CHAPTER 5

HEPATOPROTECTIVE ACTIVITY

5.0 INTRODUCTION

Liver injury caused by toxic chemicals and certain drugs has been recognised as a toxicological problem. Herbal drugs are playing an important role in health care programmes world wide, and there is a resurgence of interest in herbal medicines for treatment of various ailments including hepatopathy (Venukumar *et al.,* 2004). Liver is the main organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation (Gram *et al.,* 1971).

5.1 Functions of Liver

The functions of the liver are:

a) Detoxication of bilirubin by conjugating it as bilirubin diglucuronides and excreting through bile.
b) Epimerisation of galactose as UDP derivative.
c) Synthesis of proteins like albumin.
d) Synthesis of prothrombin.
e) Control of enzymes alkaline phosphatase and release of transaminases i.e. Aspartate amino transferase and Alanine aminotransferase.

From practical point of view bilirubin, total and conjugated, alkaline phosphatase activity, and ALT (GPT) are the important investigations generally carried out in blood (Waugh *et al.,* 2006).

5.1.2 Detoxication of bilirubin

Bilirubin is formed daily from hemoglobin (Hb) in the reticulo-endothelial system chiefly of bone marrow and spleen. About 6.25 g of haemoglobin is degraded every day. Bilirubin is transported in the plasma in combination with albumin to the liver, where it is conjugated with UDP-glucuronic acid to form bilirubin glucuronide. Bilirubin is insoluble in water but bilirubin diglucuronide is soluble in water. This is an important difference in physical property. The glucuronide is excreted in the bile and through the bile goes to the intestine. There, it is reduced by bacterial enzymes to urobilinogen. Some urobilinogen is oxidized to urobilin. The urobilinogen not oxidized in the intestine, is returned to the liver (entero-hepatic circulation) and is oxidized to bilirubin which is re-excreted in to the bile. Normal urine therefore contains very little urobilinogen, i.e., 1 to 4mg per 24 hours. Bilirubin metabolism is deranged in three important diseases. They are (i) hemolytic jaundice, (ii) hepato-cellular jaundice, and (iii) obstructive jaundice.

5.1.2.1 Hemolytic jaundice

The liver is normal in this disease and can conjugate the usual amounts of bilirubin efficiently. But when there is hemolysis, there is extensive degradation of heme and over-production of bilirubin. There is subsequently a rise of bilirubin in the blood as the liver cannot remove it efficiently. This bilirubin is not soluble in water. Hence it will not give a direct positive reaction with the van den Bergh reagent (The van den Bergh reagent is a mixture of equal volumes of van den Bergh solution A, i.e. sulthanilic acid in dilute HCL, and van den Bergh solution B, i.e., Sodium nitrite). If there is bilirubin diglucuronide which is water-soluble, it will give a pink colour immediately. Such a reaction is called the van den Bergh direct positive reaction. There may be no positive reaction but on adding methanol, the serum may respond to the van den Bergh test. This is because methanol dissolves bilirubin. Once a solution is obtained with methanol, the serum will give a pink colour with the van den Bergh reagent. This is called the van den Bergh indirect test. In hemolytic jaundice, the van den Bergh test will be indirect positive. As the liver is normal, there is no regurgitation of whatever bilirubin that has been conjugated. Hence there will be relatively greater amounts of bilirubin than normal going to the intestine. As a result, there will also be an increased excretion of urobilinogen in urine. There will not be any direct-reacting bilirubin (i.e. bilirubin glucuronide) in hemolytic jaundice as there is no regurgitation of bile into the blood. The jaundice therefore is due to increased water-soluble bilirubin in blood.

In short the following are the results of the blood and urine tests in hemolytic jaundice:

(I) Bilirubin total elevated; normal values are 0.1-0.8mg/dL;
(ii) Bilirubin glucuronides not detectable;
(iii) Van den Bergh test – indirect positive; and
(iv) Urinary urobilinogen – increased.

5.1.2.2 Obstructive jaundice

The increased level of bilirubin is due to regurgitation of bile. The regurgitation takes place due to obstruction in the bile duct (Cholestasis). So, the bile instead of flowing into the gastro-intestinal tract regurgitates into the blood. As the obstruction is outside the liver, the condition is called extra–hepatic or post–hepatic obstruction. As the liver is normal except in chronic conditions, conjugation goes on so that there is both water-insoluble bilirubin and water-soluble bilirubin glucuronides in the blood. The later gives the van den Bergh direct positive reaction. There is increased bilirubin glucuronides and total bilirubin. In view of the obstruction in the bile duct, there is no formation of urobilinogen. Hence the urine does not contain urobilinogen. Due to the regurgitation of bile, the activity of the enzyme alkaline

phosphatase is quite high (<30-35 KA units/dL) in the blood. The transaminase like Alanine aminotransferase may not be elevated significantly as there is no damage of the liver except in chronic conditions.

The following are the diagnostic findings generally observed in obstructive jaundice:

(i) Total bilirubin – elevated;
(ii) bilirubin glucuronides (direct-reacting bilirubin increased);
(iii) van den Bergh test direct positive;
(iv) alkaline phosphatase activity-above 30 – 35 KA units /dL.]
(v) Serum Alanine amino transferase- slightly elevated; and
(vi) Urinary urobilinogen negligible.

5.1.2.3 Hepato cellular jaundice

In this condition the jaundice is due to the increased level of bilirubin and bilirubin glucronides in blood. This also is due to the regurgitation of bile but such regurgitation is not due to post-hepatic obstruction but to intra- hepatic obstruction (obstruction from within the liver) because of inflammation caused by infection. The presence of bilirubin glucuronide in serum renders the van den Bergh test direct positive. Total bilirubin is elevated. Alkaline phosphatase activity is also increased but is less than 30-35 KA units /dL. Normal values are 3-8 KA units/ dL).

So long as the obstruction is there, urinary urobilinogen is negligible. This is because the obstruction, intra hepatic or post- hepatic, prevents bilirubin from reaching the intestines for reduction to uroblinogen. However, when the inflammation subsides during recoverythere is no obstruction. Under such circumstances urinary urobilinogen excretion may increase.

In view of infection, there is liver damage. Hence, the enzyme Alanine amino transferase from the necrosed cells mixes with the blood. So, there is increase of this enzyme Alanine amino transferase (normal value is 2 – 15 IU/L); double if assay is done at 37°C.

