Liver is the largest organ in vertebrate body, is a major site of intense metabolic activities. Despite the fact that the liver is an important organ for the detoxification and disposition of endogenous substances, its functions may readily be impaired by viruses, hepatotoxins and xenobiotics. Hepatic damage in such cases is associated with distortion of many vital metabolic functions. Management of liver diseases is still a challenge to modern medicine. Unfortunately conventional or synthetic drugs used in treatment of liver diseases are inadequate and cause deleterious effects (Guntupalli et al., 2006). In absence of a reliable liver protective drug in modern medicine, there are number of alternate medicinal options recommended from natural sources for the treatment of liver disorders. Bioactive natural products can be considered very promising starting points for the development of new therapeutic agents. Increased interest in plants as a source of novel pharmacophores recognizes their chemical diversity and versatility, not matched by synthetic chemistry libraries. An overview of the recent literature reveals that a great number of plant derived constituents exhibit a pleiotropic spectrum of medicinal activities. In Indian system of medicine, a large number of medicinal plants and herbal drugs have been advocated for treating liver disorders. An overview of the recent literature reveals that the family Malphighiaceae and its species *Hiptage benghalensis* exhibit a wide spectrum of medicinal activities. However the hepatoprotective potentials of *H. benghalensis* still remains unexplored which prompted me to select this plant to screen and validate its hepatoprotective activity against CCl₄ induced hepatic damage.

**7.1 PHARMACOGNOSTIC STUDIES**

A holistic interdisciplinary approach and a scientific basis of understanding plant systems is essential to overcome lack of drug standardization, inappropriate formulation and quality control which act as a major lacuna in traditional medicines (Joy et al., 2001). Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy.
**Discussion**

The process of standardization can be achieved by stepwise pharmacognostic studies (Ozarkar, 2005). Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics. According to world health organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity, purity and should be carried out before any tests are undertaken (WHO, 2002).

Organoletic study of the leaf powder of *H. benghalensis* revealed that it was green in colour, had an aromatic odour and a bitter taste (Table 2). Macroscopic and microscopic results showed that the leaf consists of a wide and thick semi-circular midrib and a thick lamina which was dorsi ventral. The adaxial epidermis was thick with rectangular or squarish cells and with thick cuticle. The abaxial epidermis was narrow and cylindrical. Epidermal trichomes were frequently seen on the epidermal cells. The abaxial epidermis was stomatiferous. The stomata were paracytic in type. Stomata is the main factor responsible for the physiological activities of the plant, abnormal stomata is responsible for behavior and hormonal imbalance in plants (Kridemann *et al*., 2000). In calyx both rosette and cluster type of calcium oxalate crystals were found; these could be used to distinguish the species. The vascular strands consisted of thin walled xylem elements and thin layer of phloem on the outer border. Flowers were observed with five petals characterized with white, pink and a yellow blotch, Fruit is a samara with 3 unequal wings characteristic to this species (Table 3). Previous pharmacognostic studies on this plant also reported similar features (Chenthurpandy *et al*., 2009).

### 7.2 FLOURESCENCE ANALYSIS

Fluorescence analysis is an important qualitative parameter of pharmacognostical evaluation of a plant material (Bigoniya *et al*., 2012) and is a phenomenon exhibited by the phytoconstituents of a plant. Some constituents fluoresce in daylight while some require UV light (Kala *et al*., 2011). A non-fluorescent compound may exhibit fluorescence if mixed with impurities or other reagents. In the current study the fluorescence property of the leaf powder and various solvent extracts were observed in daylight and UV light and has been reported in tables 4 and 5.
The physicochemical evaluation of a plant is an important parameter in detecting adulteration or improper handling of drugs (African Pharmacopeia, 1986). The ash value of the plant gives an idea of the inorganic composition and other impurities present in the plant species. Total ash is the measure of the total amount of material left after burning and includes ash derived from the part of the plant itself and acid insoluble ash. Higher values of total ash content (6.3%) and less acid insoluble ash values (1.3%) in the present study clearly indicated the presence of higher inorganic content and the purity of the test leaf sample.

Extractive values are useful for the determination of exhausted or adulterated drugs and also help in estimation of specific constituents soluble in a particular solvent (Ozarkar, 2005). The variation in extractable matter in various solvents is suggestive of the fact that the formation of the bioactive principle of the medicinal plants is influenced by number of intrinsic and extrinsic factors. In the present study the hexane (1.95%) and chloroform (2.32%) extractive values were more when compared to ethyl acetate (1.15%); this may be due to the presence of essential oil and terpenes. High alcohol soluble and water soluble extractive values reveal the presence of polar substance like phenols, tannins and glycosides (Sharma et al., 2009; Baravalia et al., 2010). In accordance to this the alcohol extractive value (11.02%) of H. benghalensis leaf powder was found to be higher when compared to water extractive values (6.14%) (Table 6, 7 and 8).

