INTRODUCTION:

The liver has a pivotal role in regulation of physiological processes. It is involved in several vital functions such as metabolism, secretion and storage. Furthermore, detoxification of a variety of drugs and xenobiotics occurs in liver. The bile secreted by the liver plays an important role in digestion. Liver diseases are among the most serious ailments. They may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatitis (non-inflammatory diseases) and fibrosis (degenerative disorder resulting in cirrhosis of the liver).

Liver diseases are mainly caused by toxic chemicals (certain antibiotics, chemotherapeutics, peroxidised oil, aflatoxin, carbon tetrachloride, chlorinated hydrocarbons, etc.), excess consumption of alcohol, infections and autoimmune/disorders. Most of the hepatotoxic chemicals damage the liver cells mainly by inducing lipid peroxidation and other oxidative damages in the liver (Recknagel and Glende, 1977). Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis (Smuckler, 1975). It has been estimated that about 90% of the acute hepatitis is due to viruses like hepatitis B, A, C, D (delta agents), E and G. Of these, Hepatitis B infection often results in chronic liver diseases and cirrhosis of liver. Primary liver cancer has also been shown to be produced by these viruses. It has been estimated that approximately 14-16 million people are infected with this virus in South-east Asia region and about 6% of the total population of the region are carriers of this virus. A vaccine is now available for immunization against Hepatitis B virus. Hepatitis C and Hepatitis E infections are also common in countries of South-east Asia region (WHO, 1997).

In spite of the tremendous advances being made in allopathic medicine, no effective hepatoprotective medicine is available. The available therapeutic agents bring about only symptomatic relief without any influence on the curative process, thus, causing the risks of relapses and danger of untoward effects. A large number of populations still suffer from hepatic diseases due to various reasons. The development of hepatoprotective/anti-hepatotoxic drugs is a major thrust area in the field of natural product research. There are
numerous plants and polyherbal formulations claimed to have hepatoprotective activities. Nearly 150 phytoconstituents from 101 plants have been claimed to possess hepatoprotective activity (Doreswamy, 1995; Handa et al., 1986). However, majority of these have not been studied in the light of modern methods. We do not have readily available satisfactory plant drugs/formulations to treat severe liver disease.

WHO has approved the use of traditional medicines as a part of health program. To pursue research in these systems of medicine, several USA agencies and institutions such as FDA and National Institute of Health have set up separate wings. According to the WHO survey 80% of the population living in the developing countries rely almost exclusively on traditional medicine for the primary health care needs. In almost all the traditional systems of medicine, the plants play a major role.

The potential of plant as a source for new drugs is yet to be unexplored systematically. Among the estimated 250,000-400,000 plant species, only 6% have been studied for biological activity, and about 15% have been investigated phytochemically (Verpoorte et al., 1998; Cragg et al., 1997; Balandrin et al., 1985). India has an ancient heritage of traditional medicine. Materia Medica of health provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products. Indian traditional medicine is based on various system including Ayurveda, Siddha and Unani. There are about 45,000 plant species with several medicinal properties. In many countries it is traditional to use medicinal plants, either a single herb or a polyherbal formulations, to control liver disorder.

Different products are marketed in India as well as globally as natural agent for the treatment of different liver disorders and several liver complaints. The reputed ones among the formulations are Acilvan Hepa-10, Liva-16, Livodin, Livosin, Livotrit, Livomap, Livocin, Vimliv, Livomycin, Liv-52, Liv-100, Amylcure, Sanliv, etc. These formulations are typically the combination products (polyherbal formulation) containing either individual components or the crude extracts. Various cultures have remedies unique to their own population. Among several remedies available from the traditional medicine, andrographolide from Androgaphis paniculata and picrrosides from
Picrorrhiza kurroha (India), silymarin from Silybum marianum and catechin from Anacordium occidentalis (Europe), glycyrrhizin from Glycyrrhiza glabra (Japan) and schizandrin from Schizandra chinesis in China (Hikino and Kiso, 1988) are the most widely used and scientifically evaluated hepatoprotective agents (Handa et al, 1986).