The following are the diagnostic findings in hepato-cellular jaundice:

(i) Total bilirubin – increased;
(ii) Direct reacting bilirubin, i.e., bilirubin glucuronides increased;
(iii) Van den Bergh test direct positive;
(iv) Akaline phosphatase activity increased upto 30-35KA units/dL;
(v) Urinary urobilinogen increased or decreased depending on the condition; and
(vi) Alanine amino transferase- increased.

Table 5.1 The blood and urine tests for the differential diagnosis of the three types of jaundice

Condition	Total bilirubin	Bilirubin Glucuro-nide	Van den Berg test	Alkaline Phosphatase	Alanine Amino transfer ase	Urinary Urobiiinogen
Hemolytic Jaundice	Increased	Nil	Indirect positive	Normal	Normal	Increased
Obstructive Increased	Increased	Increased	Direct positive	Increased above 30-35 KA units/dL.	Slightly elevated	Negligible
Hepato-Cellular Jaundice	Increased	Increased	Direct positive	Increased but below 30-35 KA units/dL	Significa ntly elevated	Initial increase, but is lowered as the disease progresses

As hepato-cellular jaundice is due to infections like viral infection, it can be treated with medicines, and no surgery is needed. Hence, it is called medical jaundice. On the other hand, obstructive jaundice, if the obstruction is due to stone in the bile duct that cannot be cured with medicines. Surgery is the only way and hence it is called surgical jaundice.

The van den Bergh's test is based upon the formation of purple colour due to azobilirubin obtained by the reaction between bilirubin and van den Bergh's diazo reagent. The reagent A is sulphanilic acid in hydrochloric acid while the reagent B is dilute sodium nitrite solution. The reagents are mixed fresh in the prescribed proportions just prior to the performance of the test. A purple colour formed within thirty seconds of mixing indicates direct reaction. Sometimes it may be delayed direct, if there is a partial conjugation defect. If the reaction is not direct positive, methanol is used as a solvent to dissolve the water- insoluble bilirubin and the colour developed is measured after letting it stand for thirty minutes (indirect positive).

In hemolytic conditions the load of bilirubin is beyond the capacity of the conjugating ability of the liver, while in hepato-cellular jaundice, since the liver itself is diseased; it is not able to handle even the normal amounts of bilirubin reaching it.

The normal serum bilirubin level ranges between 0.1 to 0.8 mg/dL. The normal urinary urobilinogen output ranges between 1–4 mg per twenty four hours. A collection of postprandial urine is necessary for the estimation of urobilinogen. Icteric index done on earlier days is not estimated as the bilirubin levels are precisely analyzed.

5.1.3 Alkaline Phosphatase

The normal serum level of this enzyme ranges between 3-11 KA units/dL. The enzyme is excreted by the liver via the bile and hence when the liver is in disorder, the serum enzyme level goes up due to defective, excretion but all the non hepatic conditions which can cause a similar rise have to be scrupulously eliminated. Such conditions are rickets, osteomalacia, hyper-parathyroidism, post hepatic obstruction, Paget's disease and bone tumours. In infective hepatitis with intrahepatic obstruction when van den Bergh's reaction with serum is direct positive, the serum alkaline phosphatase levels are moderately raised up to about 35 KA units / dL. Though not as high as in post hepatic obstruction in which it will be greater than 35 KA units/dL.

5.1.4 Transaminases and dehydrogenases

Jaundice is a contra indication for the determination of serum transaminase levels as high values are obtained even in the absence of liver damage. Transamination is not the monopoly of any particular tissue in the body, so that in the absence of second tissue damage, it is valuable in the diagnosis of liver dysfunction. Serum Alanine amino transferase (originally called serum glutamate pyruvic transaminase) raised early in liver disease even in the preicteric phase. Serum Aspartate amino transferase (originally called serum glutamate oxalo-acetate transaminase) though diagnostic of myocardial infarction, can also be a useful liver function test, provided the liver involvement is not secondary to the involvement of the heart. Determination of serum isocitrate dehydrogenase is believed to be a specific liver function test. Specificity is also seen in the estimation of the serum levels of the heat-labile hepatic isozymes of lactate dehydrogenase, i.e. LD4 and LD5. The serum amylase levels are low in liver disease. The activity of pseudo – choline esterase may also be lowered. High levels of serum leucine amino peptidase are seen in liver damage though it is not specific for liver dysfunction.

5.1.5 Plasma protein levels and liver dysfunction

Hypoalbuminemia and hence hypo-proteinemia as well as hypo-fibrinogenemia are met within gross liver diseases, as the liver is concerned with the biosynthesis of these proteins. However, hypo-proteinemia can be seen in protein malnutrition as well as after hemorrhage so that a reversal of the ratio between albumin and globulin in serum is a more useful diagnostic test, the normal ratio being 3:2. A reversal of the ratio, which is a compensatory effort on the part of the extra-hepatic situations where 20 percent of globulins are synthesized normally, is the basis of the several tests for liver function though it is said that

the quality of albumin in liver disease also differs from the albumin in normal serum. Electrrophoretic separation of serum proteins can, for example, establish conditions like cirrhosis of liver characterized by hypo-albuminemia and hyper-gamma globulinemia. However, there are non hepatic conditions where one finds a similar feature.

5.1.6 Bromosulphalein excretion test

This is the dye of choice for assessing liver function. It is non-toxic and is practically and almost exclusively excreted by the liver. Occasionally, allergy to this substance may be encountered. Extra vacation of the dye outside the vein should be avoided as it is a bad irritant. The dye is injected slowly intravenously, in a dose of 5 mg per kg body weight in a five per cent solution. Blood is drawn through the opposite median cubital vein, 30-45 minutes after the injection of the dye. With a normal liver, the retention of the dye is less than ten percent at 30 minutes and less than six percent at 45 minute. The presence of jaundice vitiates the results. Hence this test is not to be done in the presence of jaundice but is valuable in the screening of pre icteric phase of conditions like infective hepatitis and it also has prognostic value in the assessment of residual damage of the liver, if any, during the convalescent period even after the patient's bilirubin in serum reaches normal levels and the patient is apparently clinically normal. This test is superior to all other tests as it caters to specificity as well as to sensitivity.

