6. **DISCUSSION:**

The traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. A lot of medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) and proposed for their interesting multilevel activities. Among the medicinal plants used in ayurvedic preparations for their therapeutic action, some of these have been thoroughly investigated and some of are still to be explored. One such plant, *Gmelina arborea* Roxb. (Verbenaceae) is well reputed in Ayurveda and roots of this plant (as one of the major *panchmula* or *Bruhadmula*) are used in many well known ayurvedic preparations like Dashmula (Tewari, 1995). In the present study, this plant was viewed and studied for its historical, etymological, pharmacognostical, phytochemical and pharmacological aspects. Chemical constituents of *G. arborea* include lignans (Anjaneyulu et al., 1977), flavonoids (Nair and Subramaniam, 1975), alkaloids (Barik et al., 1992) and various phenyl propanoid glycosides (Hosny and Rosazza, 1998). Lignans are now days reported in treating many ailments specifically in liver disorders (Hikino and Kiso, 1988). Roots have been reported to contain various lignans and to our knowledge, no report is available regarding hepatoprotective or antioxidant activity and the type of constituents responsible for the same. Present study was undertaken to check and validate the hepatoprotective and antioxidant potentials of roots that can explain and justify their use in traditional medicine in the past as well as at present.

6.1 **PHARMACOGNOSTICAL STUDIES**

The root of *G. arborea* was authenticated by morphological and microscopical studies. (Satyavati et al., 1976; Kirtikar and Basu, 1999; API, 2001) and further the identity was confirmed by a taxonomist of Gujarat Ayurveda University, Jamnagar. The roots were found as segments cylindrical to tapering, with secondary and tertiary branches, external surface was uneven, dull, grayish brown and longitudinally wrinkled. Occasionally it showed rootlet scars, vertical cracks, fissures and numerous lenticels; fracture short and granular. The freshly fractured surface showed a thin layer of grayish
yellow bark, and the pale yellowish white wood constituted about 80% of the radius. Mature root bark when fresh, yellowish in colour; dry pieces curved and channeled, thinner ones forming single quills. The wood was light to moderately heavy, hard, strong, elastic and lustrous with a smooth feel. The fracture was somewhat tough in bark, brittle and predominant in woody portion. Root odour was indistinct and taste was mucilaginous, sweetish with slight bitterness.

Root samples were evaluated microscopically by studying detailed transverse and longitudinal sections and powder characteristics. The smoothly cut transverse section of root showed tangentially elongated rectangular and lignified cork cells sometime broken towards upper layers, 15-20 layers in young roots and 30-35 layers in mature roots. Phellogen was not distinct. The secondary cortex composed of several rows of tangentially elongated thin wall parenchymatous cells, densely filled with starch grains and oil globules. It also showed scattered resin ducts and stone cells either solitary or in group of 2-4 cells occasionally pitted and highly thickened (60-100 µ). Cortical parenchyma occasionally showed presence of prisms of calcium oxalate crystals. Stone cells were occasionally found pitted and highly thickened. Phloem was relatively narrow and made up of phloem parenchyma (occasionally, with resin masses in inner cells) interlaid with scattered sieve tubes with companion cells, and transversed by uni to bi seriate ray cells. Cambium was indistinct. The secondary xylem region represented almost two third bulk of the root and consisted of simple pitted wood parenchyma, numerous xylem vessels (130-250µ by 50-100µ), xylem fibers (with simple pits), tracheids (175-300µ by 30-50µ) and medullary rays (uni to bi seriate and 60-90 cells deep, filled with abundant starch grains and occasionally pitted), all with lignified walls. Microscopical study of powder revealed presence of fragments of isolated lignified cork cells elongated, up to 90 µ in length, xylem vessels with bordered pitted thickening, pitted tracheids and abundant pitted fibers, Stone cells found scattered or in group of 6-10 cells, and highly thickened. Powder showed abundant starch grains and oil globules in fragments of parenchyma. Starch grains numerous, simple (4-6 µ) as well as compound (20 µ), spheroid, ovate or irregular. Powder when treated with ruthenium red, showed presence of mucilage in parenchymatous fragments.
The improvement in quality control and standardization of herbal drugs has led to the development of effective quality medicines from plants. In the present study roots were evaluated qualitatively as well as quantitatively by studying various physicochemical parameters, phytochemical screening and by estimating for the presence of secondary metabolites. Root powdered was evaluated for its ash values, extractive values and loss on drying. Water soluble ash, petrol ether soluble extractive and moisture content data of roots were not reported and evaluated first time. In the present study, the detailed physicochemical parameters were developed and determined first time for roots of *G. arborea* and to our knowledge it has not been reported earlier. Hence this analysis aids to set up certain standards and contributed towards development of quality parameters for this drug.

6.2 **PHYTOCHEMICAL SCREENING:**

Root powder was found rich in flavonoids and lignans along with sterols, carbohydrates, alkaloids, tannins and coumarins as other important constituents. Crude methanolic extract of root powder was prepared and revealed remarkable presence of phenolics, flavonoids and lignans. Based on results of preliminary analysis, crude extract was subjected to fractionation with ethyl acetate, n-butanol and distill water successively. Preliminary phytochemical screening revealed the presence of phenolics; in particular flavonoids and lignans in crude extract and these results led us to estimate total phenolics and total flavonoids content in the sample. Root powder was found to have total phenolics 1.89% w/w and flavonoids 0.1694%w/w respectively.

6.3 **HEPATOPROTECTIVE ACTIVITY:**

In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function and offer protection to the liver from damage or initiated help in regeneration of hepatic cells. There are however, a number of drugs employed in traditional system of medicine for liver affections. Several medicinal plants have been used worldwide in various traditional herbal recipes for the prevention and treatment of liver disease (Patrick, 1999). The traditional healers approach is to manage liver disease, to regulate body metabolism, strengthen the liver and ultimately inhibit further liver cell
damage in the favor of their regeneration (Bean, 2002). Silymarin, a lignan, has been investigated clinically and found useful in the treatment of liver disease (Ferenci et al., 1989; Sailer et al., 2001). Further, various studies have revealed that flavonoids are able to reduce xenobiotic-induced hepatotoxicity in animals (Carini et al., 1992; Paya et al., 1993). The roots of G. arborea have been reported to contain high content of lignans (Anjaneyulu et al., 1977) and our preliminary phytochemical screening revealed presence of phenolics (1.89% w/w) and flavonoids (0.1694% w/w) in considerable amount. Moreover, flavonoids (Middleton, 2000) and phenolics (Toda et al., 1985; Larson, 1988) are reported to possess both free radical scavenging and hepatoprotective activity. In spite of rich content of lignans and phenolics in the roots of G. arborea, no reports are available till date regarding the hepatoprotective and antioxidant activity; the present study was undertaken to evaluate for the same.