It has been widely observed and accepted that the medicinal value of plants lies in its bioactive phytocomponents (Veeramuthu et al., 2008). The data of the results of the preliminary phytochemical screening of various extracts of H. benghalensis leaf powder are given in table 9. Terpenes and sterols were present in hexane, chloroform, and ethanol extracts. Whereas, coumarins were present in all extracts except water; tannins were present in all extracts except chloroform extract; flavonoids were present in ethyl acetate, ethanol and water extract. Proteins and alkaloids were present in all extracts while saponins were present in water and hexane extracts (Table 10). The phytochemical results of the present study are in accordance with previous studies on the same plant.
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(Chenthurpandy et al., 2009). The presence of alkaloids, tannins, phenolic compounds and flavonoids has been associated with various degrees of anti-inflammatory, analgesic (Wang et al., 2008) and antioxidant activities (Molina et al., 2003; Gholivand et al., 2010). The hepatoprotective potential of monoterpenoids (Pandy and Chaturvedi, 1970), di and triterpenoids (Valan, 2010), sesquiterpenoids (Doreswamy and Sharma, 1995) has been well documented over the years.

The quantitative analysis of important inorganic substances were determined and tabulated in Table 11. The calcium (4.20%) and iron contents (14.23 ppm) were found to be more. The amount of potassium (3.62%), magnesium (2.63%) and manganese (5.63 ppm) were present in moderate levels whereas boron (0.04 ppm), molybdenum (0.02 ppm), copper (0.04 ppm), phosphorous (0.32%) and sodium (0.28%) were present in lesser quantity.

The presence of high and moderate levels of calcium, iron, manganese, potassium and magnesium may contribute towards maintaining the normal metabolic pathways. Calcium imparts strength and rigidity and also needed in neuromuscular transmission, in excitability of nerves for normal excitability of heart, in clotting of blood and promoting muscular contraction. It also acts as an activator of the enzymes phospholipase, arginine kinase, adenosine triphosphatase and adenyl kinase. Iron is the most well known in biological system. It performs a wide range of biological functions. Many of these functions are connected with oxidation-reduction and processes by which energy is conserved in the body. It forms an integral part of cytochromes, haemoglobin, myoglobin, metallo-flavoproteins and certain enzymes such as catalase and peroxidases.

Potassium is important as diuretic and it takes part in ionic balance of the human body and maintains tissue excitability. It is the principal intracellular cation and also considered as a very important constituent of the extracellular fluids (Venkataraman and Gopala Krishnan, 2002). In muscles and other tissues, intracellular magnesium ions function as activators for many of the enzymes involved in carbohydrate metabolism and synthesis of nucleic acids (DNA and RNA). It also acts as an important binding agent of ribosomal particles where protein synthesis takes place. Manganese is essential for haemoglobin formation. Phosphate ions are the major anions of intracellular fluids,
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phospholipids and the coenzyme NAD and NADP and especially of ATP and other high energy compounds (Indrayan et al., 2005).

Sodium along with potassium takes part in maintaining the ionic balance of the human body, tissue excitability and also plays an important role in the transport of metabolites. Copper is an important component of many enzyme systems such as cytochrome oxidase. Besides, all these functions, inorganic constituents also play a vital role in maintaining antioxidant status.

Phytochemical profiling is of special significance since it has a direct bearing on the activity of the herbal drugs and its quantification would serve as an additional parameter in assessing the quality of the sample (Calixto, 2000). The quantitative analysis of important phytoconstituents of *H. benghalensis* were estimated and given in table 12. The total flavonoids (2.52 mg/kg) and terpenoids (3.14 mg/kg) contents were found to be more compared to alkaloids (1.53 mg/kg) and tannins (2.42 mg/kg). The presence of these compounds may attribute to the pharmacological activity of the plant of interest.