5.1.7 Prothrombin time of Plasma

This is a very useful liver function test not only for diagnostic purposes but slso as a measure of safety before one undertakes liver biopsy and operative procedures. Obviously, all the non-hepatic causes of prolongation of prothrombin time must be scrupulously excluded. The conditions are: deficient intake of vitamin K, defective absorption of vitamin K, Post-hepatic obstruction, steatorrhoea, administration of anticoagulants and administration of antibiotics. Isotonic sodium citrate solution (3.8 percent) is the anticoagulant of choice for the collection of blood as it serves the purpose of converting ionic into the non-ionic form of calcium. Suitable thromboplastic material is used, and controls are done simultaneously along with every test. The normal values vary according to the type of the thromboplastic material used.

5.1.8 Serum enzymes and isozymes in diagnosis of diseases

Clinical Enzymology is a branch of biochemistry dealing with the diagnostic value of enzyme estimation in serum and tissues in diseases. Rona was the first to introduce serum lipase estimation in pancreatic disease. Subsequently, estimations of many serum enzymes have

become popular in the clinical biochemical laboratory. In fact, estimations of alkaline phosphatase and Alanine amino transferase have become almost a daily routine.
The enzymes present in circulating plasma are of two types: 1) the plasma specific or functional plasma enzymes, and 2) the plasma non specific or non – functional plasma enzymes. The former type of functional plasma enzymes has their substrates in the plasma. These enzymes, though synthesized in the liver, are found in circulating plasma at higher concentrations than in tissues. Examples of functional plasma enzymes are lipoprotein lipase, pseudo-choline esterase, and enzymes associated with the clotting of blood. In these cases the blood levels are greater than the tissue levels.

There are many other enzymes circulating in plasma but do not have any substrate in plasma and have no role. These are usually present in small amounts but their levels are altered in pathological conditions. In many cases there is increase in their concentration and only in a few cases there is a decrease; examples of plasma non–specific enzymes are alkaline phosphatase, acid phosphatase, transaminases, viz., glutamate-oxalo acetate-transaminase (G.O.T., presently known as AST, Aspartate aminotransferase) glutamate-pyruvate-transaminase (G.P.T presently known as ALT. Alanine amino transferase), creatine phosphokinase (presently known as creatine kinase), lipase, amylase, etc. In these cases, the tissue levels are greater than the blood levels.

The increase in serum levels of an enzyme may be due to (1) obstruction in the normal flow and regurgitation; (2) Seepage of the enzymes from necrosed cells and (3) Slow rate of renal or other modes of clearance of serum enzyme.

Whatever may be the mechanism, a rise or fall in serum enzyme levels has been of immense value to a clinician. Some serum enzymes which are altered in diseased conditions are listed in the **Table 5.2**.

In any particular disease before arriving at a diagnosis, it is worthwhile to estimate three or four enzymes in serum rather than a single enzyme. Thus in heart disease, it is advisable to estimate AST, creatine kinase, CK-MB and hydroxyl butyrate dehydrogenase activity (HBD) (which reflects the levels of LDH_1 and LDH_2) while in liver diseases, the enzymes alkaline phosphatase, ALT, isocitrate dehydrogenase, LDH 4 and LDH 5 isozymes could be investigated. Likewise in pancreatic diseases, serum amylase and lipase estimations (also urine amylase) will be of value. In primary myopathies, aldolase, creatine kinase (CK) and CK-MM might be investigated.

While interpreting the values of the serum enzyme levels, it has to be taken into account that some enzymes are altered even in physiological conditions. Amylase is absent in the new born. CK is low at rest but increases after exercise; alkaline phosphatase is raised in the newborn and during pregnancy. Acid phosphatase, aldolase, and AST are raised in children and infants.

In addition to variation of serum enzymes and urine enzyme (e.g., amylase) there are reports about variation of enzymes in cerebrospinal fluid and in the tissues and about the usefulness of their studies in diseases.

5.1.9 Alkaline Phosphatase

There are six isozymes of alkaline phosphatase. They are from liver, bone, skeletal muscle, kidneys small intestines and placenta. Adult serum contains the isozyme of liver while in children; it is a mixture of liver and bone isozymes. Placental isozyme behaves like that of bone.

A distinct form was noticed in lymphatic leukemia and infectious mono nucleosis and new bands in malignancies. Electrophoretically, the isozymes are designated as alpha, beta, pre beta and gamma.

Alpha is elevated in liver damage, pre beta and beta increased in osteoblastic activity and gamma in intestinal involvement. Alpha is divisible into alpha 1 and alpha 2. Alpha 1 increase when there is an element of obstruction eg. Regurgitation of bile and alpha 2 in liver cell damage. Alpha 1 band is found only rarely in viral hepatitis. It is elevated in metastatic carcinoma of liver, obstructive jaundice and cholangitis.

Absence of alpha-1 with exaggerated alpha-2 suggests hepatitis. A sudden decrease of placental isozyme suggests placental insufficiency.

Table 5.2 Name of the enzyme with their commission number and normal levels in serum (Ramakrishnan *et al.*, 1995)

Name of the Enzyme with Enzyme commission Number and normal levels in serum	Clinical conditions in which its levels are altered	Remarks
1. Asparatate amino transferase AST (G.O.T)E.C.No.2.6.1.1 4 to 17U/1(25'C) 8 to 34 U/1 (37'C)	Myocardial infaraction (specific), liver diseases, crushing muscle injury, muscular dystrophy (but not in muscular disease of neurogenic orgin) acute pancreatitis and pulmonary embolism.	(I) increase begins at 3-8 hours after the onset of the episode; highest values at about 24hrs, returns to normal by 3 to 6 days (ii) normal in angina pectoris
2. Alanine amino transferase A.L.T(G.P.T) E.C.No.2.6.1.2 3 to 15U/1(25'C) 6 to 30U/1(37'C)	Infective hepatitis and toxic hepatitis (specific), myocardial infarction, infarction, infectious, mononucleosis, malignancy	(i) In hepatitis and other diseases associated with liver necrosis, ALT and AST increase as both of them are in high concentration in liver. ALT rises even before the onset of jaundice; its increase is parallel to necrosis. (ii) ALT content of content of heart is 1/20th of AST hence rise of ALT is less marked than AST in myocardial infarction (iii) Normal in cirrhosis of liver, obstructive jaundice without necrosis and hemolytic jaundice.
4. Alkaline phosphatase (bone forming enzyme) E.C.No.3.1.3.1 3 TO 13 KA units/dL (22 to 92 U/1) for adults 7.5 to 33.5 KA units for children (two and a half times adult value)	Physiological rise (i) in children due to osteoblastic activity (ii) in pregnant women from placental origin. Pathological : (iii) highest level in Paget's disease and hyperpara-thyroidism with skeletal muscle involvement, moderate rise in osteomalacia, considerable increase in renal rickets associated with secondary hyper para thyroidism, in bone damage due to metastatic carcinoma, myeloma, Hodgkins disease if the bones are invaded, Gauchers disease with bone resorption. (iv) increased in hepato cellular disease and bile duct abnormalities as serum alkaline phosphatase is cleared by liver. It is less than 30-35KA/dL in infective hepatitis but more than 30-35 KA/dL in obstructive Jaundice. Alkaline phosphatase increases in early obstructive disease even before bilirubin levels are increased. Considerable increase in inflammatory disease and metastatic carcinoma of the liver, bilary cerrhosis and cholangiolytic hepatitis. (v) Decrease in childhood hypothyroidism.	(i) Very helpful in differential diagnosis of jaundice; below 30-35 KA/dL in hepato cellular due to inflammation and regurgitation in to blood; above 30-35 KA/dL in obstructive jaundice due to regurgitation of bile into blood caused by post hepatic obstruction say due to stone and normal in hemolytic . (ii) 1 KA unit /dL = 7.1 U/1