Crude extract of roots (CE) and its ethyl acetate (EAF), n-butanol (BF) and aqueous fraction (AF) were prepared and evaluated for their hepatoprotective effects against CCl₄ and paracetamol-induced hepatotoxicity in in vitro as well as in vivo conditions. CCl₄ and paracetamol are known to produce hepatotoxicity, both in experimental animals as well as in human beings (Davidson and Estham, 1966; Mitchell et al., 1973). Paracetamol and CCl₄-induced hepatic injuries are commonly used models for hepatoprotective drug screening (McLean, 1975; Plaa et al., 1982; Kiso et al., 1983; Allis et al., 1990). The extent of hepatic damage was assessed by increased serum enzymes levels (SGOT and SGPT) and the hepatoprotective effect was compared with the effect of silymarin which is known to be hepatoprotective against CCl₄ and paracetamol-induced liver damages (Hikino et al., 1984; Sallie et al., 1991; Plaa and Charbonneau, 1994; Chrugoo et al., 1997). Freshly isolated rat hepatocytes are also a very useful and common tool for study of cytotoxicity and metabolic studies in this area, as they keep enzymatic activity similar to in vivo for several hours (Sweeney and Diasio, 1991). It is well known that hepatocytes are damaged by CCl₄, and cytosolic enzymes in the injured hepatocytes are leaked out of the cells due to an increase in cell permeability, membrane damage and cell necrosis (Tezuka et al., 1995). CCl₄ is reported to accumulate in the hepatocytes, which undergo formation of
CCl₃⁻ radical in the endoplasmic reticulum. The CCl₃⁻ radical is mainly responsible for the hepatotoxicity produced by CCl₄ (Williams and Burk, 1990). Exposure of hepatocytes with CCl₄ (10mM) resulted into significant reduction in the hepatocytes viability (50-55%) and increased level of serum GPT (67.39±3.26) GOT (46.42±3.35). The crude extract (1mg/ml) and EAF (1 mg/ml) exhibited hepatoprotective effects against CCl₄-intoxication as evidenced from the significant reduction in GPT and GOT release into the medium when compared with CCl₄-treated cells. EAF (1 mg/ml) was found to decrease the elevated levels of GPT (36.25±3.76) and GOT (26.58±1.94) and provided comparable results with silymarin (0.012mg/ml, 30.36±3.87 for GPT and 23.19±1.61 for GOT). Moreover, in trypan blue exclusion assay, both CE (1mg/ml) and EAF (1mg/ml) were significantly able to reduce cell death, induced by CCl₄ and produced 88.24 % and 88.33% protection respectively, which was comparable to silymarin (0.012mg/ml, 94.71 % protection). However, BF and AF did not reduce significantly the release of GPT and GOT in the medium, and were not able to achieve high cell survival rate at concentration of 1mg/ml.

Protective effects of different fractions and crude extracts in in vitro studies on isolated rat hepatocytes led us to extend our study to check protective activity of roots of G. arborea in vivo using CCl₄ and paracetamol as hepatic toxicants.

Liver injury induced by CCl₄ is the best-characterized system of the xenobiotic-induced hepatotoxicity and is a commonly used model for the screening the anti-hepatotoxic or hepatoprotective activity of drugs.

It is now well accepted that the hepatotoxicity of CCl₄ is the result of reductive dehalogenation, which is catalyzed by cytochrome P₄₅₀ and forms the highly reactive trichloromethyl free radical. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical. Both radicals (CCl₃⁻ and/or CCl₃OO') can react with sulphhydryl groups and are capable of binding to proteins or lipids, or of abstracting a hydrogen atom from an unsaturated lipid, and thus initiate lipid peroxidation, liver damage and play a significant role in the pathogenesis of various diseases. Although several isoform of cytochrome P₄₅₀ can metabolize CCl₄, most attention has been focused on the cytochrome P₄₅₀ 2E1 isoform, which is ethanol inducible. Natural compounds that reduce
the chemical activating enzymes might be good candidates for protecting against chemically induced toxicities (Brattin et al., 1985; Recknagel et al., 1989, 1991; Williams and Burk, 1990; Brent and Rumack, 1993; Brautbar and Williams, 2002).

In the present study, crude extract of roots of *G. arborea* and its ethyl acetate, n-butanol and aqueous fractions, at a concentration of 300mg/kg were evaluated for hepatoprotective effects. Being damaged by CCl$_4$, cytosolic enzymes SGPT, SGOT, ALP and total bilirubin leaked out of the cells due to an increase in cell permeability of hepatocytes, hence significant rise in the transaminases levels could be taken as an index of liver damage. This fact was evidenced by elevation of serum enzymes significantly in the CCl$_4$-intoxicated rats. This damage to the structural integrity of the liver was also observed in homogenate as evidenced by significant increased levels of MDA and by a significant decrease in SOD, catalase, GSH and total protein tissue levels in the present study. Determination and evaluation of these parameters in the serum and tissue samples helped to assess hepatotoxicity and inhibitory effects of the test drugs as an indicator of hepatoprotective activity.

Crude extract of roots of *G. arborea* and EAF used in the present study preserved the structural integrity of the hepatocellular membrane in a dose dependent manner as evident from the reduction in enzyme levels as compared to the enzyme levels in CCl$_4$-treated rats. The crude extract and EAF (300 mg/kg) significantly reduced SGOT and SGPT levels when compared with CCl$_4$-treated group, achieving the comparable protective potency as of silymarin (50mg/kg).

Alkaline phosphatase (ALP) enzyme levels reflect the pathological alteration in biliary flow (Ploa and Hewitt, 1989). CCl$_4$-induced elevation of this enzymatic activity in serum was in line with high level of serum bilirubin content (5 fold increase compared to control) in CCl$_4$-treated rats. The crude extract and the EAF (300mg/kg) mediated suppression of the increased ALP activity with the concurrent depletion of raised bilirubin levels, was comparable with silymarin (50mg/kg). It is possible that the crude extract and
the EAF were able to stabilize biliary dysfunction in rat liver during chronic hepatic injury with CCl₄.

In order to evaluate the effect of crude extract and EAF on lipid peroxidation, malondialdehyde (MDA) concentration in the liver homogenate was determined. Formation of a red chromophore, which absorbs at 532 nm, formed by the reaction of TBA with MDA and other breakdown products of peroxidized lipids formed the basis of this method. Highly significant increase was observed in MDA concentrations after CCl₄ administration, when compared to control. Also significant inhibition was observed by the administration of CE, EAF and BF from roots of G. arborea, the highest inhibitory rates being obtained with CE and EAF when compared with silymarin which suggests its potent antilipid-peroxidase activity.