7.4 ANTIOXIDANT ASSAY AND TLC PROFILING

Several epidemiological studies suggest that rich anti-oxidant content show a strong anti-oxidant activity which has a direct influence in the pharmacological potential of a chosen plant. Selective extraction from natural materials by an appropriate solvent is important for obtaining fractions with high anti-oxidant activity. However the anti-oxidant activity of plant extracts varies with assay methods. Therefore a single assay may be inadequate (Sun and Ho, 2005). For this reason anti-oxidant activity of aqueous and ethanolic leaf extracts of *H. benghalensis* (ELEHB) was assayed with three methods based on different mechanisms namely: DPPH assay, reducing power assay and nitric oxide assay to evaluate the extract with better anti-oxidant activity. Our results indicated that ELEHB possessed prominent anti-oxidant activity than aqueous extract when compared with standard (Table 13 & Fig. 2). Hence ethanol was chosen as the optimal solvent for extraction than aqueous for further studies. The results of anti-oxidant potential of *H. benghalensis* are the first of its kind and have not been documented earlier.
TLC in phytochemical evaluation of herbal drugs is being employed extensively because it enables rapid analysis of herbal extracts with minimum sample clean-up requirement, it provides qualitative and semi quantitative information of the resolved compounds and it enables the quantification of chemical constituents (Priti Patil and Rajani Shettigar, 2010). In the present study, TLC profiles of the ELEHB (Table 14, Fig. 3.1 & 3.2) on silica gel plates using toluene: ethyl acetate (93:7) as mobile phase showed 4 spots under UV (254nm) and 8 spots under UV (366nm) which may indicate the presence of terpenes as this is the most suitable mobile phase to detect terpenes. Qualitative and quantitative tests in the present study also confirmed the presence of terpenes as retrieved in TLC profiling.

7.5 HPTLC and GC-MS

HPTLC technique is widely employed in identification and detection of adulterants in herbal products, quality control of herbs (Soni, et al., 2010) and authentication of various species of plants. HPTLC analysis was carried out for ELEHB in the present study with toluene: ethyl acetate (93:7) as mobile phase and scanned at 366 nm. The results (Table 15, Fig. 4 & 5) showed that ELEHB showed 6 peaks at 254 nm and 8 peaks at 366 nm.

GC and GC-MS are unanimously accepted methods for the analysis of volatile constituents of herbal medicines, due to their sensitivity, stability and high efficiency. Especially, the hyphenation with MS provides reliable information for the qualitative analysis of the complex constituents (Guo et al., 2006 and Teo et al., 2008). In the present study GC-MS was carried out for ELEHB to analyze its chemical constituents and the results revealed 55 constituents which has been tabulated and illustrated in table 16 and fig. 6 along with their chemical name, molecular formula, molecular weight, retention time and percentage area. Identification of the chemical constituents were based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns (Sermakkani and Thangapandian, 2011). A detailed literature survey on the constituents were done which revealed that most of the constituents possessed good pharmacological properties and the constituents with
good antioxidant and hepatoprotective were chosen for in silico analysis to prophesize that the pharmacological potential of the chosen extract may possibly be due to the presence of these constituents.

7.6 ACUTE TOXICITY STUDIES

Acute toxicity studies in animals are essential for any therapeutical substance intended for humans as the results of these studies may be used in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity and occasionally, revealing delayed toxicity. It aids in the selection of starting doses for Phase 1 human trials, provides information about over dosage and could also be used for the determination of therapeutic index (LD50/ED50) of drugs (Rang et al., 2001; Maikai et al., 2008).

ELEHB was subjected to acute toxicity studies. A single dose of 1, 2, 3, 4, 5 g/Kg bw. was given to five different groups of animals and observed continuously for 21 days. Administration of plant extract caused no significant change in general behaviour of the experimental animals. Food and water intake were normal. Body temperature, state of stools, initial and final body weight were not altered significantly on treatment. The liver marker enzymes SGPT, SGOT and ALP were not altered significantly compared to normal control. Other biochemical parameters such as urea, uric acid, protein, glucose, bilirubin and creatinine levels were observed to be within normal limits indicating the healthy status of the experimental animals. The plant extract did not show any toxicity and mortality up to a dose of 5 g/Kg bw. in experimental animals (Table 17-21 & Fig.7-11). The observations of the present study matched with the findings reported by Kulkarni et al., 1986; Janorious winka et al., 2012.

7.7 HEPATOPROTECTIVE STUDIES

Liver diseases such as cirrhosis, fatty liver and chronic hepatitis are important world health issues. Treatment of diseases associated with hepatic injury is very vital, and must be done with importance and extensive care. Inspite of the tremendous scientific advancement in the field of hepatology in recent years, liver problems are on the rise. In view of several undesirable side effects of synthetic agents there is growing focus to
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follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity.

*H. benghalensis* popularly known as Madhavi lata is a commonly available high climbing liana claimed to possess anti-inflammatory, antimicrobial, wound healing and hepatoprotective activity. However methodical investigation on the hepatoprotective potential of this plant has been far and few. Therefore the present investigation is designed to evaluate the hepatoprotective efficacy of ethanolic extract of *H. benghalensis* against CCl₄ induced hepatic damage in male albino wistar rats.

### 7.7.1 CCl₄ AS INDUCING AGENT

Hepatotoxicity induced by CCl₄ is the most commonly used model system for the screening of hepatoprotective activity of plant extracts/drugs (Srivastava and Shivanandappa, 2010). The changes associated with CCl₄ induced liver damage are similar to that of acute viral hepatitis (Rubinstein, 1962). The hepatotoxicity of CCl₄ is attributed to the formation of trichloromethyl and trichloromethyl peroxyl radicals, initiating lipid peroxidation and resulting in fibrosis and cell necrosis (Kadiiska *et al.*, 2000). Long-term administration of CCl₄ causes chronic liver injury (Hernandez-Munoz *et al.*, 1990).

