver weight after carbon tetrachloride dosing are a valuable index of the extent of subacute hepatic damage. However, liver to body weight ratio has been used in the present study since the method has been shown to be a more sensitive indicator of CCl_4 toxicity than absolute liver weight. Therefore, observed increase in relative liver weight or liver to body weight ratio in CCl_4 administered rats have been found to be consistent with those already reported by (Kadiri *et al.*, 2007). It has been widely accepted that during CCl_4 induced oxidative stress condition, there is accumulation of fatty substances like triglycerides and thus attributes for the higher relative liver weight in comparison to normal treatment group (Castro, 1997). The antioxidant constituents demonstrated statistically comparable protective effects to bring the relative liver weight to near normal in comparison to the silymarin treated animal group. It has been observed that disturbances in hepatic lipid homeostasis are one of the multifaceted alterations caused due to CCl_4 toxicity in liver organ (Cessarato, 2004). Thus, the fatty degeneration and accumulation is one of the clear symptom developed during CCl_4 intoxication in rat liver and possibly may be due to alteration in fatty acid metabolism by free radical toxicity condition (Ara *et al.*, 2005; Manibusan *et al.*, 2007). In present study, the

hepatoprotective potential of FFB from *Ficus bengalensis* was evaluated using parameters like changes in liver weight / body weight ratio and body weight gain of rats since they're significant indices of CCl_4 toxicity. The reversal of the CCl_4 induced increase in liver/body weight ratio of rats by the pretreatment of FFB is considered direct evidence of the extracts protective effect in preventing carbon tetrachloride induced subacute hepatic injury and its hepatoprotective role is attributed by its free radical scavenging ability.

In the present investigation, the dose of CCl_4 used, caused liver injury in rats. The rats treated with an overdose of CCl_4 developed significant hepatic damage, which was observed through a substantial increase in the concentration of serum parameters. Pretreatment of the rats with FFB at (50 and 100 mg per kg. bw) prior to CCl_4 administration resulted in a significant protection against CCl_4 induced elevation of serum marker enzymes and was found to be in agreement with the recognized plant derived hepatoprotective agents. The elevated levels of serum marker enzymes (AST, ALT), ALP and LDH in rats treated with CCl_4, indicate cellular leakages and loss of functional integrity of cell membrane in liver. These enzymes are originally present in higher concentration in cytoplasm of liver parenchymal cells. During hepatopathy, they are released into the blood stream due to membrane disruption of hepatic parenchymal cells (Drotman and Lawhorn., 1978; Jaeshke *et al.*, 2004). Oral treatment with silymarin and FFB extract attenuated these increased enzyme activities produced by CCl_4 and a subsequent recovery towards normalization of these enzymes strongly suggests the possibility of FFB being able to improve the condition of the hepatocytes so as to cause accelerated regeneration of parenchymal cells, thus protecting against membrane fragility and decreased leakage of marker enzymes into the circulation. Thus, it is evident that FFB preserved the structural integrity of the hepatocellular membrane and liver cell architecture damage caused by CCl_4, which is further confirmed by histopathalogical studies. Therefore, it may be concluded that the tendency of hepatospecific serum parameters to return towards a near normal level in groups treated with Silymarin and FFB is a clear manifestation of their protective effects against CCl_4 induced oxidative stress related liver pathologies.

In the present study pretreatment with FFB demonstrated increased activity of antioxidant enzymes in rat liver tissue compared to CCl_4 treated animals indicating the efficiency of the extract to act as an antioxidant by preventing the oxidative damage

inflicted on antioxidant system under CCl_4 induced toxicity. Biological systems protect themselves against the damaging effects of oxidants by different means. These include free radical scavengers and chain reaction terminators; enzymes such as SOD, CAT and Glutathione. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical mediated cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Recent studies on the antioxidant properties of flavonoids from various plant extracts revealed their stimulatory action on antioxidative enzymes (Anila and Vijayalakshmi, 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) and reduction in the activity of these enzymes may result in a number of deleterious effects due to accumulation of superoxide radicals and hydrogen peroxide. Oral administration of FFB restored the activities of both catalase and SOD in CCl_4 intoxicated rats. The observed increase in enzyme activities suggests that the FFB have an efficient protective mechanism in response to ROS. Our results also revealed that FFB decreased oxidative stress by preventing excessive accumulation of free radicals and protected the liver from CCl_4 intoxication. CCl_4 intoxication produced significant depletion of GSH content and resultant imbalance in GSH/GSSG ratio. The ratio of GSH to GSSG is considered a more sensitive marker of oxidative stress, and significant decrease in this ratio in animals intoxicated with CCl_4 indicates early stages in oxidative stress phenomenon. The reduced form of GSH becomes readily oxidized to GSSG on interaction with free radicals. Moreover, the depletion of liver GSH content is thought to result from inhibition of GSH efflux across the hepatocyte's membrane (Dahm *et al.*, 1991). This tissue GSH depletion was inhibited by the pretreatment with FFB extract in a dose dependent manner and our results are consistent with earlier reports that suggests 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 the decreased level of the hepatic reduced glutathione (GSH) (Bleibel *et al.*, 2007; Hamza, 2007; Hegde and Joshi, 2009).

The role of CCl_4 intoxication in development of cellular necrosis and morphological alteration is well known. Such morphological alterations during CCl_4

induced oxidative damage have been suggested due to fatty deposition, hepatocytes structural disorganization and cellular swelling (Manikbusan *et al.*, 2007). The observations in histopathological study employing light microscopy and SEM analysis clearly exhibited less incidence of alterations in rat liver surface morphology, hepatocellular necrosis, fatty degeneration and inflammatory cell infiltration in FFB pretreated groups. Histopathological studies showed that CCl_4 caused steatosis and hydropic degeneration of the liver tissue whereas study of liver morphology suggests that CCl_4 either affects the endothelial lining directly resulting in the loss of the integrity of the local sinusoidal liver architecture; or alternatively, it might activate the local liver-associated macrophage population (i.e., the Kuffer cells) that in turn release cytotoxic substances which cause the acute endothelial vascular and parenchymal tissue damage. Consistent with this view, it is generally accepted that the increased release of pro-inflammatory mediators and reactive oxygen species by stimulated liver macrophages causes severe and acute liver damage. The results from histopathological and morphological study supported the hepatoprotective activity of FFB constituents against CCl_4 induced toxicity, which is also confirmed from the results of biochemical studies. It is firmly believed that lipid peroxidation of hepatocyte membrane leads to changes in hepatic architecture; further regarded as principal cause of CCl_4 induced hepatotoxicity (Basu, 2003; Weber *et al.*, 2003). The protective potential of FFB were comparable with those of silymarin pretreated group, a proven hepatoprotective (Fraschini *et al.*, 2002). The improved histology of the liver as seen in histopathological observations on animals treated with the FFB as compared to that seen in animals administered only CCl_4 indicated the possibility of being able to induce accelerated regeneration of the liver. Pretreatment with FFB could also revert acute structural and pathophysiological damage of the liver sinusoidal endothelial lining to normal within days. It is known that some flavanoids are able to reduce xenobiotic-induced hepatotoxicity in animals (Tirkey *et al.*, 2005; Hwang *et al.*, 2007). The inhibitory activity of flavanoids on free radical production could be related to their hepatoprotective effects since exogenous antioxidants may counteract the damaging effects of oxidative stress, cooperating with natural systems like glutathione, tocopherol or protective enzymes. It is suggested that FFB may play a role in stabilizing the plasma membrane as was reported in case of several plant derived hepatoprotective agents (Bhadauria *et al.*, 2008).