Clinical conditions, such as oxidative stress have been implicated in several diseases such as diabetes, neurodegenerative disorders, liver disorders, cancer, and Alzheimer disease (Baynes, 1991; Buscigllo and Yankner, 1995; Dreher and Junod, 1996). The physical factors, like restraint stress, electromagnetic field exposure, etc (Bagchi et al., 1999; Flipo et al., 1998; Bonhomme-Faivre et al., 1998), environmental factors, like ozone, carbon monoxide, nitrogen dioxide, sulphur, lead, asbestos etc (Menzel, 1979; Sevanian et al., 1979; Campbell and Hilsenroth, 1976; Fujimaki et al., 1984) and chemicals factors like alcohol, CCl_4, DDT, pyrogallol, alloxan etc. (Nordman et al., 1992; Ereai et al., 2000; Koner et al., 1998; Sakurai and Ogiso, 1994) are known to produce oxidative damage. The over exposure to such conditions or factors or agents generates the free radical, to the extent that they overwhelm the antioxidant defence mechanisms. Under these conditions, the cell dysfunction or tissue degeneration is inevitable due to oxidative damages. Whether the free radical generation is the per se effect or the consequence of the cellular response is not clear. However, except in case of some chemicals, oxidative stress appears to be a consequence of stressful over activation of the cell function. The obvious generation of free radicals may be consequent to over activated biochemical processes.

The higher organisms have developed effective antioxidant systems. Oxidative stress in biological systems can be induced by the depletion of antioxidants and/or by an overload of oxidant species, i.e., reactive oxygen and nitrogen species (ROS, RNS) and other radicals (R*), so that antioxidant levels become insufficient. Sustained oxidative stress damages the cellular macromolecules and contributes to the pathophysiology of many diseases. An imbalanced production of free radicals plays an important role in the pathogenesis of a number of human diseases such as different liver disorders, ischemia, reperfusion injury (Chan, 1996), atherosclerosis (Terasawa et al., 2000), neurodegenerative diseases (Buscigllo and Yankner, 1995) and cancer (Dreher and Junod, 1996). The role of oxidative stress is also implicated in inflammation,
hypersensitivity and autoimmune conditions (Maurice et al., 1997). In addition, oxidative stress due to inadequate antioxidant enzymes has been related with many other specific pathologies as chronic granulomatous disease, Downs syndrome, diabetic complications, hepatitis, arthritis, influenza virus, ulcer, Pneumonia, HIV infection, cataract and glaucoma (Ames et al., 1993; Halliwell, 1994; VanDam et al., 1995; Araujo et al., 1998).

These free radicals react with several organic bio-molecules in the surroundings. Because of the high turnover of most of these small organic biomolecules, the damage is likely to be transient but not necessarily harmless. The wide variety of such organic biomolecules includes vitamins A, C, and E; uric acid; carbohydrates; amino acids; (Halliwell and Gutteridge, 1984). Many of these molecular targets for radicals have received a status of radical scavengers and considered as natural antioxidants. However, each reaction between such molecules and a radical generates a new radical, which is usually less reactive than initial one but can be potentially hazardous to the biological systems. For example, when the oxygen radical interacts with membrane bound lipids it sets in a chain of lipid peroxidation, which consequently damage the membrane (Davies et al., 1982). Activated forms of oxygen are also known to degrade the proteins and nucleic acids and these reactions are very lethal to a cell (Breimer, 1991; Stadtman and Oliver, 1991).

With respect to oxidative stress in liver it is very well known that mitochondria and cytochrome P450 enzymes are the main sources of reactive oxygen species (ROS) in hepatocytes acutely and/or chronically exposed to a toxic injury (environmental drugs, alcohol, therapeutical drugs, viruses, etc.). ROS may be generated from Kupffer and inflammatory cells, in particular neutrophils. In hepatocytes, ROS may play a role in a very large cascade of reactions, such as Ca^{++} accumulation, circulatory status and transport function, nitric oxide (NO) synthesis and metabolism, cytokine gene expression, caspase activity, growth factor synthesis and activity, DNA fragmentation, Na^{+} influx, etc. Hence, a significant and sufficiently steady increase of ROS production leads to perturbation of the normal redox state and of the metabolism of the cell, with consequent impairment of various cell functions and activities, possibly until irreversible damage.

The onset of liver damage depends on the length of the “toxic” insult. The consistent evidence of an increased level of malondialdehyde (MDA), 4-hydroxynonenal (4-HNE),
protein-adducts, etc. on liver and blood samples from patients with different degree of chronic liver disease, supports the in vivo role of ROS as mediators of liver damage (Paradis et al., 1997). More recently, ROS were also identified as involved in the response to interferon (IFN) therapy by patients with chronic HCV infection (Mutlu-Turkoglu et al., 1997).