### 7.7.2 EFFECT OF ELEHB ON THE SERUM HEPATIC MARKER ENZYMES

Serum levels of marker enzymes are very sensitive indicators employed in the diagnosis of liver diseases and to understand the extent (Ansari, 1991) and position of the liver injury. The liver marker enzymes (AST, ALT and ALP) are cytoplasmic in nature; LDH is a non-specific enzyme but is an important marker for both liver and heart. Upon liver injury these enzymes enter into the circulatory system due to altered permeability of membrane (Zimmerman and Seeff, 1970). In this study, significant increase in AST, ALT, ALP and LDH levels in the serum were observed after administration of CCl₄. GOT is associated with liver parenchyma cells and is raised in acute liver damage. GPT is an enzyme found in hepatocytes and its leakage into blood is observed in acute liver damage. ALP is excreted normally via bile by the liver. The liver injury due to toxins can result in
defective excretion of bile by hepatocytes which are reflected as their increased levels in serum (Rajesh and Latha, 2004).

In the present investigation, it was evident from the results obtained that the rise in marker enzymes level in CCl₄ induced animals is attributed to damaged structural integrity of the liver. LDH levels were also raised by almost six-and-a-half fold in the animal group intoxicated with CCl₄ when compared to the control (Mastour Al-Ghamdi, 2003). This accounts to a finding which may suggest that other organs had also been damaged by CCl₄ intoxication. However on treatment with ELEHB all enzyme activities returned to near normal which may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity (Table 22 & Fig. 12). The therapeutic efficacy of a plant drug depends on its ability to reduce the harmful effects or in maintenance of structural integrity of hepatocytes that was disturbed by the hepatotoxin.

Administration of the selected plant extract restored the enzyme levels to normalcy. An evident change was noticeable in groups treated with in 300mg/kg bw. of ELEHB. This is indicative that the plant extract exhibited good hepatoprotective potential as it contributed to the maintenance of the structural integrity of the cellular membranes of the hepatocytes by restoring the altered levels of hepatic marker enzymes. Silymarin, the standard drug chosen for the present study also exhibited excellent protective potentials and the effect of ELEHB was well comparable to the values obtained with silymarin. The results of the present investigation are in agreement to the findings of previous studies carried out by Thabrew and Joice, 1987.

7.7.3 EFFECT OF ELEHB ON THE MEMBRANE BOUND ENZYMES

The membrane bound ATPases are SH group containing enzymes and are lipid dependent (Gubdjarson et al., 1983). Sodium Potassium ATPase is an integral plasma protein involved in energy consumption in cellular metabolism (Rajat Sandhir and Gill, 1999). Calcium and Magnesium ATPases are membrane bound enzymes that play a prominent role in the active transport of electrolytes across biological membranes. The administration of the hepatotoxicant (CCl₄) altered the membrane permeability that was...
noticeable with enhanced production of active metabolites that eventually led to hepatocellular necrosis and accumulation of water in hepatocytes.

Calcium ions can decrease the lipid fluidity of hepatocyte plasma membrane by influencing membrane bound enzymes to alter the lipid composition. It has been reported that free radical enhanced calcium release from the sarcoplasmic reticulum and also they inhibited sarcolemmal Na\(^+\)/K\(^+\) ATPase, possibly causing the activation of the Na\(^+\)/Ca\(^+\) exchange mechanism in the hepatic cell membrane (Trump, 1984).

The restoration of the normal activity of the membrane bound ATPases was achieved on treatment with ELEHB which revealed its capacity to inhibit membrane lipid per oxidation thereby protecting the SH groups of the enzymes from oxidative damage. Silymarin treated groups also showed similar results offering maximum protection to the SH groups of the enzymes (Table 23 & Fig. 13). The protective role of ELEHB was significant and well comparable with the animal groups treated with silymarin.

### 7.7.4 EFFECT OF ELEHB ON THE ANTIOXIDANT ENZYMES

Free radical induced oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders as there are ample experimental and epidemiological studies supporting the involvement of oxidative stress in the pathogenesis and progression of several chronic diseases and great importance has been attributed to antioxidants in the prevention and treatment of diseases (Maxwell, 1995). Oxygen free radicals are rapidly and effectively neutralized by a series of enzymatic and non-enzymatic antioxidant systems. Aerobic organisms employ a battery of defense mechanisms such as antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) to prevent or mitigate oxidative tissue damage (Halliwell and Gutteridge, 1989).