The results of the present study showed significant depletion in the levels of SOD and catalase in CCl₄-treated group. Amongst all fractions tested, the levels were significantly restored by EAF (300mg/kg) and were found comparable with silymarin (50mg/kg) treated group.

Various studies on the mechanism of CCl₄-induced hepatotoxicity have shown that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl₄. GSH is largely mediated through the activity of glutathione-S-transferase, and forms adducts with the toxic metabolites of CCl₄ (Williams and Burk, 1990; Recknagel et al., 1991).

GSH is an important endogenous antioxidant system found rich in liver; the reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. In our study, CCl₄ treatment produced significant depletion in GSH and total protein levels in tissue homogenate. Pre-treatment of the rats with EAF significantly restored the protein level and increased the concentration of mitochondrial GSH level which was found comparable with silymarin. The hepatoprotective action of this fraction might be due to possible impairment of CCl₄-mediated lipid peroxidation, either through decreased production of free radical derivatives as evident from the ameliorated MDA levels in this study or due to the antioxidant potentials attributed to the presence of phenolics and flavonoids in the extract.
Although an inhibitory effect was observed by the administration of BF in serum ALP and elevated MDA level in tissue homogenate, other results were not in good agreement with these data. The AF was without any activity in CCl₄-induced hepatotoxicity.

The histopathological studies are direct evidence of efficacy of drug as protectant. Simultaneous treatment of EAF with CCl₄ exhibited less damage to the hepatic cells as compared to the cells treated with CCl₄ alone. The results of histopathological parameters also supported the results of biochemical parameters and confirm the hepatoprotective activity of G. arborea.

In the present study it has been hypothesized that the plant extracts under study afford protection against CCl₄ induced liver injury by impairing CCl₄-mediated lipid peroxidation, either through decreased production of free radical derivatives, as evident from the ameliorated MDA levels or due to the antioxidant potential of the extracts.

In another set of in vivo evaluation of different extracts of G. arborea, hepatotoxicity was induced by administering paracetamol. Paracetamol is known to cause hepatotoxicity in experimental animals and humans at high doses (Prescott et al., 1971; Mitchell, 1988; Kuma and Rex, 1991; Eriksson et al., 1992; Thompsen et al., 1995) and liver injuries induced by paracetamol is widely used for the screening of hepatoprotective drugs (Davis et al., 1974). It is established that a fraction of paracetamol is converted via the cytochrome P₄₅₀ pathway to a highly toxic electrophilic metabolite, N-acetyl-p-benzoquinone-imine (NAPQI) (Dahlin et al., 1984), which is normally conjugated with glutathione and excreted in the urine as conjugates (Jollow et al., 1974; Brouwer, 1993). With overdoses, these pathways become saturated and more paracetamol is available for activation to the toxic metabolite (NAPQI), which conjugated with GSH (Hinson, 1980) leading to depleted glutathione stores (Jollow et al., 1974; Rathbun et al., 1996) and sharp increase in metabolite covalent binding to proteins (Jollow et al., 1973; Mitchell et al., 1973), mitochondrial dysfunction and the development of acute hepatic necrosis (Parmar et al., 1995). Also depletion of glutathione enhances the expression of tumor necrosis factor alpha (TNFα) (Agarwal and Piersco, 1994). TNFα primes phagocytic NADPH oxidase to the enhanced production of oxygen free radicals and contributes to liver damage (Gupta et al., 1992).
Normally GSH contributes significantly to the intracellular antioxidant defensive system as it is a powerful consumer of superoxide, singlet oxygen, and hydroxyl radicals (Miesel and Zuber, 1993). The breakdown of the GSH-dependent antioxidant defensive system increases the intracellular flux of oxygen free radicals (Miesel and Zuber, 1995) creating an oxidative stress and initiating apoptosis.

The massive production of reactive species may lead to depletion of protective physiological moieties ensuing widespread propagation of the alkylation as well as peroxidation, causing damage to the macromolecules in vital biomembranes (Aldridge, 1981). The reactive species-mediated hepatotoxicity can be effectively managed upon administration of agents possessing antioxidant (Attri et al., 2000), free radical scavenger (Sadanobu et al., 1999) and anti-lipidperoxidant activities (Lim et al., 2000).

In the present study, rats treated with paracetamol alone developed significant hepatocellular damage as evident from a significant increase in the serum levels of glutamate oxaloacetate transaminase (AST), glutamate pyruvate transaminase (ALT), ALP and bilirubin when compared with control. The increase was found 7 fold for the enzyme SGOT, SGPT and bilirubin while almost 18 fold for ALP. Pretreatment of rats with CE and EAF at dose of 300 mg/kg significantly reduced the elevated serum levels of SGOT, SGPT and ALP and bilirubin. Pretreatment with BF and AF was not found to be effective in reduction of these enzymes.

Paracetamol also caused a marked increase in hepatic MDA formation compared with control group. CE and EAF treatment attenuated the elevated MDA formation to normal levels. The production of MDA was assayed to examine paracetamol-induced lipid peroxidation. The pretreatment effect of CE and EAF (300 mg/kg) significantly reduced paracetamol-induced increase in MDA production compared with paracetamol-treated rats.

In the present study, paracetamol-treated group showed significant decrease in hepatic SOD and catalase activity, when compared with control. This inhibition was significantly reduced in the rats pre-treated with EAF, retaining almost normal activity in the liver. Crude extract was also found effective in restoring the hepatic levels of SOD and catalase but EAF was found to be the most potent fraction in this study.
The protective role of GSH against cellular lipid peroxidation has been well
documented (Burk, 1983). Reaction of NAPQI with GSH is enzymatic and
yields the conjugate 3-(glutathione-S-yl) paracetamol (Hinson et al., 1982;
Coles et al., 1988) while covalent binding to proteins occurs primarily via
reaction with the cysteine groups on the proteins to form 3-(cysteine-S-yl)
paracetamol adducts (Hoffman et al., 1985). It is proposed that NAPQI is a
mediator of oxidative stress which leads to depletion of GSH, peroxidation of
lipids, DNA damage, and oxidation of proteins (Gibson et al., 1996). Similar
observations were made in the present study where the levels of glutathione
and total protein in liver homogenate were found significantly decreased in the
paracetamol- administered rats. A substantial increase in hepatic lipid
peroxidation as was evident by elevated MDA level in liver homogenate and
its suppression to nearly normal levels by CE and EAF of roots of G. arborea
treatment supports the view that the plant under investigation prevents
paracetamol-induced depletion of GSH. This gets further support from the fact
that supplementation of CE and EAF (300mg/kg) significantly restored the
depleted levels of glutathione and total protein to near normalcy as compared
to paracetamol-treated group.