A large number of evidences pointed to the potential involvement of oxidative stress in CCl_4 induced liver toxicity mediated by production of ROS like trichloromethylperoxyl etc. Exposure to CCl_4 can transform the redox state to a more oxidising environment and enhance the ROS level. Therefore, measurement of ROS level not only determine the extent of oxidative damage, but also demonstrate the efficiency of plant extract aimed at reducing ROS mediated oxidative stress (Ghosh and Sil, 2007). Our data suggest the potential capacity of the FFB to reduce basal ROS production in liver homogenate under oxidative stress condition. Although, it is suggested that the effects of FFB as well as silymarin a standard hepatoprotectant in reducing DCF fluorescence maybe associated with their scavenging ability of free radicals resulting in a conspicuous decrease in the level of ROS, however we cannot exclude the possibility that they can also scavenge the DCF semiquinone free radical intermediates (oxygen radical) produced during the formation of the fluorescent product DCF.

The bioactivation of CCl_4 can generate a large number of toxic principles; a very common one being $CCl_3^{\cdot}$ which can produce cellular injury by targeting damaging acts on target biomolecules inside liver cells (Slater, 1984). 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 *et al.,* 2006). In earlier experiments, the oxidative damage to biomolecules has been widely viewed as an important parameter to evaluate the ability of hepatoprotective agents to protect liver cells against hepatotoxin induced oxidative damage which also includes CCl_4 toxins (Orhan *et al.*, 2007; Lin *et al.*, 2008). The ability of FFB to protect against 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 and agarose gel electrophoresis have been used to assess the protective nature of FFB against CCl_4 induced oxidative DNA damage. It is well established that CCl_4 induced toxicity condition generates a number of deadly free radicals and thereby 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 parameter (Amin and

Hamza, 2007; Ahmad and Fatani, 2007). In the present study FFB exhibited significant reduction in hepatic DNA damage, suggesting their possible role as a hepatoprotectant. They have exerted their hepatoprotective activity and that is due to its free radical scavenging ability.

Lipid peroxidation, an important indicator of oxidative damage of biological tissues, was found to be induced in rats exposed to hepatotoxins. Carbon tetrachloride is one of the most widely used hepatotoxins in the experimental study of liver diseases. The hepatotoxic effects of CCl_4 is mediated by its active metabolite, trichloromethyl radical which covalently binds to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides, which in turn give products like malondialdehyde (MDA) that cause damage to the membrane. This lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl_4 (Basu, 2003). MDA a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid (Abd Ellah *et al.,* 2007) is a main marker of endogenous lipid peroxidation and its determination by thiobarbituric acid (TBA) is one of the most common methods in lipid peroxidation studies (Esterbauer and Cheeseman, 1990). 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). Similar to the earlier studies malondialdehyde (MDA) level in liver tissue increased substantially upon CCl_4 intoxication in present study (Shi *et al.*, 2006; Koneri *et al.*, 2008). However, it was observed that that if the liver were pretreated with FFB, MDA level decreased distinctly as evident from spectrophotometric determination and Schiffs staining on liver tissue sections (Fig. 6.5 and 6.6). The pretreatment with extract has prevented oxygen free radicals and thereby prevented the formation of peroxy radicals. This aspect of FFB extract also contributes to the hepatoprotectivity. The increase in MDA levels in liver of CCl_4 treated groups suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. In the present study, pretreatment with FFB significantly reduced the increased amount of MDA during CCl_4 induced toxicity. Hence it is possible that the mechanism of hepatoprotection of FFB is due to its antioxidant effect. Since most of the hepatotoxins including CCl_4 inflict oxidative damage to liver mainly by enhancing lipid peroxidation directly or indirectly;

pretreatment with FFB resulted in significant dose dependent decrease in MDA concentration and could effectively protect liver against lipid peroxidation induced by CCl_4.

The above observations and discussion clearly reveals that free radical mediated oxidative stress condition plays a pivotal role in pathology and progression of liver diseases. Therefore, the compounds or agents with ability to scavenge free radical or inhibit their formation, metal chelation property particularly Fe^{2+} may serve as effective hepatoprotective agents (Nazmi *et al.*, 2005; Ozsoy *et al.*, 2008). A large number of antioxidants from plant sources of medicinal and dietary importance with powerful antioxidant potential have been demonstrated to deliver preventive role in hepatopathies induced by CCl_4 mediated oxidative stress in liver tissue (Vitaglione *et al.*, 2004; Bhaduria *et al.*, 2008). A number of plants hepatoprotective activities was found to be due to their glycosides (Xi *et al.*, 2008; Lin *et al.*, 2008); flavonoids (Wu *et al.*, 2006; Park *et al.*, 2008) and phenolic compounds (Orhan, 2007; Dani *et al.*, 2008). Since the FFB found to have strong free radical scavenging and metal chelating ability and their active constituents found to be flavonoids. Thus it is suggested that potential hepatoprotective activity of FFB observed in the present study is also due to their flavonoid constituents.

CONCLUSION

CCl_4 induces a marked oxidative stress in rat liver which is amenable to attenuation by FFB treatment. This was confirmed from the reduction of the elevated levels of the serum marker enzymes ALT, AST, ALP and LDH; increase in the antioxidative status; inhibition of the CCl_4 induced damage to liver tissues as revealed from histopathological and SEM studies; and by inhibition of DNA fragmentation. The efficacy was almost similar to the well known hepatoprotective agent silymarin. It is suggested that the hepatoprotective effects of FFB may be due to its antioxidant potential that is its free radical scavenging and metal chelation abilities. The antioxidant potential may be attributed to the presence of flavonoid compounds. Thus FFB might play a crucial role in controlling the tissue damage caused by CCl_4 mediated by reactive oxygen species. When FFB constituents were fed alone, there was no sign of any *in vivo* toxicity effects at biochemical, histological and behavioural level. Therefore, it may be concluded that FFB from *Ficus bengalensis* could serve as a

source of potential liver protective as well as potential anti-lipid peroxidative and antioxidant agent against CCl_4 induced liver damage in rats without any *in vivo* toxicity effects.

Table 6.1 Experimental design and different treatment groups

Treatment group	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=6)	FFB (50 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2	FFB (50 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2	FFB (50 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2	FFB (50 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2
Group 4 (N=6)	FFB (100 mg per kg.bw p.o per day)+CCl_4 Dose given in Group 2	FFB (100 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2	FFB (100 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2	FFB (100 mg per kg.bw p.o per day)+CCl_4 dose given in Group 2
Group 5 (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 dose given in Group 2	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 dose given in Group 2
Group 6 (N=6)	VEH+ FFB (200 mg per kg.bw) per day	VEH+ FFB (200 mg per kg.bw) per day	VEH+ FFB (200 mg per kg.bw) per day	VEH+ FFB (200 mg per kg.bw) per day

Table 6.2 Study on body weight, absolute liver weight and relative liver weight of CCl_4 treated rats with or without the pretreatment of FFB constituents.