5.1.10 Michaelis menten constant (Km value) of serum enzymes in differential diagnosis of disease

Km value is a fundamental characteristic of all enzymes and isozymes. It shows the efficiency of binding of the substrate to the active centres of the enzyme.The greater the Km value the less the efficiency and vice versa as Km is given by substrate concentration at half the velocity maximum.

Serum contains mixture of isozymes of an enzyme. For example, the alkaline phosphatase of serum is a mixture of isozymes of alkaline phosphatase from the liver and the bone with a little contribution from the kidneys and small intestines (and placenta during pregnancy). Each isozyme has a definite Km. In the usual course of analysis in a clinical biochemistry laboratory, total alkaline phosphatase activity is assayed in serum. If it is increased, it could be infective hepatitis (Moderate elevation) obstructive jaundice (high) or bone diseases like Paget's disease osteomalacia. How to get the differential diagnosis between these diseases, If, now the Km value of serum alkaline phosphatase (hereinafter referred to apparent Km value, as serum contains a mixture of isozymes each having a definite Km) is estimated having different substrate concentrations and drawing Line weaver-Burk plots, if the apparent Km is close to that of the isozyme of bone, it is bone disease. If it is close to that of liver isozyme, it is liver disease. i.e., what an estimation of total enzyme activity can not reveal, Km determination will help (Ramakrishnan *et al.*, 1995). It was found that apparent Km of serum alkaline phosphatase was higher in cirrhosis of liver but normal in infective hepatitis.

Aspartate amino transferase (AST or SGOT) of serum could have isozymes of heart and liver. The total enzyme activity is increased in myocardial infarction and infective hepatitis and can not clinch the diagnosis. But apparent Km of serum AST is increased in advanced infective hepatitis with bilirubin above 13mg .dL while it was not elevated in myocardial infarction.

Alanine amino transferase (ALT or SGPT) of serum could be a mixture of isozymes of liver and the heart. Estimation of total enzyme activity could not help in differential diagnosis. Apparent Km of serum ALT is increased in infective hepatitis but normal in myocardial infarction (Ramakrishnan *et al.*, 1995).

Increase of apparent Km of serum AST and ALT in infective hepatitis might be due to the interaction of toxic metabolites with the active sites of the enzymes and decreasing the efficient binding of the enzyme and the substrate.

Lactate dehydrogenase of serum has isozymes of heart, liver, etc. Cancerous tissues synthesize isozyme and release into the blood. Total LDH is increased in myocardial infarction infective hepatitis, carcinomas and hematogical disorders and is not of help in differential diagnosis. But the apparent Km of serum LDH is increased in all cases of Carcinomas but not in myocardial infarction or infective hepatitis (Ramakrishnan *et al.*, 1995).

Thus the determination of apparent Km value of serum enzymes is helpful in differential diagnosis of diseases while just an assay of total enzyme activity might record an elevation in more than one disease. Relating Km with diagnostic biochemistry is a new field with prospects of tremendous advancement.

Hepatotoxicity caused by drugs or chemicals can occur due to occupational, environmental or dietary exposure. Such toxicities appear to be the consequence of the unique vascular, secretary, synthetic and metabolic features of the liver (Beris, 1991). Hepatotoxins can affect biological macromolecules such as proteins, lipids, RNA, DNA and induce several types of lesions (Yerra, 2005), of which genotoxic alterations may lead to carcinogenesis.

Diethylnitrosamine (NDEA), a known toxin as well as a potent carcinogen found in air, water and soil (Murray *et al.*, 2000; Malila *et al.*, 2002), belongs to nitrosamine group that has been established to cause hepatotoxicity in human beings. The variety of products that would result in human exposure, include mainstream and sidesstream tobacco smoke (Jayaprakash *et al.*, 2001), meat and whiskey (Kirtikar *et al.*, 1975). It can also be generated from metabolism of certain therapeutics drugs (Dutt, 1995). NDEA is extensively used as a solvent in the fiber industry, as a softener for co-polymers, as an additive for lubricants, in condensers to increase the dielectric constant and for the synthesis of 1,1-diethylhydrazine (Nadkarni, 1976; Kholkute *et al.*, 1976). NDEA has been shown to cause cancer in liver and other organs in various experimental animals (Bandara *et a.*, 1989). NDEA is known to be bio-activated by cytochrome P450, following which it forms DNA adducts rapidly, bringing about mutation and fragmentation that may lead to micronuclei formation.

In order to intervene the earlier stages of chemical mediated toxicity, use of chemopreventive agents may be considered as an effective alternate. Among various agents with chemopreventive effects, phytoproducts are gaining more and more attention gradually due to their decreased toxicity and high efficacy. Studies of natural products provide opportunities to reveal interesting biology and generate leads pertaining to specific cellular targets, activities, and therapeutic manipulations (Prashanth *et al.*, 2001; Koul *et al.*, 2007).

Liver, the major site of intense metabolism is prone to various disorders as a consequence of exposure to toxins of extrinsic as well as intrinsic origin. Most of the hepatoprotective drugs now available in the market cost much, and hence a genuine need is felt to devise some cost-effective drugs based on plant principles in this regard (Venukumar *et al.*, 2004).