ROS, in particular superoxide anion, rapidly react with nitric oxide (NO) to form peroxynitrite that may act as a dangerous molecule by influencing some SH-related enzyme activities and inducing membrane lipid peroxidation. The formation of peroxynitrite may also be considered as a “protective pathway” because through this mechanism NO acts as scavenger of ROS.

Liver contains different forms of NO-synthase: the neuronal form (nNOS) in the peribiliar plexus; the inducible form (iNOS) in hepatocytes, cholangiocytes, Kupffer, and stellate cells; and the endothelial form (eNOS) in the endothelial cells. Liver iNOS is primarily regulated at the transcriptional level by cytokines and ROS, but NO could also derive directly from cytokine-activated neutrophils and/or lymphocytes in the circulation. NO is also regenerated by the reaction of (GSH) with peroxynitrite. Through the nitrosylation of free thiol groups, NO is stabilized by forming adducts, both directly and by the action of glutathione-S-transferase (GST) on GSH and organic nitrites. These reversible addition products act as the exogenous source, storage, and transport of NO in cells and circulation (Vos et al., 1999).

In the liver, NO may have a protective role by maintaining perfusion and inhibition thrombosis and apoptosis, but can still contribute to liver necrosis, immunomedi ated liver damage, and to DNA fragmentation by blocking mitochondrial function and depleting cellular pyridine nucleotides (Fortenberry et al., 1999). In patients with chronic HCV infection, a direct correlation has been documented recently between iNOS induction and hepatitis C virus ribonucleic acid (HCV-RNA) titer, as well among hepatocyte nitrotyrosine, plasma nitrosothiols (S-NO) and histological severity of liver damage. Therefore, in addition to the “oxidative stress”, also the nitrosative stress may have a relevant role in the pathogenesis of chronic viral hepatitis (Mihm et al., 1997).
Alcohol-related liver damage partly depends on endotoxin-mediated Kupffer cells activation, with secondary production of pro-inflammatory cytokines, which induce iNOS expression. In these conditions, NO may reduce or even enhance the toxicity of ethanol by interfering with the activity of some alcohol-metabolising enzymes (Corrales et al., 1991; Griffon et al., 2000). It has been outlined recently that the toxic or protective function of this molecule is dependent on the entity of ROS production, as well as the amount of cell antioxidants, in particular GSH. Low levels of ROS and high content of GSH facilitate the protective effects of NO. In these conditions, NO exhibits lipid peroxidation and liver necrosis. In opposite conditions, i.e. high levels of ROS and low GSH, NO can be deleterious, by enhancing lipid peroxidation and apoptosis (Jones and Czaja, 1998; Vos et al., 1999).

Hepatocytes contain about 10% of total body pool of GSH (Loguercio and De Pierro, 1999). A normal homeostasis of this tripeptide is necessary for the activity of the cytochrome P450 enzyme system and of mitochondria, for the "traffic" from cytoplasm and nucleus and finally for the maintenance of a normal cellular redox status. GSH also affects the transcription of pro-inflammatory/anti-inflammatory cytokines genes, liver regeneration through cytokine mediated nuclear factor k binding (NF-kB) induction, NO bioavailability, and energy metabolism. By inducing GSH depletion, ROS induce oxidative stress and reduce the antioxidant capability of other antioxidants (Huang et al., 1998) depletion of GSH may also be a consequence of liver damage. Indeed, a large literature documented the decrease of GSH in liver and circulation in patients with alcoholic and viral cirrhosis, more recently, in those HCV-related chronic hepatitis. In this last group the bioavailability of GSH influences both the entity of liver apoptosis and necrosis and the response to the antiviral therapies (Loguercio et al., 1994).

Other antioxidant systems are involved in the induction and progression of chronic liver damage. In rats, ethanol significantly alters the GSH-peroxidase system by contributing to mitochondria dysfunction (Bailey et al., 2001). Particularly in presence of vitamin E, superoxide dismutase (SOD) is a primary defence mechanism against the damaging effect of ROS. In experimental animals, the induction by diet and alcohol of a SOD-catalase insensitive free radical species facilitates liver damage (Swart et al., 1999; Koch
et al., 2000). Selenium also represents a protective agent against lipid peroxidation, liver injury, and HCC and its circulating levels are decreased in cirrhotic patients, in which also a decrease of retinal serum levels is associated to the risk of HCC.