Group/treatment	0 days of treatment	28th day of treatment		
	Body weight a (g) a	Body weight (g) a	Absolute liver weight (g) a	Relative liver weight (g %)
Normal Control	243.33 ± 23.30	286.66 ± 16.12	4.01 ± 0.21	1.39 ± 0.03
CCl_4 Control	268.33 ± 9.27*	241.66 ± 14.2*	5.96 ± 0.48*	2.46 ± 0.16*
FFB (50 mg per kg bw)+CCl_4	211.66 ± 7.26**	260 ± 10.22**	5.29 ± 0.2**	2.03 ± 0.014**
FFB (100 mg per kg bw)+CCl_4	232.33 ± 8.33**	278.3 ± 8.3**	5.10 ± 0.15**	1.83 ± 0.11**
Silymarin (75 mg per kg.bw)+CCl_4	230 ± 5.56**	266.6 ± 5.3**	4.52 ± 0.22**	1.69 ± 0.06**
Vehicle + FFB (200 mg per kg .bw)	258.33 ± 8.33	283.33 ± 8.33	4.11 ± 0.17	1.45 ± 0.01

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 6.3 Protective effect of FFB constituents on serum oxidative stress markers AST, ALT, ALP, LDH and Bilirubin levels of CCl_4 intoxicated 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	26.48 ± 1.31	56.18 ± 4.98	13.22 ± 0.29	120.41 ± 10.73	0.92 ± 0.02
CCl_4 Control	71.81 ± 2.19*	848.32 ± 16.80*	57.61 ± 2.47*	843.19 ± 18.90*	4.93 ± 0.19 *
FFB (50 mg per kg bw)+CCl_4	40.76 ±1.15**	517.32±14.30**	24.79±1.03**	560.70±12.79**	3.37 ± 0.21**
FFB (100 mg per kg bw)+CCl_4	32.14±1.16**	251.21±13.80**	18.52 ± 0.45**	292.52±10.98**	2.28 ± 0.13**
Silymarin (75 mg per kg.bw)+CCl_4	33.92±1.02**	135.62±12.40**	17.09 ± 0.15**	188.32 ± 7.67**	1.87 ± 0.14**
Vehicle+ FFB (200 mg per kg .bw)	27.19 ± 1.58	62.18 ± 3.84	13.82 ± 0.72	105.29 ± 6.18	0.821 ± 0.02

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 6.4 Effects of FFB 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/g of liver)	Total protein (mg protein per mL)
Normal Control	92.78 ± 4.38	220.27 ± 13.31	5.44 ± 0.14	110.75 ± 4.25
CCl_4 Control	49.85 ± 3.86*	85.82 ± 6.97*	1.45 ± 0.08*	97.52 ± 5.05*
FFB (50 mg per kg.bw)+CCl_4	63.93 ± 4.37**	185.86 ±8.50**	2.65 ± 0.23**	103.23±4.75**
FFB (100 mg per kg bw)+CCl_4	76.65 ± 3.47**	205.57±7.57**	4.103±0.10**	107.30±3.22**
Silymarin (75 mg per kg.bw) + CCl_4	86.57 ± 4.38**	148.47±9.95**	4.40 ± 0.11**	112 ± 3.51**
Vehicle + FFB (200 mg per kg .bw)	90.63 ± 5.32	323.65 ± 16.30	4.63 ± 0.17	114.77 ± 3.44

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 6.5 Total protein, cholesterol and triglycerides level in serum of rat during CCl_4 toxicity and their protection in the presence of FFB constituents.

Treatment Group	Total protein (mg protein per mL)	Total triglycerides (mg per dL)	Total Cholesterol (mg per dL)
Normal Control	0.81 ± 0.03	79.44 ± 3.66	112 ± 6.90
CCl_4 Control	0.57 ± 0.03*	201.93 ± 4.78*	192.74 ± 11.40*
FFB (50 mg per kg bw)+CCl_4	0.61 ± 0.02**	93.15 ± 3.42**	139.76 ± 6.78**
FFB (100 mg per kg bw)+CCl_4	0.75 ± 0.03**	84.41 ± 4.16**	121.22 ± 4.14**
Silymarin (75 mg per kg.bw)+CCl_4	0.79 ± 0.03**	85.68 ± 5.01**	124.69 ± 5.88**
Vehicle+ FFB(200 mg per kg .bw)	0.79 ± 0.02**	61.1 ± 1.43**	31.72 ± 3.76**

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, $p < 0.05$.