CCl_4 intoxication in rats is an experimental model widely used to study necrosis and steatosis. Hence, the same method is adopted to induce hepatopathy in this experiment.

Carbon tetrachloride and Paracetamol are known to cause liver damage (Recknagel, 1983; James *et al.*, 2003). When administered to rats, they act by inducing oxidative damages to liver cells which leads to cellular necrosis, resulting in increase in serum enzymes SGOT and SGPT. These models of hepatotoxicity has been widely used to study the antihepatotoxic activities of exogenous drugs in experimental animal models (Shenoy *et al.*, 2001; Bisshayi *et al.*, 2002; James *et al.*, 2003)

Paracetamol-and CCl_4 – induced hepatitis are usually used as experimental models in the search for new antihepatotoxic compounds (Parthasarathy *et al.,* 2004). Once introduced in the organism, CCl_4 is converted in the liver into a radical which reacts with molecular oxygen to form a trichloromethyl peroxyl radical. This compound attacks membrane polyunsaturated fatty acids and causes membrane lipid peroxidation (Recknagel, 1983) which leads to impairment of membrane function. When Paracetamol is introduced in excess in the body, this compound is also metabolized in the liver to a reactive metabolite which reacts with enzymes and membrane components of liver cells which results in cellular lesion (Rang *et al.*, 1999). In both cases, an increase in the serum of some liver enzymes such as ALT and AST is observed. An extract is said to be antihepatotoxic if it prevents the increase in the level of these serum enzymes in animals in which hepatitis has been experimentally induced.

Biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (ALP), bilirubin, total serum protein, lipid peroxide and glutathione content of the liver were estimated to determine liver function and metabolism.

5.1.11 Biochemical estimation

The AST, ALT (Oser, 1965), ALP (Bergmeyer, 1974), bilirubin (Oser, 1965) and total serum protein (Lowry *et al.*, 1951) can be estimated in serum of rats. Lipid peroxide (Ohakawa *et al.*, 1979) and glutathione (Mulder *et al.*, 1995) content of the liver are measured in liver tissue to determine liver function and metabolism.

5.1.11.1 Hepatotoxicity in laboratory animals

Paracetamol induced hepatotoxicity in rodents is a widely used animal model to assess the hepatoprotective activity of new compounds (Parthasarathy *et al.,* 2007). The rise in serum levels of SGOT, SGPT and ALP has been attributed to the damaged structural integrity of the liver. Pretreatment with plant extracts prior to the administration of Paracetamol significantly prevent the increase in ALP and aspartate aminotransferase activity in a dose dependent manner (Kyung *et al.*, 2001). The reversal of increased serum enzymes in paracetamol induced liver is observed in hepatoprotectve plant extracts may be due to prevention of leakage of the intracellular enzymes by its membrane stabilizing activity (Thabrew *et al.*, 1987).

The development of fibrosis is critical for progression of all chronic liver diseases. In the last 20 years, the molecular and cellular mechanisms regulating liver tissue scarring have been investigated in detail, and additional pieces of the puzzle are being added now that several research groups are investigating this aspect of liver pathophysiology. A major acquisition has been the identification of liver fibrosis as a tissue-specific counterpart of the "wound-healing" response, a process whereby damage triggers a series of events, involving multiple cell types, aimed to limit damage and to preserve tissue function and integrity (Friedman *et al.*, 2000). These events include inflammation, activation of fibrogenic myofibroblasts (e.g., hepatic stellate cells, HSC), deposition of fibrillar extracellular matrix, and possibly neo-angiogenesis. As data on the role of leptin in the regulation of these steps accumulate, it is important to establish their possible relevance to the pathophysiology of human chronic liver diseases (Fabio, 2007).

5.1.12 Plants in the treatment of liver and biliary tract diseases

Liver is the principal organ of metabolism and excretion in subject to a number of diseases which may be classed as liver cirrhosis (cell destruction and increase in fibrous tissue), acute chronic hepatitis (inflammatory disease) and hepatitis (non-inflammatory condition).

In 1989 it was estimated that there were some 200 million chronic carriers of the hepatitis B virus of which 40% were expected eventually to die of hepato-cellular carcinoma and 15% of cirrhosis. Causative factors for liver disorders include virus infection; exposure to, or consumption of, certain chemicals, chemotherapeutic agents and possibly plants materials such as those containing pyrrolizidine alkaloids, ingestion of industrial pollutants, including radioactive materials.

In such liver damage the serum level of the liver enzymes, particularly serum glutamic pyruvic transminase is raised and extent of its control by the antihepatotoxic drug used as a basis for estimation. Other effects of included liver damage which can also be used in the evaluation of the plant extracts are: the prolonged lengthening of the time of lost reflex induces by short acting barbiturates, reduction of prothrombin synthesis giving an extended prothrombin time.

Some researchers reported that about 170 phytocostituents isolated from 110 plants belonging to 55 families were stated to possess liver protective activity; about 600 commercial herbal formulations with claimed hepatoprotective activity are being marketed world-wide. The terminal events in the attack on the liver by carbon tetrachloride involved in the production of a highly reactive radical leading to lipid oxidation and the inhibition of the calcium pump of the microsome giving rise to liver lesions.

A number of plant drugs used for treating biliary disorders are cholagogues. Herbalists prescribe such drugs either singly or more commonly as mixtures; a cholagogue tea, for example, may consists of a mixture of Peppermint leaves 50.0%, Melissa leaves 20.0% (sedative adjuvant), Fennel fruits 20.0% (complementary carminative), Frangula bark 10.0% (gentle laxative).