It has recently been suggested that measurement of plasma levels of GSH, vitamin A, β-carotene, SOD, GSH-peroxidase, catalase and the markers of lipid peroxidation may represent a useful marker to monitor the progression of liver damage and the response to therapy in patients with viral and alcoholic disease (Newsome et al., 2000). The GST family represents one of the main detoxifying systems in the hepatocytes, because these enzymes bind GSH with a large series of hydroperoxides, toxic substances, etc. GST regulates apoptosis by influencing the lipid peroxidation pathway (Hayes et al., 1991). Ethanol and virus infections cause a liver induction of GST and their plasma variations are a good predictive response index to IFN therapy (Vanhaecke et al., 2000).

There are various plants reported to possess antioxidant activity associated with hepatoprotective activity (Date et al., 1997). There are scanty reports suggest that Ficus bengalensis and Hemidesmus indicus possess antioxidant activity. F. bengalensis Linn. belonging to family Moraceae is a large tree with spreading branches attaining at a times a height of 100 ft. It grows everywhere in India including sub-Himalayan region, deciduous forests of Deccan and south India. It is hardy and drought resistant and can withstand mild frost. It develops from and can also propagate from its cuttings (Kirtikar and Basu, 1996; Chopra et al., 1956). In India the plant is known by different local names in different languages like, Bargat in East India (Bengal), Vad in Gujarati, Paeral in Malayalam, Vata-vruksha in Marathi, Bera in Punjabi, Vata in Sanskrit, Vada in Tamil, Marichettu in Telugu. F. bengalensis has been used as folk medicine for treatment of inflammation of liver and other liver complaints. However no scientific study has been reported to our knowledge about its hepatoprotective activity.

F. bengalensis contains flavonoids, glycosides, saponins, sterols, triterpenoids and tannins. Some important chemical constituents include leucopelargonidin and leucocyanidin, Bengalenoside, a flavonoid glycoside and glycoside of pelargonidin. The antioxidant effect of aqueous extract of the bark of F. bengalensis has been evaluated in
hypercholesterolaemic rabbits. Moreover, in hyperlipidemic rats, two flavonoid compounds dimethyl ether of leucopelargonidin and 5-3'–dimethyl ether of leucocyanidin 3-O-α-D galactosyl cellbioside obtained from the bark of *F. bengalensis* were found to showed significant antioxidant effect when compared with the activity of structurally similar flavonoid, quercetin, a known antioxidant (Daniel et al., 1998; Geetha et al., 1994; Cherian et al., 1992; Cherian and Augusti, 1993).

Another important reputed well-known plant in Ayurvedic system of medicine, *Hemidesmus indicus* belonging to family Asclepiadaceae, known as *Indian Sarsaparilla* or *Anantmul*. This plant is distributed throughout India in plains and low hills. In India the plant is known by different local names in different languages like, *Anantmoola* in East India (Bengal), *Sariva* in Gujarati, *Magrabi* in Hindi, *Namadaberu* in Malyalam, *Anantmool* in Marathi and Punjabi, *Sugandhi* in Sanskrit, *Nannari* in Tamil, *Gadhisugandhi* in Telugu.

Roots of *H. indicus* contain flavonoids, coumarinolignoids, glycosides, saponins, sterols, triterpenoids and tannins. Some important chemical constituents of the root include hemidesmin1, hemidesmin2, amyrins, lupeol 2-hydroxy 4-methoxy benzoic acid and some triterpenes (Das et al., 1992; Alam et al., 1994; Roy et al., 2001) from aerial parts of the plant, several pregnan steroids and glycosides have been isolated (Prakash et al., 1991; Chandra et al., 1994; Deepak et al., 1997; Sigler et al., 2000). Traditionally it is used as blood purifier, diuretic anti-rheumatic and antidote in snakebite (Satyavati et al., 1987). Roots are reported to have anti-microbial (Sivarajan and Balchandran, 1994), anti-inflammatory (Dutta et al., 1982; Alam and Gomes, 1998) and antihepatotoxic activity (Prabhakan et al., 2000). Methanolic extract of root bark has been reported to possess antioxidant activity (Ravishankara et al., 2002).