Superoxide dismutase (SOD) removes the toxic superoxide radical (O\(^2-\)) formed by the partial reduction of oxygen in tissues. The function of SOD is to protect aerobic organisms against the potential deleterious effects of superoxide anion (O\(^2-\)). Catalase is an enzyme prevalent in erythrocytes, liver and also found in small bodies as peroxisomes containing the enzymes (oxidases). It deals effectively with a large amount of hydrogen...
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Peroxide generated in peroxisomes, removes two hydrogen atoms from one molecule of hydrogen peroxide (the substrate), liberating oxygen, and gives them to another molecule of hydrogen peroxide, forming water thus protecting from oxidative stress (Popovici Irina, 2000). GPx is an important constituent of the glutathione redox system that is not only capable of utilizing hydroperoxides but also for metabolizing the same in both the cytosolic and mitochondrial compartments.

Perturbation of the GSH status of a biological system has been reported to increase the lipid peroxidation. When the liver cell plasma membrane is damaged, many of the enzymes normally located in the liver cytosol are released into the blood stream. Their estimation in blood is a useful quantitative marker of the extent and type of hepatocellular damage (Uday et al., 1999). GST binds to lipophilic compounds and acts as an enzyme for glutathione (GSH) conjunction reaction. The redox system works effectively to reduce the production of ROS which is responsible for lipid peroxidation and membrane damage.

The reactive metabolites such as trichloromethyl (CCl₃) and trichloromethylperoxy radicals emanated from CCl₄, initiated peroxidation of membrane unsaturated fatty acids. This resulted in lipid peroxidation of membrane that seriously impaired the function of liver. The antioxidant enzymes SOD, CAT, GSH, GPx and GST constitute a mutually supportive team of defense against ROS (Uday et al., 1999). The decrease in activity of SOD in the liver of CCl₄ induced rats may have attributed to the increased lipid peroxidation or inactivation of the enzyme by cross-linking with malondialdehyde. This further caused accumulation of superoxide radicals instigating increased lipid peroxidation. GST, which binds to lipophilic compounds and acts as an enzyme for glutathione (GSH) conjunction reaction was also found to be reduced due to CCl₄ toxicity may be due to decreased availability of GSH. Depletion of GSH also enhanced lipid peroxidation which inturn caused decrease in its levels (Anandan et al., 1999). There was also a notable depletion in the levels of GPx in CCl₄ induced rats indicative of damage to membranes and other cell components. In the present study the activities of SOD, CAT, GSH, GPx and GST were found to be significantly decreased in the CCl₄ induced rats compared with normal. Administration of ELEHB restored the
activities of the above anti-oxidant enzymes to near normal when compared to the CCl₄ administered rats. The changed activity of these marker enzymes observed in CCl₄ induced rats in our study correspond to the extent of liver damage induced by the toxin. The tendency of these enzymes to return towards a near normal level in groups treated with silymarin and ELEHB is a clear manifestation of their anti-hepatotoxic effect (Table 24 & Fig. 14).

7.7.5 Effect of ELEHB on Biochemical markers

Liver is the main site of synthesis for albumin and globulin. A significant decrease in the levels of protein is observed after CCl₄ intoxication. This may probably be due to loss of structural integrity of liver cells causing transfer of protein to other parts of the body. In the present study CCl₄ induced experimental animals showed reduced levels of protein which is indicative of liver damage and impaired synthetic function of the liver. However on treatment with ELEHB a noticeable elevation in protein levels was observed which may be due to regeneration of liver tissues (Table 25 & Fig.15). The results obtained corroborated with the histopathological studies (Plate 10) and supported by the data of results on the DNA content of the hepatic tissues.

A/G Ratio

Liver is the main site of synthesis of albumin which plays a key role in maintaining colloidal osmotic tension. Decreased level of albumin is an indicator of liver impairment. Globulin is a simple protein which is comprised of different subtypes namely alpha, beta, and gamma. They play an important role in the defense mechanism of the body by eliciting immunological response. Globulin levels were found to be increased with liver dysfunction indicated by increased concentrations of gamma globulin that are produced by the immune system.

In the present investigation, there was a reversal of A/G ratio in CCl₄ intoxicated groups which may be due to decreased synthesis of albumin in liver. The ratio was restored close to normal on treatment with ELEHB indicating that the synthetic functions
Discussion

of liver was resumed. The results obtained were on par with the silymarin treated groups that also showed significant reversal in A/G ratio (Table 25 & Fig 15).

Hepatic Glycogen

Glycogen is the major energy reserve of the body and is synthesized and stored in the liver. There is a marked decrease in the level of glycogen on liver impairment due to induction of excessive glycogenolysis. CCl₄ intoxication damages the active hepatocytes that are involved in the synthesis of glycogen resulting in its reduction. A noticeable improvement in the levels of glycogen was found in the animals treated with ELEHB which may be due to its ability to rejuvenate the hepatocytes thereby improving its synthetic abilities. The silymarin treated animal groups showed nearly normal synthetic function of the liver. The group treated with ELEHB at a dose level of 300mg/kg bw. revealed similar effects as silymarin (Table 25 & Fig.15).