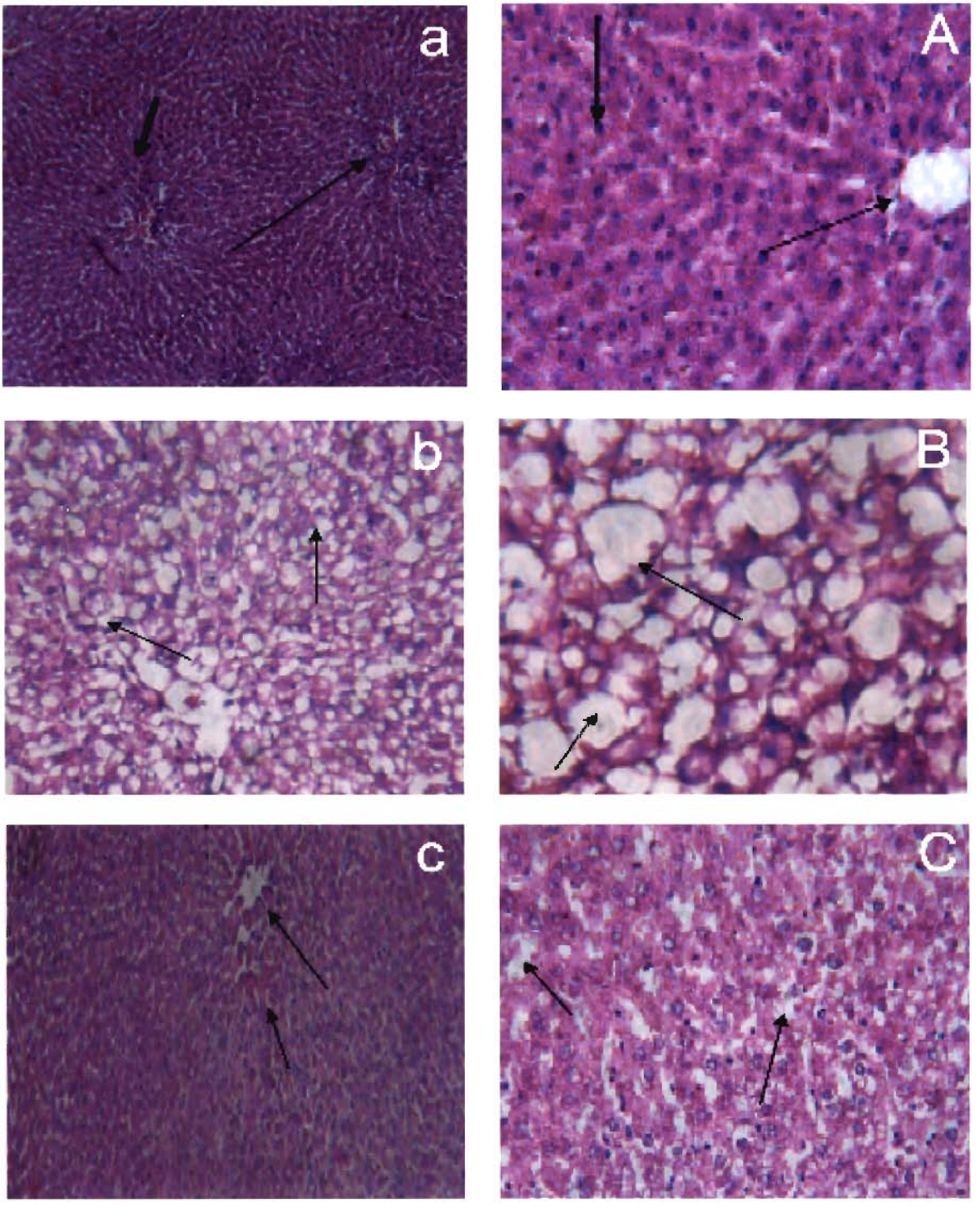


Fig 6.1 Light microscope photomicrograph of H&E stained rat liver tissue showing protective effects of FFB on CCl_4 intoxicated rat. Photograph [(a) Normal control (100X), (A) Normal control (400X)] Normal arrangement of hepatocytes and sinusoids; [(b) CCl_4 treated control (100X), (B) CCl_4 treated control (400X)] centrilobular necrosis, fatty deposition, hepatocellular ballooning degeneration with multiple vacuolation, enlargement and collapse of sinusoidal walls, and inflammatory cell infiltration; [(c) CCl_4+silymarin 75 mg per kg.bw (100X), (C) CCl_4+silymarin 75 mg per kg.bw (400X)] less symptom of hepatocellular damage with improved hepatocellular arrangement.

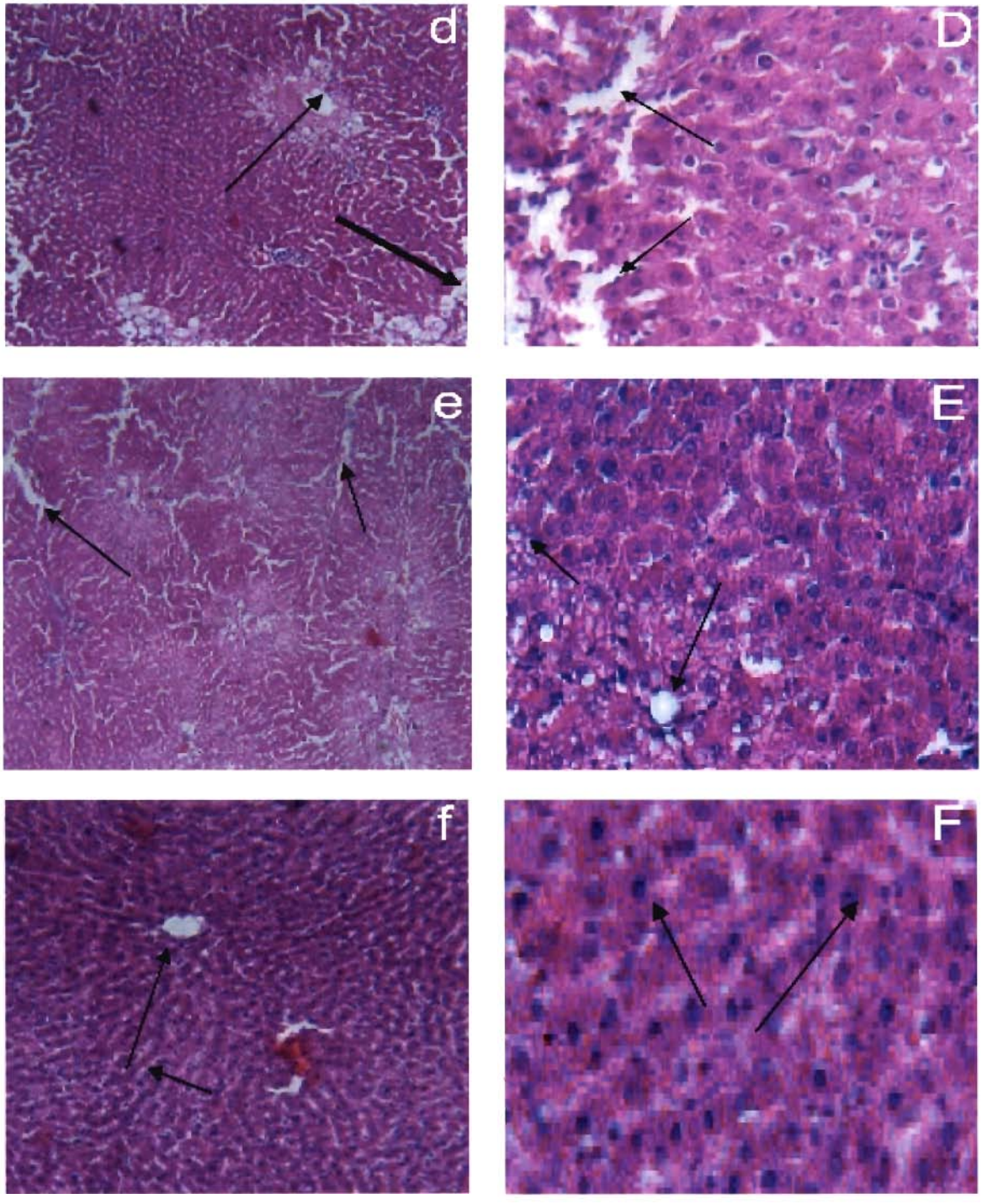


Fig. 6.1 Continued. In the continuation of histological study, photograph [(d) CCl_4+FFB 50 mg per Kg bw (100X), (D) CCl_4+FFB 50 mg per Kg.bw (400X)] mild ballooning, moderate necrosis and mild inflammatory infiltration; [(e) CCl_4+FFB100 mg per kg.bw (100X), (E) CCl_4+FFB100 mg per kg.bw (400X)] improved hepatocytes arrangement and mild observation of cellular necrosis and ballooning and fatty deposition; [(f) Only FFB 200 mg per kg.bw (100X), (F) Only FFB 200 mg per kg.bw (400X)] Normal hepatic architecture without any hepatocellular damage.