Lipid peroxidation is a free radical-inducible process in which membrane
polyunsaturated fatty acids are oxidatively degraded into a variety of products
including MDA. The MDA is most abundant individual aldehyde resulting
from lipid peroxidation breakdown in biological systems and used as an
indirect index of lipid peroxidation (Draper and Hadley, 1990). Fortunately,
there are several antioxidant mechanisms that can neutralize free radicals in
living organisms. Antioxidant defense mechanisms can be grouped into
enzymatic antioxidants (mainly SOD, GSH and catalase) and non-enzymatic
antioxidants that can neutralize free radicals (Parra et al., 2003). In other
words, lipid peroxidation can be initiated when oxidative stress overcomes the
antioxidant defenses. Therefore it is conceivable that paracetamol-induced
depletion of hepatic GSH and its associated increase of MDA originated as a
result of the paracetamol-induced elevation of free radicals which in turn speed
up lipid peroxidation and cause irreversible cell damage. Histological
evaluation of paracetamol-induced damage in the paracetamol treatment group
showed that 50–60% of the liver was damaged, with large zones of
degenerating and necrotic hepatocytes. When EAF was given to rats prior to the paracetamol treatment, the area of liver damage was reduced to 40–50% compared to paracetamol-treated group. Other extracts have not exhibited significant protection.

Our results from the hepatoprotective studies provide strong evidence that CE and EAF of roots of *G. arborea* significantly inhibit the acute liver toxicity induced by high doses of CCl₄ and paracetamol in rats, as shown by a reduction of serum liver enzyme activities and hepatic lipid peroxidation, as well as the preservation of the integrity of the liver cells evident from histological study. Further, the effectiveness was also observed on rat hepatocytes.

Amongst all the fractions of crude extract tested, EAF was found most effective against acute damage induced by CCl₄ and paracetamol in rats. On this basis, it was further evaluated for its effectiveness against chronic damage induced by prolonged administration of ethanol in rats. Increased formation of lipoperoxides, conjugated dienes and malondialdehyde (MDA) and reduced levels of various antioxidants and glutathione in the tissues have been demonstrated in experimental animals administered with ethanol as well as alcoholic human subjects (Pal et al., 1993).

The hepatocytes contain three main pathways for ethanol metabolism to give acetaldehyde, each located in a different subcellular compartment, namely the alcohol dehydrogenase pathway of cytosol, the microsomal ethanol oxidizing system located in the endoplasmic reticulum, and catalase located in the peroxisomes. Chronic ethanol feeding results in the appearance of a form of cytochrome P₄₅₀ also differing by its catalytic activity from other cytochrome P₄₅₀ species (Giljoly et al., 1977). Furthermore, administration of ethanol has been reported to lead glutathione depletion, which is attributed to conjugation of GSH with acetaldehyde, an ethanol metabolite. Ethanol-induced hypoxia has also been invoked as a possible cause of the potentiation of hepatotoxicity (Gulati et al., 1995).

The biochemical investigations revealed elevated levels of serum transaminases, alkaline phosphatase and serum bilirubin in ethanol treated groups as compared with the control group. Elevations in values tend to be relative to the amount of liver damage. Elevated level of bilirubin was an
indication of biliary obstruction and haemolysis while of SGOT and SGPT were indicative of hepatocellular injury. Treatment of rats with EAF at dose of 300 mg/kg after ethanol intoxication markedly reduced the elevated serum levels of SGOT, SGPT and ALP and bilirubin as compared to ethanol treated group.

Lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity. The defense of EAF against oxidative stress was expressed in terms of decreased TBRAS formation and increased levels of antioxidant enzymes in tissue homogenate. The prevention of ethanol mediated lipid peroxidation in homogenate by this fraction was found in agreement with the serum enzyme observations. Further, histopathological analysis revealed appreciably normal liver section and healthy hepatocytes in the group treated with EAF after ethanol intoxication, when compared with other groups. Hence, it could be inferred that administration of EAF might have prevented free radical generation in alcohol metabolism, and could be responsible for the healthy state of liver cell. Present study revealed hepatotoxic effect of ethanol by free radical mechanism and we observed that treatment with the EAF protected hepatocytes, and reduced the severity of damage due to ethanol toxicity.

Results from present study suggests that amongst all the fractions tested, EAF exerted antioxidative effects against toxicants-induced oxidative stress by preventing not only the elevation in MDA levels, but also the depletion in GSH level and by attenuating the depletion of the antioxidant enzyme activities (catalase and SOD). It is also possible that the effect may be as a result of decreased bioactivation of toxicants upon treatment with EAF of roots of *G. arborea* and thereby decreased production of reactive metabolites. No attempt has been made in this study to confirm this mechanism. Ethyl acetate fraction was found rich in presence of natural phenolic compounds such as flavonoids and lignans. The protective effects of this fraction against three different toxicants- induced liver damage, both *in vivo* and *in vitro* could be related to the presence of phenolic compounds. As is well known, phenolic antioxidants such as flavonoids and tannins are considered promising therapeutic agents for free radical pathologies due to their scavenging ability with ROS (Halliwell and Gutteridge, 1984). In the present study, through their
free radicals scavenging capacity, EAF and its phenolic composition have been shown to have protective effects against oxidative stress. Antiperoxidative and antihapatotoxic effects of various flavonoids and lignans (Fernandes et al., 1994) reported earlier may explain the mechanism of the hepatoprotective effect of roots of *G. arborea* against paracetamol, CCl<sub>4</sub> and ethanol-induced hepatotoxicity in rats.

### 6.4 *ANTIOXIDANT ACTIVITY:*

Natural compounds that reduce the chemical activating enzymes might be good candidates for protecting against chemically induced toxicities. Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Halliwell et al., 1992). Therefore the effective scavenger of these radical is essential for the treatment of diseases associated with free radical generation or with underlying cause of free radical formation. Herbal medicines derived from plant extracts are increasingly being utilized to treat a wide variety of clinical diseases, with relatively little knowledge on their modes of action (Kyung et al., 2004). Those containing radical scavengers are gaining importance in prevention and treatment of many diseases. Phenolic compounds and flavonoids are the major constituents of most of the plants reported to possess antioxidant and free radical scavenging activity. Several studies have shown that the hepatoprotective effect is associated with antioxidant rich plant extracts (Dwivedi et al., 1990; De et al., 1996; Emmanuel et al., 2001). In the present study, we have evaluated various fractions along with crude extract of roots of *G. arborea* for its hepatoprotective potential *in vivo* as well as *in vitro*, and the ethyl acetate fraction was found most potent. This fact can be correlated with the occurrence of various phenolics in the extract and also this led us to evaluate antioxidant activity of different extract in several *in vitro* as well as *ex vivo* systems.