Hepatic Hydroxyproline

Hepatic fibrosis is a scarring response of the liver to chronic liver injury that may lead to irreversible cirrhosis. When fibrosis progresses to cirrhosis, morbid complications can develop as a consequence of chronic or repeated liver injury caused by hepatotoxic agents like alcohol and viruses, as well as immune and congenital metabolic disorders (Albanis et al., 2003). The level of hydroxy proline in liver increases with increasing hepatic fibrosis acting as a reliable marker for detecting fibrosis (Aoto, 1984). The hydroxy proline content in the liver is related to the reduced content of Cyt P450 in liver. During CCl₄ induced liver injury the levels of Cyt P450 reduces resulting in the accumulation of hydroxy proline. As the damage progresses the solid liver tissue becomes fibrotic and hence the collagen content of the tissue gets hydrolysed to hydroxy proline.

In the present study the CCl₄ induced rats showed an elevation in the hydroxy proline content. The levels of hydroxy proline decreased on treatment with ELEHB, indicating the efficacy of ELEHB towards preventing degradation and arresting hepatic fibrosis (Table 25 & Fig.15). The results obtained were in par with that of silymarin.

Serum Bilirubin
Discussion

Bile flow appears to be largely determined by the active canalicular secretion of bile salts. The reduction in the spontaneous bile flow can be ascribed to the inhibition of active transport mechanisms of the liver cells when affected by hepatotoxins. Liver is the site of synthesis of bilirubin. It is an important marker of hepatic functioning. The increase in the levels of serum bilirubin reflects the depth of liver disease inefficiency of the liver to conjugate bilirubin and excrete it into the bile (Forker, 1969).

In the present study, the elevated levels of bilirubin concentration was observed in the disease control group which was an indication of the extent of liver impairment. The CCl₄ induction caused perivenular changes preventing the uptake of unconjugated bilirubin. Administration of the ELEHB resulted in a significant decrease in bilirubin levels thereby reducing the depth of the disease. The data of results obtained were comparable with that of the standard drug silymarin (Table 25 & Fig.15).

Synthesis of DNA

Active cells in the body undergo cell division to replace the worn out tissues. DNA synthesis occurs during the cell cycle. The cells which undergo mitosis are metabolically active and are involved in the synthesis of DNA. Cells which are damaged by toxins usually lose the capacity to regenerate.

In the present study, reduced DNA synthesis was observed in the CCl₄ induced experimental animals acting as an indication of hepatic damage in the animals. The experimental animals treated with ELEHB resulted in increased DNA synthesis indicating progress in hepatic cellular division that subsequently led to the regeneration of damaged tissues (Table 25 & Fig.15).

Liver weight

CCl₄ intoxication induced an abnormal increase in liver weight due to blocking of the secretion of the triglycerides in the plasma and fatty infiltration of the liver. In the present investigation, while the experimental animals induced with CCl₄ hepatotoxin showed a significant increase in liver weight, it was decreased in the groups treated with ELEHB. The reduction in liver weight was more prominent in ELEHB treated rats at a
dosage of 300mg/kg bw. (Table 25 & Fig.15). Similar results were observed in the rats treated with the standard drug silymarin. The results of the present study are in accordance with a similar study carried out on extracts of *Psidium guava* (Chanchal Roy *et al.*, 2006).

### 7.7.6 Effect of ELEHB on Lipid Profile in Hepatic Tissues

Lipogenesis and lipoprotein synthesis are important metabolic processes that occur in the liver. Hence damage to liver is often associated with aberrations in lipid metabolism. Any hepatotoxin or other factors contributing to liver damage often cause hepatic necrosis leading to accumulation of fat in liver of which CCl₄ induced toxicity has been reported to prominently cause fatty infiltration of liver with strong centrilobular hepatic necrosis. It is also believed that it causes translocation of fats from peripheral adipose tissue to liver for accumulation during toxicity (Roullier, 1964). In liver diseases the synthesis and metabolism of cholesterol are impaired. It leads to a significant increase in hepatic PL, cholesterol and TG level (Cicognani *et al.*, 1997).

The present study demonstrated an increase in the level of TG, cholesterol, LDL and PL with a decrease in HDL level in CCl₄ induced rats (Table 26 & fig.16). HDL plays an essential role in the transport of cholesterol to the liver for excretion into bile (Dietschy, 1997). The observed increase in cholesterol level may result from the decline in HDL level or from increased fatty acid synthesis in liver. Moreover TG accumulation in hepatocytes is caused by CCl₄ intoxication which might be mediated by the suppression of the secretion of lysosomal acid triacylglycerol lipase activity (Gans, 1973).