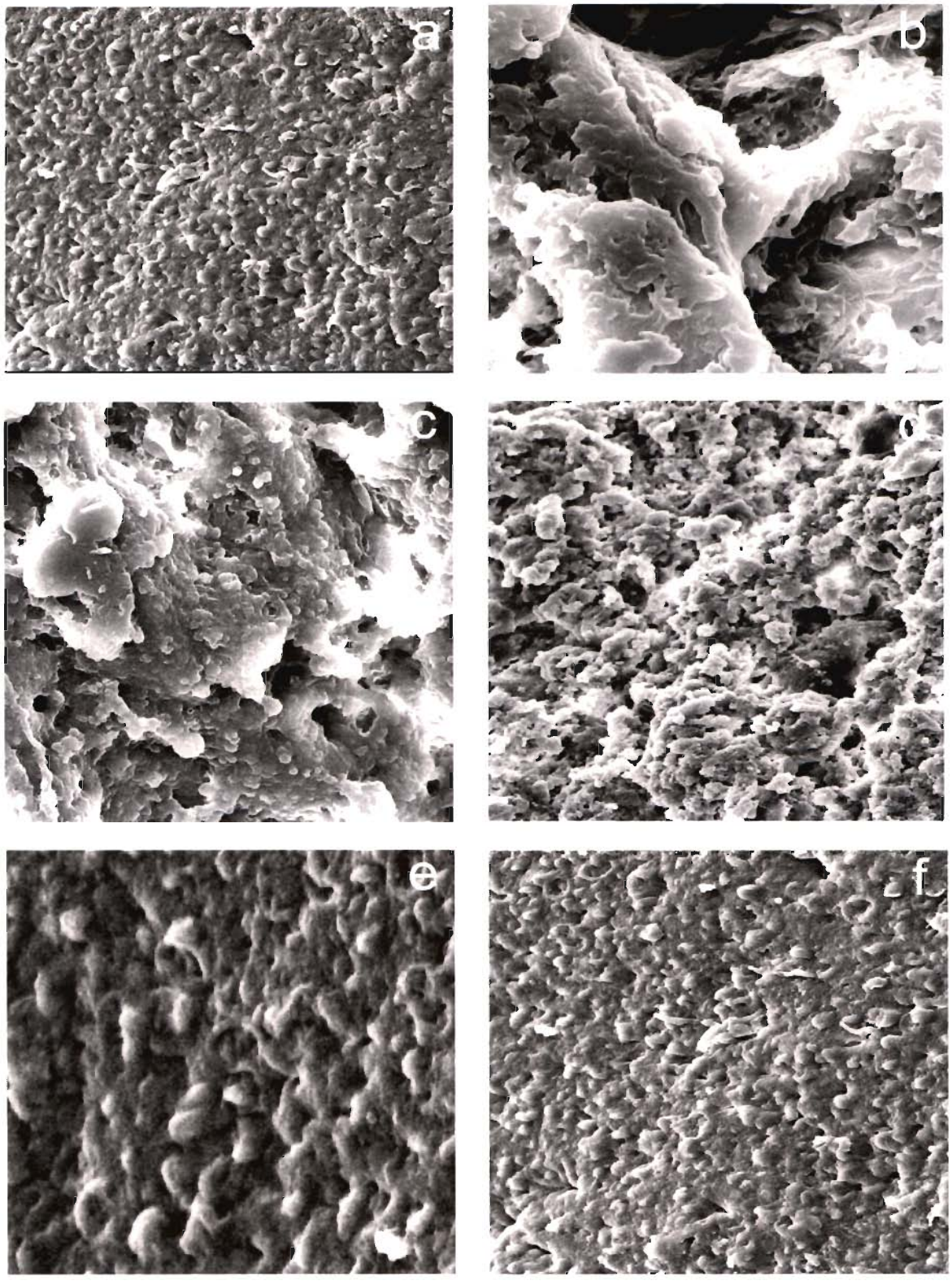


Fig 6.2.Scanning electron microscope photograph of rat liver surface under different CCl_4 toxicity model experiment at 2000X magnification. In photograph [a, (Normal control)]; [b, (CCl_4 treated control)]; [c, (CCl_4+ FFB 50 mg per kg. bw)]; [d, (CCl_4+ FFB 100 mg per kg. bw)]; [e, (CCl_4+Silymarin 75 mg per kg.bw)]; [f, (FFB 200 mg per kg•bw)], 2000 X

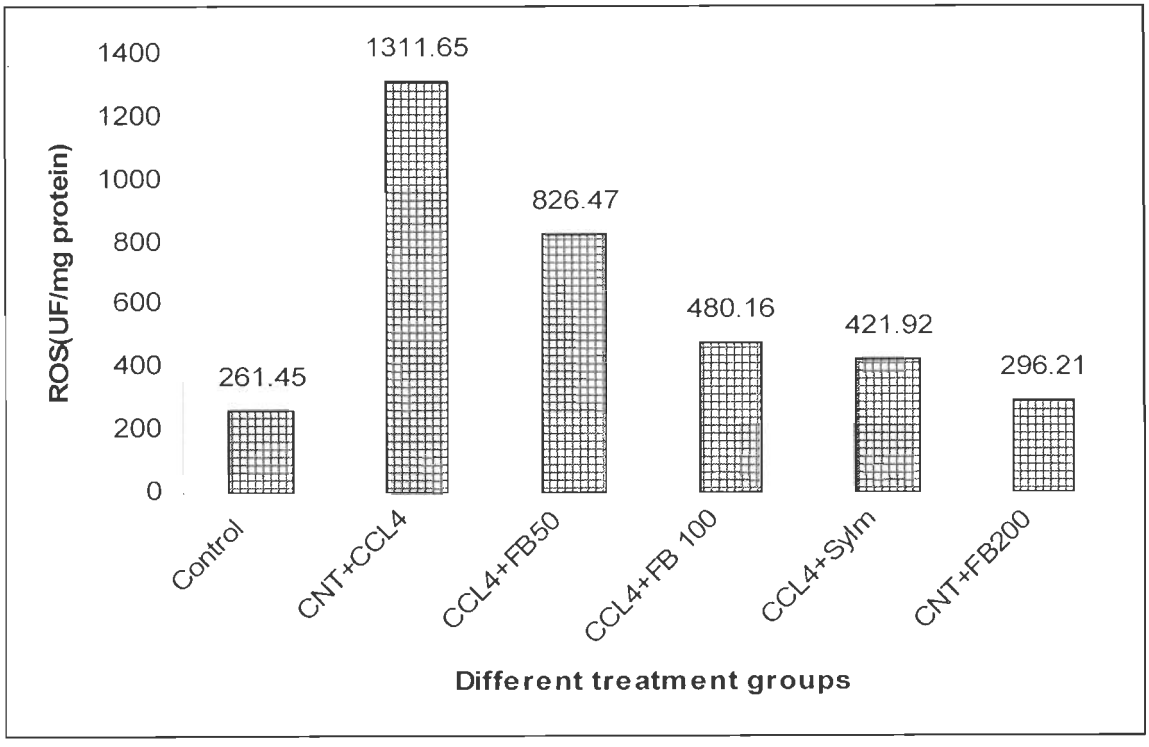


Fig. 6.3 The ROS level determination using DCFH-DA fluorescent probe *in vivo* CCl_4 intoxicated rat liver. CNT (Normal control, vehicle only); CCl_4+CNT (CCl_4 treated control);CCl_4+FFB50(50mgperkg.bw);CCl_4+FFB100(100mgperkg.bw);CCl_4+Sylm(Silymarin 75 mg per kg.bw); CNT+FFB200(200 mg per Kg bw).The ROS measurement is expressed in terms of fluorescence unit per mg rat liver protein.

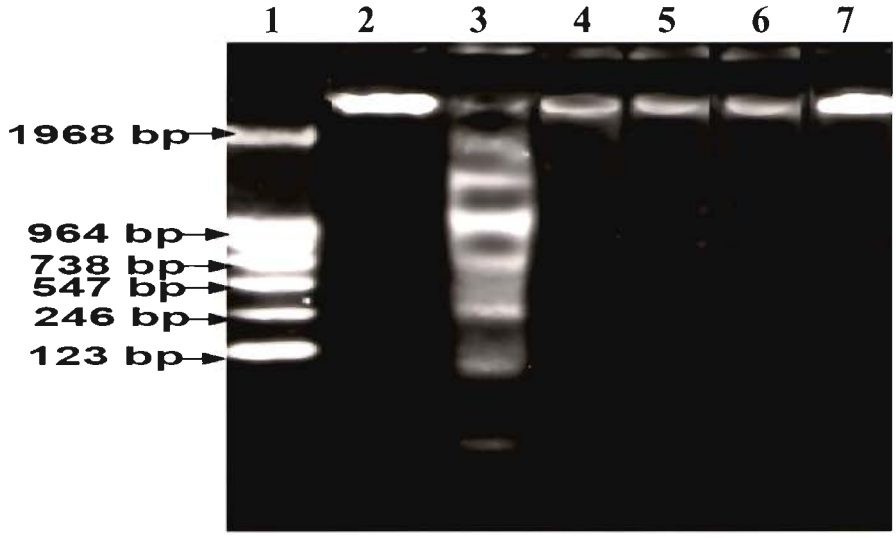


Fig 6.4 (a) DNA fragmentation pattern on rat liver genomic DNA under CCl_4 toxicity and pretreatment with FFB. Lane 1- Marker; Lane 2- Normal Control; Lane 3- CCl_4 treated control; Lane 4- CCl_4 + FFB (50 mg per Kg bw); Lane 5- CCl_4+ FFB (100 mg per Kg bw); Lane 6- CCl_4+ Silymarin (75 mg per Kg bw); Lane 7- VEH+ FFB (vehicle and FFB (200 mg per kg.bw).