DPPH is known to abstract labile hydrogen (Constantin et al., 1990; Matsubara et al., 1991) and the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (Ratty et al., 1988; Rekka and Kourounakis, 1991). In this study, we found the CE and EAF to be the efficient scavengers.
of DPPH radical with IC$_{50}$ as 120 µg/ml and 113 µg/ml respectively. This assay provided information on the reactivity of the test compound with a stable free radical since its odd electron, DPPH provides strong absorption band at 517 nm in visible spectroscopy (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. It appears from the results that EAF might have hydrogen donating capabilities and acted as an antioxidant. The scavenging effect increased with increasing concentrations of the extract. However, scavenging activity of ascorbic acid, a known antioxidant, used as positive control, was relatively more pronounced than that of EAF.

Various free radicals like nitric oxide and superoxide exhibit numerous physiological properties and they are also implicated in several pathological states. The superoxide anion radical, specifically due to its transformation into more reactive species such as the hydroxyl radical, has been implicated in several pathophysiological processes, including the liver necrosis and ischemia-induced tissue damage (Halliwell and Gutteridge, 1984). In riboflavin-light-NBT, photochemical reduction of flavins generates O$_2$, which reduces NBT, resulting in the formation of blue formazan. This formed the basis of this method. In the present study, results indicated that the ethyl acetate fraction had a notable effect on scavenging of superoxide when compared with ascorbic acid, which was used as positive control. The action may depend on hydrogen atom donation by its components leading to the formation of secondary radical species that are resonance stabilized, like many phenolics antioxidants (Slater and Eakins, 1975).

In all the extracts tested, EAF significantly inhibited the concentration of nitrite after spontaneous decomposition of sodium nitroprusside. This indicated the presence of compounds capable of scavenging nitric oxide. However, the specificity of this assay has been questioned since nitrite is one of the final products of the reaction of nitric oxide with oxygen, through intermediates such as NO$_3$, N$_2$O$_4$ and N$_2$O$_3$ (Marcocci et al., 1994). Therefore the decrease in nitrite production could also be due to interaction of the extract and with other nitrogen oxides.
For the measurement of the reducing power, we have investigated the $\text{Fe}^{3+}$–$\text{Fe}^{2+}$ transformation in the presence of all extracts of roots of *G. arborea*, using the method of Oyaizu (1986). Direct correlation between antioxidant activities and reducing power of plant extracts has earlier been reported (Tanaka, 1988; Pin-Der-Duh, 1998; Pin-Der-Duh et al., 1999) The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Gordon, 1990). Our results on the reducing power of EAF suggest that EAF is likely to contribute significantly towards the observed antioxidant effect. However, the antioxidant activity has been attributed to various mechanisms like prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997). In this study, ethyl acetate fraction was found to have maximum reducing capacity, which further contributes to its antioxidant capacity.

Oxidative damage that results in lipid peroxidation can inactivate cellular components and can have serious effects on the cells probably leading to ageing as well as several diseases (Rice and Burdon, 1993; Esterbauer, 1996; Packer and Ong, 1998). In recent years much attention has been focused on this subject, especially in the field of clinical medicine, due to its relevance in degenerative diseases, ageing, stroke and ethanol toxicity and antioxidant properties (Sies, 1996; Thomas and Kalyanaraman, 1998). Therapeutic properties of certain plant extracts used in traditional medicine have been linked to their antioxidant abilities (De-las-Heras et al., 1998; Lin et al., 1998; Prince et al., 1998). For instance protective effects of the herbal hepatoprotective formulations Liv 52 and Liv 100 have been linked to their ability to inhibit lipid peroxidation in rat liver homogenate (Suja et al., 1997).

In the present study, the antioxidant activity of different extracts was measured by the bleaching of $\beta$-carotene. The addition of the extracts and ascorbic acid at various concentrations prevented the bleaching of $\beta$-carotene to different degrees. $\beta$-carotene in this system undergoes rapid discolouration in the
absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generate free radicals. Linoleic acid hydroperoxides attack the β-carotene molecule and, as a result, it undergoes rapid decolourization. The corresponding decrease in absorbance can be monitored spectrophotometrically. The presence of different antioxidant extracts can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha et al., 2001). In the present study, EAF (91.89%) was found most effective in hindering the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system. In comparison with ascorbic acid as reference standard, CE and BF also showed an appreciable antioxidant activity. The data for oxidation rate ratio and activity coefficient also supports the antioxidant activity index of these extracts.

The antioxidant effects of different extracts prevented the peroxidation of linoleic acid (thiocyanate method), as evident by protection offered by them at various concentrations. Both the linoleic acid system and emulsion system were used to evaluate the efficacy of prepared extracts in homogeneous and heterogeneous systems. Absorbance of the control was increased due to the oxidation of linoleic acid hydroperoxides, which decomposed to many secondary oxidation products. These oxidation products further react with ferrous sulphate to form ferric sulphate which, on further reaction with ammonium thiocyanate, forms the ferric thiocyanate red colour. Antioxidants can slow down the peroxidation of linoleic acid; hence, the ferric thiocyanate formation will be slow (Hua-Ming et al., 1996). The assay included both linoleic acid and linoleic acid emulsion systems which represented homogeneous and heterogeneous systems (Osawa and Namiki, 1981). The linoleic acid system can be correlated with a homogeneous system or bulk oil system system. The linoleic acid emulsion system can be simulated with the biological lipid system or with food/fat emulsions. The extracts under study showed antioxidant activity in both methods. The percentage protection for EAF was found 85.37% in linoleic acid emulsion system and 86.34% in the linoleic acid system. Ethyl acetate fraction showed more protective effect than crude extract and butanol fraction and exhibited antioxidant activity
Radical chain reactions are generally catalyzed by transition metal ions, mainly iron and copper. Many antioxidants act by binding to metal ions. In another set of lipid peroxidation, oxidation of linoleic acid was induced by iron-ADP complex in presence of ascorbic acid. This mixture is known to stimulate lipid peroxidation through formation of superoxide anion. Linoleic acid hydroperoxides are reported to yield thiobarbituric acid reactive substances (TBARS). Here, Fe$^{2+}$-ADP chelate promotes redistribution of iron from water into the lipid system and also helps to keep the iron in the solution thereby rendering it redox active (Asakawa and Matsushita, 1979). In view of this, the effectiveness of all the extracts in reducing MDA concentration, as a result of reduction in the amount of iron available for catalytic role in the generation of oxygen related free radicals, were determined. Consistent with the earlier results, EAF showed activity comparable with ascorbic acid and exhibited 85.37% protection at a concentration of 1000 µg/3 ml.