This also resulted in a decrease in serum protein levels leading to impaired synthesis of lipoproteins, thereby leading to accumulation of lipids in the liver ultimately causing fatty liver. Administration of ELEHB reverted back the hypercholesterolemic, hypertriglyceridemic and hyperphospholipidemic profiles of CCl₄ intoxicated animals to near normalcy. On comparison with the standard drug treated animals, the results of the effect of ELEHB treated rats on lipid profile were a close match.

### 7.7.7 HISTOPATHOLOGICAL STUDIES


**Discussion**

Histopathological examination with microtome sections of liver tissues stained with Hematoxylin and Eosin showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. In the liver sections of the rat intoxicated with CCl\textsubscript{4} there was disarrangement and degeneration of normal hepatic cells with intense centrilobular necrosis with sinusoidal hemorrhages and dilatation. Treatment with ELEHB showed very mild fatty changes. The hepatic lesions produced by the toxin were reduced. The extent of tissue necrosis was reduced in a dose dependent manner. However the liver sections of the experimental animals treated with ELEHB at a concentration of 300mg/kg bw. and silymarin showed less vacuole formation, reduced sinusoidal dialation, less disarrangement and degeneration of hepatocytes indicating better regenerative activity of both ELEHB and the standard drug silymarin. The observations basically supported the results obtained from the biochemical assays and would serve as a full diagnostic support for the preclinical and clinical studies (Plate 10).

7.8 **IN SILICO STUDIES**

Attempts were made to understand the mechanism of action of molecules in the plant of interest employing *in silico* methods, using the compounds identified in GC-MS analysis namely, squalene, tetradecanoic acid, (E)-9-Octadecanoic acid ethyl ester and the standard drug silymarin.

7.8.1 Selection of target

HCV nonstructural protein 5B (NS5B) polymerase is an RNA dependent RNA polymerase (RdRp) that resides at the C-terminal domain of a polypeptide of several structural and nonstructural proteins and contains the catalytic machinery responsible for synthesis and for replication of the viral genome. NS5B can initiate RNA synthesis by two different mechanisms: primer-independent initiation from the 3 alpha terminus of the viral genome, also known as *de novo* initiation and primer-dependent initiation using either DNA or RNA as primers. The *de novo* synthesis is mainly used during virus replication in infected cells (Lindenbach and Rice, 2005).

The 3D structures of soluble forms of the HCV polymerase genotype revealed a classical “right hand” shape formed by the palm, thumb, and fingers domains as initially
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defined in the Keno fragment of *Escherichia coli* DNA polymerase I (Lindenbach and Rice, 2005) and showed the presence of an extension in the fingers, the so-called fingertip sub domain, containing two loops, delta 1 and delta 2, which anchor the fingers to the thumb. As a result, the polymerase has a relatively closed and spherical appearance, and the active site cavity, to which the RNA template and the NTP substrates have access via two positively charged tunnels, is completely encircled. NS5B polymerase is recognized as the most viable protein target for HCV drug discovery (Tramontano, 2008). NS5B inhibitors have been developed that mimic natural polymerase substrates and bind to less conserved sites outside the active site and impair the enzyme’s catalytic efficiency. Since there is no effective drug for treating the HCV, the need for developing suitable and competent natural plant compounds to inhibit NS5B polymerase has become profound as it could act as a precise and efficacious method for treating HCV infection. This can be achieved through molecular docking studies.

In the present study, three plant compounds namely squalene, tetradecanoic acid, (E)-9-Octadecanoic acid ethyl ester were retrieved from the Pubchem database based on literature studies, were drawn in Chemsketch and optimized. The structure of the HCV NS5B RdRp-was retrieved from PDB database of HCV and molecular docking studies were carried out by targeting its active amino acid residues. Silymarin was used as the reference compound for the present study.

Docking Studies

Protein preparation

AutoDock is a suite of automated docking tool. It is designed to predict the binding of small molecules, such as substrates or drug candidates, to a receptor of known 3D structure (Scott *et al.*, 1999). The protein NS5B RdRp was assigned with Kollmann charges that aided in addition of hydrogens and side chains were optimized for hydrogen bonding. The energy minimized protein was then saved in PDB format. Using MGLTools-1.4.6 the nonpolar hydrogens were merged, AutoDock atom type AD4 and Gasteiger charges were assigned and finally saved in protein.pdbqt format (Morris *et al.*, 1998).

Ligand preparation

Scientific validation and evaluation of hepatoprotective potential of leaf extract of *Hiptage benghalensis* (L.) Kurz
Structure of ligands were drawn using Chemsketch, optimized with 3-D geometry and the two-dimensional structure of squalene, tetradecanoic acid, (E)-9-Octadecenoic acid ethyl ester and Silmarin were converted into 3-D structure using the open Babel format molecule converter (Guha et al., 2006) and saved in PDB format for AutoDock compatibility. MGLTools-1.4.6 (The Sripps Research Institute) was used to convert ligand.pdb files to ligand.pdbqt files.