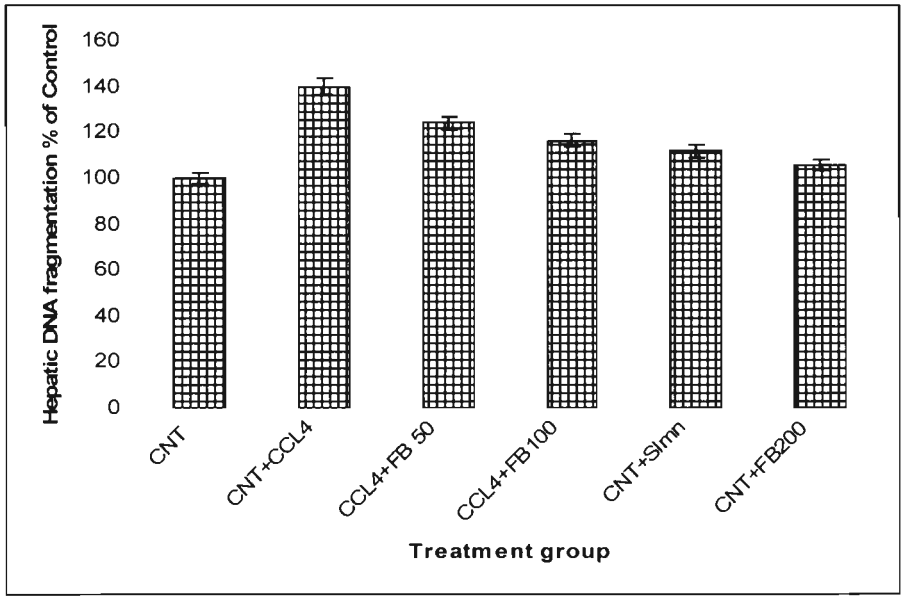


Fig 6.4 (b) Quantitative determination of DNA fragmentation using diphenylamine spectrophotometric method in different treatment groups rat liver tissue under CCl_4toxicity along with or without FFB. (Normal control, vehicle only); CNT + CCl_4 (CCl_4 treated control); CCl_4+ FFB (50 mg per kg.bw); CCl_4+FFB (100 mg per kg.bw); CCl_4+Slmn (silymarin 75 mg per kg.bw); VEH+ FFB (vehicle and FFB (200 mg per kg.bw).

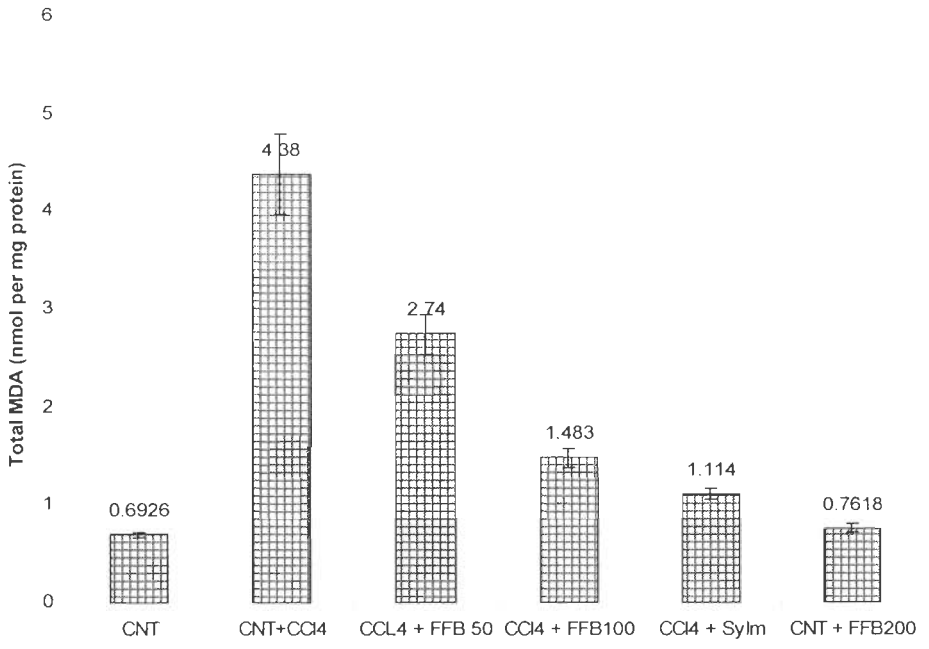


Fig. 6.5 (a) MDA production during lipid peroxidation in different rat liver tissue under CCl_4 toxicity and pretreatment with FFB constituents. CNT (Normal control); CNT + CCl_4 (CCl_4 treated control); CCl_4 + FFB (50 mg per kg. bw); CCl_4 + FFB (100 mg per kg. bw); CCl_4 + Sylm (Silymarin 75 mg per kg. bw); VEH + FFB (Vehicle + FFB 200 mg per kg. b.w).