The antioxidant activity was further evaluated by quantifying the ability of different extracts at varied concentrations to suppress iron Fe$^{2+}$-induced lipid peroxidation in rat liver homogenates and compared with ascorbic acid. At the highest concentration of the EAF, inhibition of the extent of lipid peroxidation was 81.95% which indicated its effectiveness in suppressing Fe$^{2+}$-induced lipid peroxidation.

Phenylhydrazine is auto-oxidisable substance which under aerobic conditions may react with molecular oxygen to form a variety of reactive species like superoxide radicals, hydrogen peroxide, hydroxyl radical and phenyl radical, each with capacity to initiate the peroxidation of unsaturated fatty acids in endogenous membrane phospholipids (Hill and Thornelley, 1983). Erythrocyte superoxide dismutase, glutathione peroxidase and catalase activities remove the superoxide and peroxide oxidative challenge and in doing so, reduce the likelihood formation of the highly cytotoxic hydroxyl radical (Jain and Hochstein, 1980). Phenyl radical, however represents different problem as, there is no defensive mechanism to resist phenyl radical attack except for α-tocopherol. The phenyl radical is therefore a prime candidate for mediating
phenylhydrazine related cytotoxic phenomena in phenylhydrazine-induced haemolysis (Cazana et al., 1990). In the present study, CE and EAF inhibited haemolysis in a dose-dependent manner with 78.54 % and 81.49 % protection respectively at a concentration 500µg/3ml, while BF was found to be less active.

In hepatoprotective activity of various fractions of crude extract from the roots of *G. arborea*, we found that EAF showed potent protective effects against three well known toxicants-induced (CCl₄, paracetamol and ethanol) liver injuries in rats. EAF also showed potent antioxidant activity in various *in vitro* as well as *ex vivo* systems. In the present study for antioxidant activity, EAF exhibited maximum free radical scavenging and antiperoxidative activity amongst all fractions tested. Further, the results of phytochemical analysis of EAF showed presence of varied range of compounds like flavonoids and lignans. In the light of the above, we conclude that the protective effect observed with EAF was due to the phenylpropanoid rich composition of this extract and the observed effects would be as a consequence of the synergism or interaction between these different components.

The hepatoprotective activity can at least partly be associated with their antioxidant properties, acting as a scavenger of free radicals (such as superoxide and alkoxy radicals) even at low levels of GSH. The improvement in hepatic injury and liver functions by EAF may be due to the presence of phenolics, which may effectively protect against liver plasma membrane alterations.

### 6.5 IDENTIFICATION OF ISOLATED COMPOUNDS:

Based on the results of pharmacological activity, ethyl acetate fraction of crude extract was subjected further to isolate and characterizes the possible bioactive principles by applying different chromatographic techniques. Gradient elution of crude ethyl acetate extract was performed which yielded fraction A. A portion of fraction A was then rechromatographed and purification was done by gradient elution with heptane: dichloromethane (100:0 to 98:2) at a flow rate of 0.5 ml/min. Elutes from heptane: dichloromethane (98:2), up on concentration and recrystallization from
methanol, yielded yellowish white crystals (compound A, 80-82°C). Compound A resolved at Rf 0.3±0.02, in mobile system, hexane: ethyl acetate (9:1), when detected with UV 254 and 366nm. Combined elutes from heptane: dichloromethane (99:1) yielded white shiny crystals. Further, recrystallization from methanol gave whitish crystals (compound B, 78-80°C), resolved at Rf 0.49±0.03, in mobile system, hexane: ethyl acetate (9:1) when detected with UV 254 and 366nm. Isolated compounds A, and B were then subjected to various spectral analysis like UV, IR, NMR and Mass spectroscopy for structure determination.