Some antihepatotoxic and cholagogue drugs are listed below: (Evans, 2006)

1. *Silybum marianum*: This plant, syn. *Carduus marianus* is one of the milk thistles. Indigenous to the Mediterranean region, it has been introduced to most areas of Europe, North and South America and Southern Australia.
2. *Peumus boldus*: Leaves of this plat are collected from the small tree, *Peumus boldus* (Bolodo), indigenous to Chile.
3. *Taraxacum officinale* root (Compositae)
4. *Chionanthus virginicus* (Oleaceae)
5. *Euonymus atropurpureus* (Celastraceae)
6. *Hydrasitis canadenis* (Ranunculaceae)
7. *Juglans cinereae* (Juglandaceae)
8. *Veronicastrum virginicum* (Scrophulariaceae)
9. *Aralia elata* (Araliaceae)
10. *Eclipta alba* (Compositae)
11. *Picrorrhiza kurroa* (Scrophulariaceae)
12. *Schisandra chinenis* (Magnoliaceae)
13. *Javanese turmeric* (*Curcuma xanthorrhiza*)
14. *Wendelia calendulaceae* (Compositae)

Medicinal herbs are significant source of plant drugs. Latest trends have shown increasing demand of phyto-drugs and some medicinal herbs have proven hepatotprotective potential. Silymarin, a flavonol lignan mixture extracted from the *Silybum marianum* (Milk thistle) is a popular remedy for hepatic diseases (Pradhan *et al.,* 2006). Today every herbal company is marketing formulations for liver disorders but the actual scene is that only selected medicinal herbs have been tested for hepatotprotective activity. Some herbal formulations claiming to be hepatoprotective may actually contain chemical constituents having hepatotoxic potential. Andrographolide (*Andrographis paniculata*), Glycyrrhizin (*Glychyrrhiza glabra*), Picrrorihzin (*Picrorrhiza kurroa*) and Hypo-phyllanthin (*Phyllanthus niruri*) are potential candidates with hepatoprotective activity.

Alternative systems of medicine viz. Ayurveda, Siddha, and Traditional Chinese Medicine have become more popular in recent years in treating liver diseases (Eisenberg *et al.*, 1993). Medicinal herbs and extracts prepared from them are widely used to treat hepatitis, cirrhosis and loss of appetite (Cupp, 1999). Medicinal herb is a biosynthetic laboratory, for chemical compounds like glycosides, alkaloids, resins, oleoresins, etc. These exert physiological and therapeutic effect (PDR, 1998). The compounds that are responsible for medicinal property of the drug are usually secondary metabolites.

A number of recent reviews have focused on the adverse effects of herbal products (De Smet, 1997). Some herbs known to be hepatoprotective, their mechanisms of hepatoprotectivity and clinical documentation are available (Malhotra, 2001). In fact some herbal products claiming to be hepatoprotective may actually be having some components with hepatotoxic potential. *Silybum marianum, Picrrorhiza kurroa, Andrographis paniculata, Phyllanthus niruri,* and *Eclipta alba* are proven hepatoprotective medicinal herbs, which have shown genuine utility in liver disorders (Bisset, 1994). These plants are used widely in hepatotprotective preparations and extensive studies have been done on them (Malhotra *et al.*, 2007). Some of the medicinal plants with proven hepatoprotective activity are:

Taraxacum officinale

Traditionally, *Taraxacum officinale* has been used as a remedy for jaundice and other disorders of the liver and gallbladder, and as a remedy for counteracting water retention. Generally, the roots of the plant have the most activity regarding the liver and gallbladder. Oral administration of extracts from the roots of *Taraxacum officinale* has been shown to act as a cholagogue, increasing the flow of bile. Bitter constituents like taraxecerin and taraxcin are active constituents of the medicinal herb (Cordatos, 1992).

Cichorium intybus

Cichorium intybus is a popular Ayurvedic remedy for the treatment of liver diseases. It is commonly known as Kasni and is part of polyherbal formulations used in the treatment of liver diseases. In mice, liver protection was observed at various doses of *Cichorium intybus* but optimum protection was seen with a dose of 75 mg/kg given 30 minutes after CCl_4 intoxication.In preclinical studies an alcoholic extract of the *Cichorium intybus* was found to be effective against chlorpromazine-induced hepatic damage in adult albino rats. A bitter glucoside, Cichorin ($C_{32}H_{34}O_{19}$) has been reported to be the active constituent of the herb (Luczaj *et al.,*2007).

Solanum nigrum

In *Ayurveda*, the drug is known as *Kakamachi.* Aromatic water extracted from the drug is widely prescribed by herbal vendors for liver disorders. Although clinical documentation is scare as far as hepatoprotective activity is concerned, but some traditional practitioners have reported favorable results with powdered extract of the plant (Sultana *et al.,* 1995).

Glychyrrhiza glabra

Glychyrrhiza glabra, commonly known as liqoirice contains triterpene saponin, known as glycyrrhizin, which has potential hepatotprotective activity. It belongs to a group of compounds known as sulfated polysaccharides. Several studies carried out by Japanese researchers have shown glycyrrhizin to be for anti-viral and it has potential for therapeutic use in liver disease (Sanwa, 1985).

Experimental hepatitis and cirrhosis studies on rats found that it can promote the regeneration of liver cells and at the same time inhibit fibrosis. Glycyrrhizin can alleviate histological disorder due to inflammation and restore the liver structure and function from the damage due to carbon tetrachloride. The effects including: lowering the SGPT, reducing the degeneration and necrosis and recovering the glycogen and RNA of liver cells. Effect of glycyrrhizin has been studied on free radical generation and lipid peroxidation in primary cultured rat hepatocytes (Zhao, 1983). Favorable results have been reported in children suffering from cytomegalovirus after treating with glycyrrhizin (Numazaki, 1994).

Wilkstroemia indica

W. indica is a Chinese herb and has been evaluated in patients suffering from hepatitis B. A dicoumarin, daphnoretin is the active constituent of the herb. The drug has shown to suppress HbsAG in Hep3B cells. It is said to activator of protein kinase C (Chen *et al.*, 1996).

Curcuma longa

Like *Silymarin*, turmeric has been found to protect animal livers from a variety of hepatotoxic substances, including carbon tetrachloride, (Srinivas *et al.*, 1991) galactosamine, pentobarbitol, 1-chloro-2,4-dinitrobenzene,7 4-hydroxy-nonenal, (Selvam *et al.*, 1995) and paracetamol. Diarylhepatonoids including Curcumin is the active constituent of the plant.

Tephrosia purpurea

In Ayurveda, the plant is known as Sharpunkha. Alkali preparation of the drug is commonly used in treatment of liver and spleen diseases. In animal models, it offered protective action against carbon tetrachloride and D-galalactosamine poisoning (Murthy *et al.*, 1993). The roots, leaves and seeds contain tephrosin, deguelin and quercetin. The hepatoprotective constituent of the drug is still to be proved.