Active site prediction
The active site of the protein is its binding site or usually a pocket at the surface of the protein that contains residues responsible for substrate specificity which often act as proton donors or acceptors. Identification and characterization of binding site is the key step in structure based drug design. The binding site was identified by computational and literature reports. The active site region of the protein was identified by Q-site (Alasdair et al., 2005). These servers analytically furnished the area and the volume at the probable active site of each pocket to envisage the binding site.

Docking protocol
Grid parameter files (protein.gpf) and docking parameter files (ligand.dpf) were written using MGLTools-1.4.6. Receptor grids were generated using 90x60x60 grid points in xyz with grid spacing of 0.375 Å. Grid box was centered and co crystallized ligand map types were generated using autogrid4. Docking of macromolecule was performed using an empirical free energy function and Lamarckian Genetic Algorithm, with an initial population of 250 randomly placed individuals, a maximum number of 106 energy evaluations, a mutation rate of 0.02, and a crossover rate of 0.80. One hundred independent docking runs were performed for each ligand. Results differing by 2.0 Å in positional root-mean square deviation (RMSD) were clustered together and represented by the result with the most favorable free energy of binding.

ADME Toxicity prediction
Plant compound inhibitor can also be optimized by computational ADME (absorption, distribution, metabolism, excretion) prediction as it is crucial in inhibitor designing. Inadequate ADME properties are the cause of many drug development
Discussion

failures (Smith et al., 2004). Hence, in the present investigation care was taken to study if the selected compounds exhibited appreciable ADME properties to take it further for inhibitor designing. The results so obtained possessed all the characteristics of the plant molecule such as molecular weight, computed dipole moment of the molecule, IP, EA, PISA, WPSA, PSA, volume, #rotor, donorHB, acceptHB which were very essential to determine if the selected ligand possessed the desired biological activity. The results showed that the values of the ADME properties fell within the acceptable range proving the plant of choice to possess good efficacy.

Docking Studies

Study on domain region of a protein forms the platform of understanding the positioning of amino acid residues in its active sites. Interestingly, amino acid residues in the domain region claim to play a central role in its functions and establish a structure-function relationship of a protein in its tertiary conformation. Thus the analysis of the binding site or the active site in RNA dependent RNA polymerase showed amino acid residues LEU392, ALA396, ILE424, HIS428, LEU492, VAL494, ARG503.

Docking analysis of the selected ligands and the target showed that the amino acid residue ARG 503 was involved in interaction with squalene in the active site of RNA dependent RNA polymerase (NS5B). The length of hydrogen bond formed was 1.76511 Å with an IC$_{50}$ value of 2.5 (µm). The second ligand, tetradecanoic acid showed two interactions with the amino acid residue ARG 503 in the active site of RNA dependent RNA polymerase (NS5B) with a bond length of 2.117 Å and 1.96 Å and the IC$_{50}$ value of this compound was found to be 3.6 (µm). The amino acid residue ARG 503 established interactions with (E)-9-Octadecenoic acid ethyl ester, the third ligand chosen for the present study in the active site of RNA dependent RNA polymerase (NS5B).

The bonding of the ligands with the active sites of NS5B RdRp were compared with the reference drug Silymarin which showed three interactions with the amino acid residues HIS428 and THR399 in the active site of RNA dependent RNA polymerase (NS5B). The length of hydrogen bond formed were 2.185 Å, 2.062 Å and 2.037Å with an IC$_{50}$ value of 5.1(µm). The results obtained showed that there was effective hydrogen bonding
established between the ligands and target which fell within the acceptable and effective range of >1.5 Å and <3.2 Å. The IC\textsubscript{50} values of the ligands chosen were lesser than the reference drug which proved its efficacy in exhibiting a strong hepatoprotective activity.

The bonding energy score observed in the docking studies of the ligands present in the selected plant clearly revealed that the hydrogen bonds formed between the ligand and NS5B RdRp protein is a stable one thereby suggesting that the plant chosen possess anti-hepatotoxic properties.

Thus the data of the results of the in silico, in vitro and in vivo studies carried out in the present work corroborated well with each other and also depicted that ELEHB possessed hepatoprotective activity. The data of the results were on par with that of the standard drug Silymarin (25mg/kg bw.). It was observed that the 300mg/Kg bw. dose level of ELEHB was the most effective dose. The active principles identified in the ethanol extract of \textit{H. benghalensis} might have probably been responsible for its hepatoprotective activity. However, further studies would help in specifying compounds against hepatitis in order to confirm the present hypothesis.