Structure of compound A was elucidated and molecular formula was determined as C_{38}H_{66}O_{4} by ESI-MS. The spectrum displayed a weak peak, M+1 peak at 587.1 and a base peak at 177.5 corresponding to the major fragment ion (C_{10}H_{9}O_{3}). This data suggested that molecular weight of compound A must be around 586. UV (\lambda_{max}) analysis showed 247 nm and 327 nm as the maximum absorption peaks for compound A, indicated presence of phenolic functional group in the structure. The IR spectra of compound A showed absorption bands (cm^{-1}) for hydroxyl groups (3407.98), unsaturated esters (C = O, 1710.74, C = C 1640), aromatic ring (1517.87 and 1600), aromatic ether (1170.71, 1244.06 and 1325.01) C-O stretching (1120.56) and CH_{2} long chain (721.33). This suggested the possibility of unsaturated acid ester of long chain alcohol. The \textsuperscript{1}H-NMR spectrum of compound A displayed a triplet at 0.8801 δ for the presence of -CH\textsubscript{2}-CH\textsubscript{3} (5H), a broad singlet at 1.2533 δ for -CH\textsubscript{2} (52H, long chain proton), a singlet at 3.9309 for one -OCH\textsubscript{3} group (3H), a broad multiplet at 4.2032 δ for -CH\textsubscript{2}O (2H) and a broad hydroxyl signal at 5.834 δ (-OH,1H). Further, it also indicated two olefinic proton signals appearing as doublets at 7.6272 δ and 6.2720 δ for β-proton (1H) and α-proton (1H) of cinnamoyl moiety respectively. The multiplet signals that appeared at δ 7.0378, 6.9973 and 6.9058 were assigned to 3 aromatic protons (H-2, H-5 and H-6). Thus, from the \textsuperscript{1}H-NMR data, compound A has been confirmed to have ester of long chain alcohol and hydroxycinnamic acid. The structure of compound A was determined as ferulic acid ester of cluuytyl alcohol which was again confirmed by comparing the data of compound A with that of the reported data for cluuytyl ferulate (Govindachari et al., 1971). Isolated compound A closely resembled to the
data reported for cluytyl ferulate with respect to all spectral analysis and identified as cluytyl ferulate. Structure of compound B was elucidated and molecular formula was determined as C\textsubscript{37}H\textsubscript{64}O\textsubscript{4} by ESI-MS. The spectrum displayed the M+1 peak at 573.6 and a base peak at 163.4 corresponding to the major fragment ion (C\textsubscript{9}H\textsubscript{7}O\textsubscript{3}). This data suggested that molecular weight of compound B must be around 572. The base peak present at 163.4 could be related to the presence of caffeic acid moiety in place of ferulic acid (Wang et al., 1999). Melting point of compound B was found 78-80°C and UV absorption spectrum showed maxima at 287 nm, strongly indicated its phenolic nature. IR spectral study revealed different peaks (cm\textsuperscript{-1}) including 725 (CH\textsubscript{2} long chain), 1115 (C-O stretching), 1160, 1255 and 1350 (aromatic ether), 1515 and 1600 (aromatic ring), 1640 (C=C) along with 1710 (ester) and a broad peak at 3400 for phenolics hydroxyl group. The IR spectrum data of compound B found similar as of cluytyl ferulate indicated similar substitution pattern in both the esters. The \textsuperscript{1}H-NMR spectrum of compound B indicated two olefinic proton signals appearing as doublets at δ 6.2720 and 7.5877 for α-proton and β-proton of cinnamoyl moiety respectively. The signals that appeared as multiplets at δ 7.0876, 6.9259 and 6.9056 were assigned to aromatic protons of a 3,4-dihydroxylcinnamoyl group (H-2, H-5 and H-6) respectively. Further, spectrum showed a triplet at 0.88 δ for the presence of -CH\textsubscript{2}-CH\textsubscript{3} (5H), a broad singlet at 1.2533 δ for -CH\textsubscript{2} (52H, long chain proton), a broad multiplet at 4.2036 δ for -CH\textsubscript{2}O (2H) and a hydroxyl signals at 5.8598 δ (OH, H-3, singlet) with a broad signal at 6.0720 (-OH, H-4). Resemblance of spectral data of compound B with cluytyl ferulate indicated possibility of another ester of cluytlyl alcohol. Further, a singlet that appeared at 3.9309 for one -OCH\textsubscript{3} group (3H) in cluytlyl ferulate was found to be absent in compound B and in place of that it showed two broad singlet signals for 3,4-dihydroxylcinnamoyl group which revealed the presence of caffeoyl moiety in the structure of ester. Further, the results of \textsuperscript{1}H-NMR data and a base peak found at 163.4 was found to be in good agreement for the data of caffeic acid moiety being present in structure of compound B. A long chain ester characteristic was reflected in all spectral data for compound B and on that basis, compound B was determined as caffeic acid ester of cluytyl alcohol (cluytyl caaffeite). Thus, isolated
compound A and B were identified as cluytyl ferulate and cluytyl caffeite respectively. Cluytyl ferulate was reported earlier in heartwood of *G. arborea* but in present study it was isolated from the most active hepatoprotective and antioxidant fraction (ethyl acetate) of roots. Besides cluytyl ferulate, cluytyl caffeite was also isolated from the same fraction of roots of *G. arborea* and this is the first instance of isolation and characterization of cluytyl ferulate and cluytyl caffeite from the bioactive fraction of roots of *G. arborea*. Cluytyl caffeite has not been reported earlier in any literature from roots of *G. arborea*.

**6.6 HPTLC Analysis:**

**6.6.1 Estimation of isolated compound A (cluytyl ferulate) and compound B (cluytyl caffeite) by HPTLC method:**

Ethyl acetate extract (EAE) and hydrolyzed ethyl acetate extract (HEAE) of root powder was prepared and analyzed for presence of isolated compounds cluytyl ferulate and cluytyl caffeite. A simple and sensitive HPTLC method was developed for the estimation of both the isolated compounds in EAE and HEAE.

In the TLC densitometric method it was observed that the best resolution of cluytyl ferulate, avoiding interference from the other components of the extracts, was obtained in the solvent system of hexane: ethyl acetate (9:1) at $R_f$ 0.3±0.02. The spot corresponding to the same $R_f$ as that of cluytyl ferulate was also resolved in EAE chromatogram. Linear relationship was obtained for cluytyl ferulate in the range of 400-1200 ng/spot with a correlation coefficient of 0.999. The method was validated in terms of precision, repeatability and accuracy. The limit of detection and limit of quantification were found to be 100 ng/spot and 400 ng/spot respectively. The scanning wavelength of 327 nm was the absorption maximum of the in-situ spectrum of cluytyl ferulate zone measured by use of the spectral mode of the Camag scanner 3. Other four zones were also found to be present in EAE chromatograms resolved at $R_f$ 0.09, 0.45, 0.68 and 0.8. The % content of cluytyl ferulate estimated in the EAE of roots of *G. arborea* was found to be 0.236823±0.0095 by proposed HPTLC method. Cluytyl ferulate was found to be absent in HEAE.
Cluyltyl caffeite resolved as a flat, band-shaped zone at $R_f 0.49 \pm 0.03$, on the silica gel HPTLC plate, developed with hexane: ethyl acetate (9:1) as mobile system and the corresponding spot was also found to be resolved at the same $R_f$ in EAE chromatogram. The densitometric detection of cluyltyl caffeite was carried out at UV 287 nm, which was the absorption maxima found for cluyltyl caffeite in our study. Linear relationship was obtained for cluyltyl caffeite in the range of 400-1200 ng/spot with a correlation coefficient of 0.998. The method was validated in terms of precision, repeatability and accuracy. The limit of detection and limit of quantification were found to be 150 ng/spot and 400 ng/spot respectively. Other four zones were also found to be present in EAE chromatograms resolving at $R_f 0.09$, 0.30, 0.68 and 0.8. The % content of cluyltyl caffeite in the EAE of roots of *G. arborea* was found to be $0.0542 \pm 0.0044$ by the proposed HPTLC method. Cluyltyl caffeite was also found to be absent in HEAE.

6.6.2 Estimation of quercetin, $\beta$-sitosterol and lupeol by HPTLC method:

The positive results in preliminary phytochemical screening for flavonoids and sterols, led us to check for the presence of known flavonoids and sterols in both the extracts. In preliminary study, lupeol was found to be present in HEAE and $\beta$-sitosterol was found to be present in EAE while quercetin was found to be present in both the extracts. A simple and sensitive HPTLC method was developed for the estimation of quercetin, $\beta$-sitosterol and lupeol in EAE and HEAE.