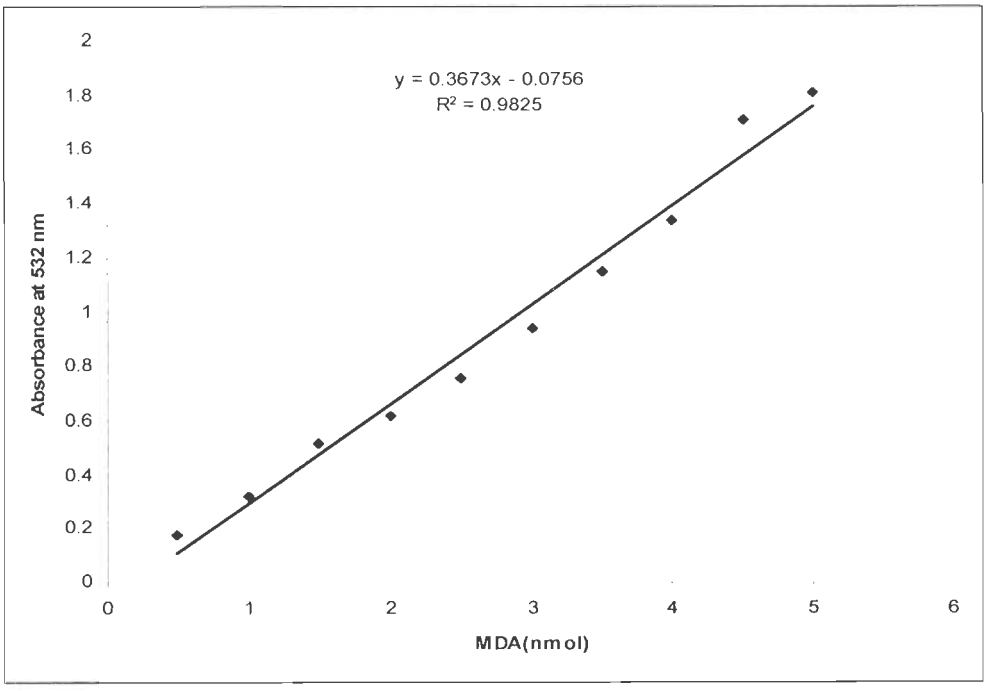


Fig. 6.5 (b) Calibration curve for TBARS determination in biological tissues

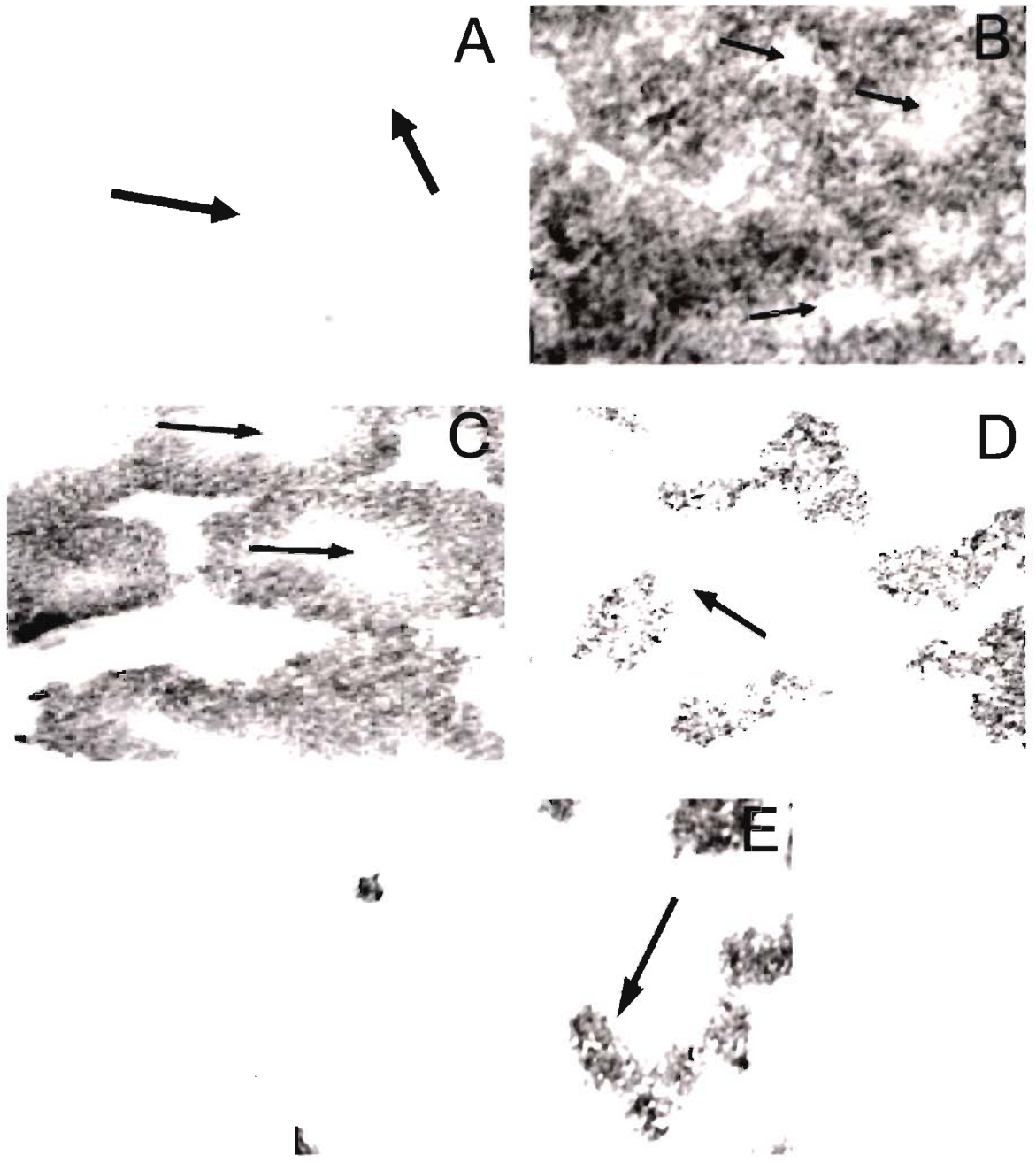


Fig. 6.6 Schiffs stained liver tissue section showing extent of lipid peroxidation under CCl_4 intoxication with or without treatment with FFB constituents. A-Normal control, B-CCl_4 treated control, C-CCl_4+FFB (50 mg per kg.bw), D-CCl_4+FFB (100 mg per kg.bw), E-CCl_4+Silymarin (75 mg per kg.bw).

Chapter 7

Summary

Liver is a vital organ playing pivotal role in detoxification and metabolic control of many toxins which are further excreted out of the body. In due course liver faces oxidative stress condition which leads to hepatotoxicity and associated with a number of pathophysiological events in the liver. Oxidative stress induced hepatotoxicity has now become a significant cause of acute liver failure and therefore, liver protection has been a subject of intense importance.

Since oxidative stress is implicated in the etiology of several chronic and degenerative diseases including liver diseases, uses of antioxidants have being suggested to be a promising strategy for therapy. Antioxidants are defined as those compounds that can overcome or reduce oxidative stress and therefore antioxidants have been one of thrust areas of research in biomedical sciences and nutraceuticals. Attempts have been made to search potential antioxidant agents from various sources. Plants due to their diversified phytochemical constituents and less toxicity have been the preferred choice. Therefore, in order to search for a valuable source of potent antioxidant and hepatoprotective agent there is need to explore the rich diverse flora with its diversified medical potential and identify the active constituents. There are several reports regarding evaluation of antioxidant potential of various plants and herbal preparation globally. Several herbs and herbal products are known to possess antioxidant principles and may be useful as organ protective agents.

In the present study an attempt has been made to screen and evaluate fifteen different medicinal and dietary plants for their antioxidant property by means of reliable *invitro* based assays. Efforts have also been made for fractionation and identification of active constituents. The study mainly focused on exploring the antioxidant and hepatoprotective potential of *F. bengalensis.*

Screening, extraction and fractionation of active antioxidant constituents

Methanolic and aqueous extracts of fifteen different plants described earlier were prepared and screened for their antioxidant activity using standard methods. Since the phenolics, flavonoid glycosides and related components are suggested to account for antioxidant activity, the total phenolic and sugar content of both aqueous and methanolic preparation were also determined in order to see if there is any correlation. It was observed that much variation occured in antioxidant activity among aqueous as well as methanolic preparation of plants used in the present study. However, it was confirmed from the evaluation of the radical scavenging and antioxidant activity that *F.*

bengalensis stem bark is conferred with highest antioxidant potential among them. Therefore, further studies were focussed on this plant. It was also observed that stem bark of *F.benghalensis* possessed maximum flavonoid content. Though based on such observations it was difficult to interpret at this stage whether presence of this phytochemical may attribute for the high antioxidant activity of *F. bengalensis*. However based on earlier studies linking antioxidant activity with presence of phenolic and flavonoid content, it was logical to assume that high antioxidant activity observed in this plant maybe correlated with its high flavonoid content.