Oxidative stress is major cause of liver disease. In all types of liver damage there is consistent evidence of enhanced production of free radicals and/or significant decrease of antioxidant defence. As a consequence, a large number of studies have focused on the pathogenic significance of oxidative and nitrosative stress in liver injury as well as on therapeutic intervention with antioxidant and metabolic scavengers (Jones and Czaja,
Although *F. bengalensis* has been tested for its antioxidant activity in hypercholesterolaemic rabbits and in hyperlipidemic rats, a detailed investigation with respect to its antioxidant effect in hepatic damage and correlation of both the activities had not been attempted. Flavonoids and their glycosides are widely distributed class of natural product and have shown encouraging biological activities including hepatoprotective, antioxidant activities (Pier-Giorgio, 2000). Leucopelargonidin, leucocynidine, flavonoid glycosides are major phytoconstituents found in bark of *F. bengalensis*. Thus, the objective of the present investigation was to perform phytopharmacological evaluation of *F. bengalensis* and *H. indicus* with special reference to the antioxidant and hepatoprotective effect systematically using different in-vitro ex-vivo and in vivo models.

Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening; chemo profiling and marker compound analysis using modern analytical techniques like HPTLC and HPLC. Although several formulations available for liver disorder, very limited attempts have been made to evaluate their pharmacological aspects and verify therapeutic efficacy. Moreover, because of resurgence of interest in herbal drugs, it is also important to ensure that the only quality products enter the market. Efforts are being made by various government agencies and research laboratories to maintain the quality of herbal drugs by proper identification and detailed pharmacognostic, phytochemical investigations and standardization. However, in spite of the continuing efforts, there are no standard methods available for quality control of herbal drugs, which is the main hurdle for India to enter into the multi-million dollar international market. Further, the composition of plant material can vary and it is known to be influenced by the place of origin, soil, climate, season, time of collection, post harvesting conditions, temperature changes, moisture which affect tremendously the quality and therapeutic efficacy of the drug. Therefore, the quality and efficacy of the herbal drugs need to be established through systematic pharmacognostic, phytochemical and pharmacological evaluation and standardization of the drug. Before undertaking pharmacological work, we also
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carried out pharmacognostic and phytochemical standardization of *H. indicus* and *F. bengalensis* crude extract and its fractions using TLC fingerprinting.

Superoxide radicals are formed normally in the body. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (Poli, 1991). Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states (Sen, 1995). DPPH is one of the free radical generally used for testing preliminary radical scavenging activity of a compound or plant extract (Aldridge, 1981). Therefore *in vitro* models selected for testing free radical scavenging activity are DPPH superoxide scavenging nitric oxide scavenging assay. Certain *ex vivo* models like phenylhydrazine-induced haemolysis (erythrocyte membrane stabilization study) and different lipid peroxidation assays are carried out in the present study.

*In vitro* liver preparations (isolated hepatocyte) are increasingly used for the study of hepatotoxicity of chemicals. The advantages of *in vitro* model for screening are ability to carry out numerous samples at one time at low cost, requirement of small sample sizes, good reproducibility of results with little variation and overcomes differences between animals. Hence, we also studied the effect of different extracts and fractions using *isolated hepatocytes*.

Based on the results obtained using *in vitro* and *ex vivo* experiments we found methanolic extract of *F. bengalensis* and its ethyl acetate fraction while methanolic extract of *H. indicus* and its toluene fraction to have potent and desirable antioxidant activity as well as hepatoprotective activity. These fractions were then studied for *in vivo* models that exhibit both hepatoprotective activity and antioxidant activity simultaneously.

Paracetamol, an analgesic and antipyretic agent is safe in recommended doses but produces hepatic necrosis when ingested in very large doses. It is established that at these relatively large doses paracetamol is bio transformed into a reactive metabolite N-acetyl p-benzoquinoneimine (NAPQI) by cytochrome P-450 mixed function oxidase. Similarly it is well documented that carbon tetrachloride triggers hepatic and renal damage in
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animals and man (Aldridge, 1981). Hepatotoxicity induced by carbon tetrachloride is attributed to generation of trichloromethyl free radical during metabolism by hepatic microsomes. This radical binds covalently to neighbouring proteins and lipids, initiates lipid peroxidation that causes severe membrane alterations (Poli, 1991). Transaminases, phosphatases and other enzymes leak out through damaged membrane elevating plasma/serum level. Inhibition of CCl₄ bioactivation could reduce this toxic effect. Many compounds exhibit liver protection against CCl₄ induced damage either by decreasing the production of CCl₃ free radicals (Sen, 1995) or by impairment of CCl₄ induced lipid peroxidation (Poli, 1991). Thus paracetamol and CCl₄ share a common property of being converted into their respective reactive metabolites N-acetyl p-benzoquinoneimine and halogenated free radical by hepatic cytochrome P-450 (Packer et al., 1978). Therefore, we also studied the hepatoprotective and antioxidant effect of F. bengalensis and H. indicus against paracetamol and CCl₄ induced hepatic damage.