HPTLC analysis for quercetin revealed that the best resolution, with minimum interference from the other components of the extracts, was obtained in the solvent system of ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26) at $R_f 0.38 \pm 0.03$ in EAE and HEAE. Linear relationship was found in the range of 100-500 ng/spot with a correlation coefficient of 0.992. Detection and quantification limits were found 50ng and 100 ng respectively. Three other zones were also resolved at $R_f$ 0.12, 0.18 and 0.95 in chromatograms of EAE and HEAE. The densitometric detection of quercetin was carried out at 366 nm (absorption maxima) and the identity was confirmed by overlain spectra and comparing $R_f$ of corresponding spot with standard quercetin. The % content of quercetin estimated was found 0.021672±0.0021 in EAE and 0.060554±0.0033 in HEAE of roots of *G. arborea*. 
HPTLC method for estimation of β-sitosterol and lupeol was also developed for EAE and HEAE of root of *G. arborea*. β-sitosterol was estimated with very good resolution and minimum interference with other constituents in mobile system toluene: methanol (9.4:0.6), and detected as violet coloured band with 0.5 % anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 10 mins at Rf 0.31±0.03. It was then scanned at its absorption maxima 523 nm after derivatization. Three other bands resolved at Rf 0.12, 0.22 and 0.43 were found to be present in both, EAE and HEAE (spot at Rf 0.22 found absent). The identity was confirmed by co-chromatography and overlain absorption spectra with reference standard, when scanned at 523 nm. The method was precise and found to be linear in the range of 500-7500 ng/spot with the correlation coefficient 0.992. Limit of detection and limit of quantification was found 100 ng/spot and 500 ng/spot respectively. The % content of β-sitosterol estimated was found 0.120±0.018 and 0.110±0.019 in EAE and HEAE respectively.

Lupeol was also checked for the presence in EAE and HEAE and estimated by proposed HPTLC method. The spot of lupeol resolved at Rf 0.9±0.02 in the mobile system, toluene: methanol (9.4:0.6) and detected as bluish pink coloured spot after derivatization with 0.5 % anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 10 mins. The spot corresponding to the standard in the samples track and of a standard was scanned at 529 nm. Further, the identity was confirmed by good overlain spectra of standard lupeol with the corresponding spot in the HEAE chromatogram. The method was found linear in the range of 500-7500 ng/spot and with the correlation coefficient 0.995 and LOD and LOQ was found 100 ng/spot and 500 ng/spot respectively. Four other bands at Rf 0.14, 0.25, 0.35 and 0.40 were resolved in sample extracts. Lupeol was found to be present and estimated as 0.116259±0.00437 % w/w, in HEAE only.

Thus, in the present study crude EAE and hydrolyzed EAE, both were checked for the presence of various constituents and the % content of each was determined in respective extracts. A quantitative method was developed using silica gel HPTLC plates, automated band-wise sample application, detection with specific reagent solutions for each component and automated densitometric determination for a variety of constituents present in roots of *G.*
The new HPTLC methods developed for isolated compounds as well as quercetin, β-sitosterol and lupeol, were validated for specificity, linearity, accuracy, and precision. Accuracy was validated by analysis of spiked blank and standard addition samples and precision by performing replicate analyses on a single day and on different days. It is apparent from the results that validation data for these new quantitative HPTLC methods for analysis of cluytyl ferulate, cluytyl caffeite, quercetin, β-sitosterol and lupeol meet the acceptance criteria for accuracy, precision, linearity, and for detection and quantification limits.

G. arborea roots were reported to contain cluytyl ferulate, but cluytyl caffeite was isolated and estimated first time by the proposed HPTLC method. Further, no data was reported for the estimation of cluytyl ferulate, quercetin, β-sitosterol and lupeol in roots of G. arborea. This is the first instance of developing simple and specific HPTLC methods for the estimation of various constituents from the roots of G. arborea.

Two hydroxycinnamic acid esters of cluytyl alcohol, cluytyl ferulate and cluytyl caffeite were isolated from the hepatoprotective and antioxidant fraction of roots of G. arborea. The hydroxycinnamates are reportedly known intermediates in the phenylpropanoid synthetic pathway. Phenolic compounds have been reported to act as free radical scavengers by virtue of their hydrogen-donating ability and forming aryloxy radicals. The stabilization of such radicals by other functional groups in the structure enhances the antioxidant activity. Ferulic acid, due to its phenolic nucleus and unsaturated side chain, readily forms a resonance-stabilized phenoxy radical, which accounted for its potent antioxidant activity reported (Graf, 1992). The antioxidant activity of ferulic acid in scavenging superoxide anion (Toda et al., 1991), and the ability to protect against malondialdehyde generation in peroxidizing membrane systems (Sharma, 1976) has been reported earlier. Further, ferulic acid and caffeic acid esters have been reported to exhibit free radical scavenging activity (Zhaohui et al., 2003). Ferulic acid ethyl ester (FAEE) is known for its anti-inflammatory and antioxidant properties (Yan et al., 2001; Kikuzaki et al., 2002) as it showed hydroxyl and peroxyl radical scavenging activity (Sultana et al., 2005). Further, in the mechanism for the protective effects of various phenolics in liver injury, α, β- unsaturated
carboxyl ester moiety has been reported as the essential part to exert hepatoprotective activity (Eun et al., 2002). Taking into account the fact that hydroxycinnamate derivatives have demonstrated hepatoprotective activity, their presence in the ethyl acetate fraction of *G. arborea* could explain the protective effects observed in present study. Quercetin is another powerful antioxidant well known for metal-ions, such as iron and copper binding properties, as well as radical scavenging abilities (Inal and Kahraman, 2000; Szeto and Benzie, 2002). Further, the possible protection provided by quercetin against hepatic injury in rats was also reported and underlying mechanism is found to be associated with improved hepatic antioxidant capacity and decreasing tissue MDA content (Su et al., 2003). Quercetin, a known flavonoid was also found to be present and estimated in EAE and HEAE of roots of *G. arborea*, which further indicated that the effectiveness of EAF of roots of *G. arborea* may be associated with the presence of phenolics and flavonoids constituents.

In the light of above, we conclude from the present study, significant antioxidant and antilipidperoxidative activity exhibited by ethyl acetate fraction of roots of *G. arborea* can be attributed to the presence of phenolics and flavonoids in the roots. These constituents from roots of *G. arborea* may be possible active compounds, having themselves antioxidant activity or showing synergism with other bioactive compounds present in the roots but not either isolated or characterized by the techniques used in the present study (e.g. lignans). This antioxidant activity may be at least partly, responsible for significant hepatoprotective activity observed in the present study. Further, the new developed HPTLC method is likely to be valuable for qualitative and quantitative analysis of roots of *G. arborea* and thus can help in the standardization of this drug, which is highly valued in traditional system of medicine for its multiple range of activities.