Therefore, an attempt was made to purify the active constituents of the methanolic extract of this plant. Bioactivity guided extraction and fractionation of antioxidant constituents present in the methanolic extract of *Ficus benghalensis* was performed by employing liquid- liquid partitioning using hexane, dichloromethane and ethylacetate and silica gel column chromatography. Also, the antioxidant activity and total flavonoid content of each fraction was determined. Fractions which have high antioxidant activity were also found to have high flavonoid content and were pooled together. This clearly indicates that more likely the flavonoid content are responsible for the antioxidant activity. The pooled active fraction was named flavonoid enriched fraction of *F. bengalensis* (FFB). The components in the FFB were further identified by TLC, UV-VISIBLE spectrophotometry, FTIR, ESI-MS and H^1 NMR analytical techniques. The identification of constituents in FFB active fraction confirmed the presence of leucopelargonidin derivative, chrysin and luteolin as major contributor of antioxidant.

Validation of the antioxidant potential of crude FBS extract (CFBS) and flavonoid enriched fraction (FFB) from *F. bengalensis* stem bark

Since much variation was observed in the antioxidant activity of the given compound depending on the nature of the assay and damaging potential of the free radical. Therefore no single assay would accurately reflect the true antioxidant potential of a compound. Thus an attempt have been made to validate the antioxidant potential of flavonoid enriched fraction (FFB) from *F. bengalensis* stem bark by employing a battery of *in vitro* based biochemical assays towards the different nature of free radicals generated *in vitro* and *in vivo* system. The different *in vitro* antioxidant assays include DPPH and ABTS free radical scavenging, hydroxyl radical scavenging, superoxide anion scavenging, ferric reducing antioxidant power (FRAP), reducing assay, total

antioxidant capacity and metal chelating assays. FFB antioxidant constituents showed promising levels of antioxidant activity displaying excellent scavenging efficacy towards superoxide, hydroxyl, DPPH and ABTS free radicals with minimum inhibitory concentration IC_{50} value comparable to those of standard antioxidants like Gallic acid and BHT. FFB also showed very high metal chelating potential. Antioxidant potential was also evaluated by investigating their protective effects towards oxidative damage to biomolecules. Protective effects of FFB constituents against metal catalyzed (MCO) oxidative damage to calf thymus DNA and pUC18 plasmid was studied using spectrophotometric determination of MDA generation and agarose gel electrophoresis technique. To investigate protective mechanisms against oxidative damage to protein, bovine serum albumin was selected as model protein and two different oxidant system including metal catalyzed oxidation (MCO) and HOCl oxidation system were used to induce oxidation. Spectrophotometric DNPH based method for carbonyl detection and SDS-PAGE was employed. The inhibitory role of FFB antioxidant constituents against ascorbate/Fe^{2+} oxidizing system induced lipid peroxidation in rat liver homogenate was studied by using determination of MDA production by spectrophotometric technique. FFB demonstrated significant level of concentration dependent protection against oxidative damage to DNA, protein and lipid biomolecules, which is statistically equivalent in comparison to standard compounds like quercetin and glutathione. It is suggested that this protective activity can be very well correlated with its metal chelation as well as radical scavenging abilities.

***In vivo* hepatoprotective effects of FFB antioxidant constituents in CCl_4 induced rat liver oxidative stress model**

It is well established that oxidative stress plays a central role in the development of acute liver toxicity by CCl_4 mediated free radical generation. Since free radical mediated oxidative stress condition plays a pivotal role in liver disease pathogenesis and progression, use of dietary antioxidants have been proposed as therapeutic agents to counteract liver damage. Therefore the antioxidant mechanism is a major defense system that converts active oxygen molecules into non-toxic compounds and consequently revert cellular damage. Thus an antioxidant may also serve as good hepatoprotective agent. An attempt has been made to study the hepatoprotective potential of FFB antioxidant constituents in CCl_4 intoxicated rat liver toxicity model by employing biochemical, histological and molecular biology technique. To study the

biochemical aspects, liver damage marker enzyme level like AST, ALT, ALP, LDH and bilirubin in serum of different treatment groups was analyzed by means of biological kits. The protective effect of FFB constituents was evaluated by determining the antioxidant status of liver tissue. The morphological and histolopathological changes in experimental rats was investigated by performing H&E staining using light microscopy and Scanning electron microscopy on rat liver tissue sections of different treatment groups. To study the extent of oxidative damage to DNA, diphenylamine based spectrophotometric assay and genomic DNA fragmentation method was employed. The extent of lipid peroxidation using TBARS method with MDA estimation in liver tissue homogenate and histochemical detection of lipid peroxidation on rat liver tissue sections. In biochemical study of present work, it was observed that increased level of marker enzymes ALT, AST, ALP, LDH and bilirubin was attenuated to a considerable extent after treatment with FFB (50, 100 mg per kg.bw) constituents in comparison to CCl_4 treated animal group. Also it was noted that the reduced antioxidant enzyme level in CCl_4 treated group was improved significantly in pretreated groups with FFB constituents. Administration of FFB (50 and 100 mg/kg.bw/day) constituents as a pretreatment to CCl_4 treated groups during histopathological study demonstrated maintenance of structural integrity of hepatocytes with less incidence of cellular necrosis, swelling and fatty degeneration in comparison to the condition observed in CCl_4 treated animal group. On the basis of SEM observations, FFB constituents demonstrated significant hepatoprotection with recovery up to 75 and 80 percent, respectively, which supported the biochemical based hepatoprotective results. On the basis of results obtained from oxidative damage to biomolecules including DNA, and lipid, it was observed that FFB constituents treated groups showed lesser extent of damage demonstrating reduction in DNA fragmentations and lipid peroxidation mediated MDA production. The combined hepatoprotective efficacy of FFB constituents was found to be equivalent in terms of percentage recovery in CCl_4 induced liver damage as shown by standard silymarin (75 mg/ kg .bw).

Conclusions

Among fifteen Indian dietary and medicinal plants screened, the methanolic extract of stem bark of *F. bengalensis* found to have maximum antioxidant activity. A flavonoid enriched fraction (FFB) accounting for the antioxidant activity was obtained

by bioactivity guided fractionation and purification. The identification of constituents in flavonoid enriched fraction from *F. bengalensis* showed the presence of leucopelargonidin derivative, chrysin and luteolin as their major components. Active antioxidant constituents FFB demonstrated concentration dependent antioxidant activity in a battery of assays and were equivalent to the recognized synthetic and plant derived antioxidant. A significant level of protection by FFB was also observed against oxidative damage to DNA and lipid. These results indicate that FFB constituents have an antioxidant effect against CCl_4 induced hepatic oxidative damage and is useful as a hepatoprotective agent against various liver diseases induced by oxidative stress. The probable mechanism in hepatoprotective role of FFB could be due to their free radical scavenging potential or, metal chelation property; which may have attributed for its protective role against oxidative damage to macromolecules and strengthening of antioxidant enzyme status. Thus it is suggested that the FFB fraction from *F. bengalensis* stem bark could serve as a potential source of antioxidant and hepatoprotective agent without any toxic effect.

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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 various International Journals of repute and Conferences.

A. Papers in Journals:

1. Sharma NK, **Dey S**, Prasad R. 2007. *In vitro* antioxidant potential evaluation of *Euphorbia hirta L.* plant. PharmacologyOnline 1: 91-98.

B. Book Chapter.

1. Sharma NK, **Dey Sreela**, R Prasad. 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, **Dey S**, Prasad R. Antioxidant potential from dietary and medicinal plants. National Biotechnology Conference on Current trends and Future perspectives, Deptt of Biotechnology & Deptt of management studies, IIT Roorkee & Indian Federation of Biotechnologists (IFB-India) .Sept 2-3, 2006. pp. 134.

2. Sharma NK, **Dey S**, Prasad R. 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.