5.1.13 Use of hepatoprotective plants in traditional medicine

***Treadway,* (1998)** reported the traditional use of medicinal plants like *Terminalia chebula, Cichorium intybus, Piper longum, Terminalia arjuna, Emblica officinalis, Boerhaavia diffusa* and *Phyllanthus niruri* in revitalizing the liver and treating liver dysfunction and disease. Many of these herbs have been evaluated in clinical studies and are currently being investigated phytochemically for better understanding of their actions.

***Gupta et al.,* (2006)** investigated the effect of aqueous ethanolic extract of *Chamomile capitula* on blood and liver glutathione, Na+ K+- ATPase activity, serum marker enzymes, serum bilirubin, glycogen and thiobarbutiric acid reactive substances against paracetamol induced liver damage in rats.

***Madani et al.,* (2008)** had investigated the hepatoprotective effect of polyphenolic extracts of *Silybum arianum* and *Cichorium intybus* on thioacethamide-induced hepatotoxicity in rat.

***Deepak et al.,* (2007)** reported that the chloroform and methanolic extracts of *Ichnocarpus frutescens* have hepatoprotective and antioxidant effects on paracetamol (750mg/kg) induced acute liver damage in Wistar albino rats.

***Samudram et al.,* (2008)** evaluated the hepatoprotective effect of biherbal formulation of ethanolic extracts of *Eclipta alba* leaves and of *Piper longum* seeds at a dose level of 50 mg/kg body weight, administered orally daily once for 14 days against CCl_4 induced hepato-toxicity in albino rats.

5.2 MATERIALS AND METHODS

5.2.1 Plant Materials

The leaves of *Urtica parviflora* Roxb. was collected from Majhitar,East Sikkim, India in March 2006. The plant was identified by the Botanical Survey of India (BSI), Gangtok, Sikkim. The voucher specimen has been retained in our laboratory for future reference. The collected leaves were air dried and pulverized in a mechanical grinder. In the pilot study the other two plants namely methanol fraction of, leaf of *Callicarpa arborea* and root bark of *Morinda citrifolia* did not showed any hepatoprotective activity, thus excluded from the study. Also in this study the ethanolic fraction of *Urtica parviflora* was chosen because of its higher hepatoprotective activity as compared to other fractions.

5.2.2 Preparation of extracts and phytochemical study

The leaves (500 g) were coarsely powered and subjected to successive solvent extraction with petroleum ether (60-80°C), benzene, chloroform, ethanol and water. The solid extracts obtained after complete removal of the solvents under reduced pressure were stored in desicator. The ethanolic extract was suspended in aqueous Tween 80 solution (0.5 %). The chemical constituents of the extracts was identified by qualitative chemical tests and further confirmed by thin layer chromatography for the presence of alkaloids, sterols, tannins, reducing sugars and flavonoids (Trease, 1996).

5.2.3 Animals

Swiss Albino male rats of Sprague Dawley strain, weighing 150-175 g each, were used. They were housed under standard conditions of temperature (23 ±10°C) and relative humidity (55±10%); 12h/12h light/dark cycle and fed with standard pellet feed and water *ad libitum*. The Institutional Animal Ethics Committee reviewed the entire animal protocols prior to the experiments.

5.2.4 Experimental induction of liver damage and treatment

Liver damage was induced in rat by administering CCl_4 subcutaneously (SC) in the lower abdomen at the dose of 1ml /kg body weight except the animals of first group. CCl_4 was administered on every first and fourth day of the week up to 13 weeks (Venukumar *et al.*, 2004).

The rats were divided into 6 groups, 8 animals in each. Group I served as control, receiving Tween 80 solution (0.5%) orally. Group II received only CCl_4. The ethanol extract of *U. parviflora* Roxb leaves was administered orally to groups III, IV and V at a dose of 250, 500 and 750 mg/kg body weight respectively. Reference drug Silymarin (100 mg/kg) was administered orally to Group VI animals in Tween 80 solution (0.5%) (Pradhan *et al.*, 2006). Every day at 9.00 am a known quantity of food was replenished. The animals kept starved

over night one day before the last day of the experiment. On the next day they were sacrificed and blood was collected making an incision on jugular vein.

5.2.5 Enzyme assay

The serum was separated from blood for biochemical estimation by centrifugation at 2500-3000rpm. Different parameters like serum alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) activity were measured according to method of Reitmen and Franckel (Reitman 1957).

5.2.6 Estimation of total protein and bilirubin

The level of total protein (TP) was estimated in serum of the animals by Biuret method (Kingsley *et al.*, 1964). The level of bilirubin was estimated by the method of Mallory *et al.*, (1939).

5.2.7 Histopathologic examination

Liver lobes of the animals were removed and washed with normal saline. Small pieces of liver tissue were preserved in 10% formalin solution for histological analysis. The pieces were dehydrated with 90% ethanol, embedded in paraffin, cut into thin sliced sections (7 µm thick), stained with haematoxylin-eosin dye and observed under a light microscope, for cell necrosis, vascular degenerative changes, inflammation and fibrosis.

5.2.8 Statistical analysis

The data were analyzed statistically using one-way analysis of variance followed by Dunnett's't' test. The data are expressed as mean ± SEM. P values less than 0.05 indicate significance.

5.3 Results of hepatoprotective activity

The microphotographs of the histopathological examination of the liver of the animals under study are presented in the **Fig 5.1 to 5.6**. **Fig 5.1**, exhibits normal architecture of group I rat liver. Single dose of CCl_4 caused centrolobular necrosis extending to midzone with neutrophilic collection in liver of group II animals shown in **Fig 5.2**. Central to central bridging was seen. The cells of centrolobular region showed vacuolated cytoplasm. Vacuolar size showed variations from spherical to large droplet like structures. In most of the necrotic cells centrally placed nuclei were suspended in small amount of cytoplasm, which remains continuous by cytoplasmic strands that traverse through the vacuoles connecting peripheral of cytoplasm. Many of them were dead. Kupffer cells and sinusoidal cells showed arrest in distribution. The administration of ethanol extract at 250 mg/kg body weight protected the

liver partially which is shown in **Fig 5.3**. The extent of the necrotic region was reduced significantly. Numbers of necrotic cells located in this region were considerably reduced and were retained in immediate vicinity of the vein. Most of the cells on the boundary of the necrotic region showed small vacuoles indicating preliminary stage of necrosis. Necrotic region showed the pathological architecture as described above and in the region of healthy cells normal histological structure was evident. Centrolobular region of rats treated with ethanol extract at 500 mg/kg body weight along with CCl_4 showed normal cellular architecture without any necrotic cells that show any type of stress, **Fig 5.4**. Clear bile canaliculi were noted. Distribution of Kupffer cells and sinusoidal cells was normal. The livers of rats were totally normal when treated with ethanol extract and standard drug Silymarin at 750 mg/kg and 100 mg/kg body weight respectively, **Fig 5.5** and **5.6**.

To elucidate the biochemical mechanism of the hepatoprotective activities of the *U. parviflora* extract, the levels of ALT, AST, ALP, total protein and bilirubin were estimated. CCl_4 extensively studied liver toxicants and its metabolites such as trichloromethyl peroxy radical ($CCl_3O_2^-$) are involved in the liver damage (Kamalakkannan, 2005; Sherlock, 2002; Subramonium, 1999). The toxic chemical caused peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes (Mankani *et al.*, 2005). The increase in the levels of serum bilirubin reflected the depth of jaundice and the increase in the transaminases and alkaline phosphatases was clear indication of cellular leakage and the loss of cellular integrity of the cell membrane (Sarawat *et* al., 1993). The results depicted in **Table 5.3** showed that administration of animals with the test formulation at dose of 750 mg/kg body weight returned the enzymes levels, total protein and bilirubin to near normal, which were significantly lower than only CCl4 and close to Silymarin treated animals. Oral administration of *U.parviflora* ethanol leaves extract seems to reverse the hepatic cell damage in a dose dependent manner providing significant protection with a dose 750 mg/kg body weight.

The findings of our study provide some scientific basis for traditional use of *U. parviflora* leaves for managing hepatic disorders. The data obtained are consistent with literature report on hepatoprotective activity of *U.parviflora* leaf using enzyme assays and histopathological examination of liver in rats (Gurung, 1999; Kar *et al.,* 2007).

Table 5.3 Effect of ethanol leaves extract of *U. Parviflora* on CCl_4 induced hepatotoxicity in rats.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
ALT (IU/l)	30.63 ±3.32	120 ± 8.81[a]	118.3 ± 6.44[b]	96.7 ± 6.43[b]	47.9 ±4.98[b]	30.23 ± 1.82[b]
AST (IU/l)	60.12 ± 0.05	65 ± 6.40[a]	148.9 ± 7.76[b]	81.2 ± 5.18[b]	58.3 ±7.41[b]	58.1 ± 4.56[b]
ALP (IU/l)	58.29 ± 0.12	108.2 ± 3.39[a]	89.7 ± 1.73[b]	71.4 ± 4.81[b]	63.7 ±3.67[b]	57.9 ± 1.89[b]
Bilirubin (IU/l)	0.49 ± 0.05	2.67 ± 0.13[a]	1.15 ± 0.12[b]	0.86 ± 0.09[b]	0.74 ± 0.04[b]	0.56 ± 0.02[b]
Total protein (g/dl)	8.15 ± 0.27	5.21 ± 0.13[a]	6.17 ± 0.27[b]	7.56 ± 3.32[b]	9.54 ± 2.13[b]	9.23 ± 1.13[b]

Values are expressed as mean ± SEM from 6 rats in each observation. [a]$P<0.05$ compared to control group, [b]$P<0.05$ compared to CCl_4 treated group.

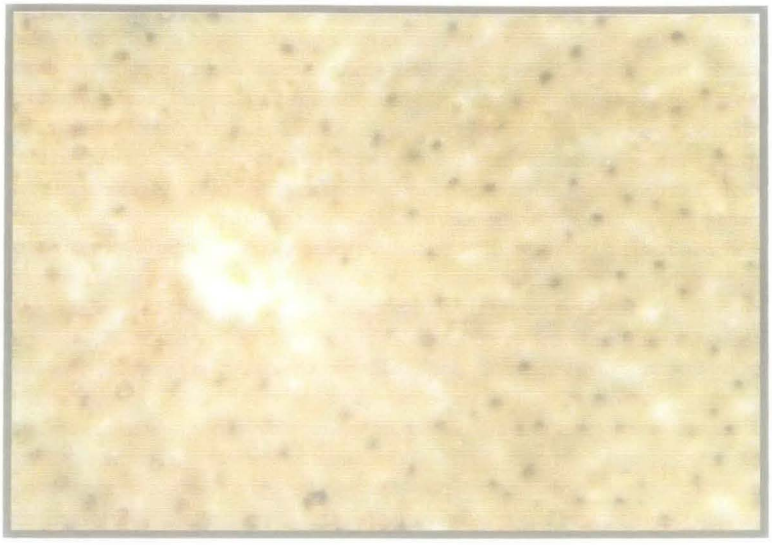


Fig 5.1 Normal rat liver section (M×400), haematoxylin–eosin stain. Liver section of the rat showing normal cellular architecture with distinct hepatic cells, sinusoidal spaces, central vein and vacuole.

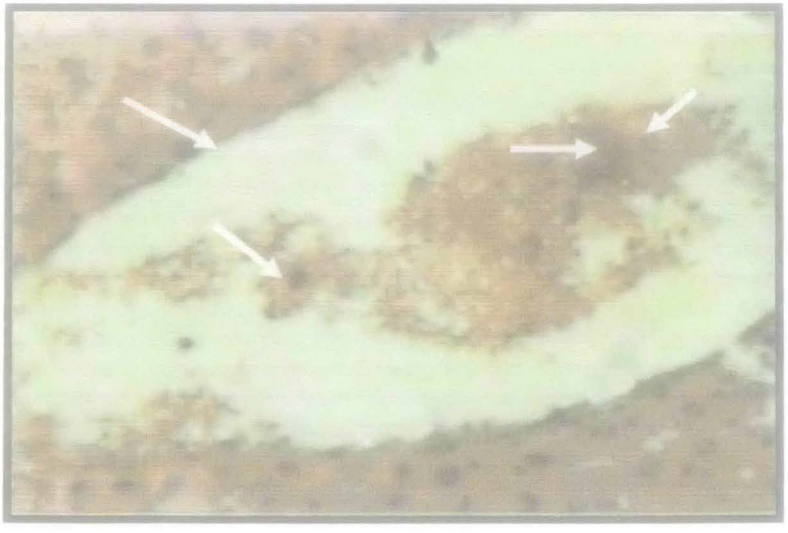


Fig 5.2 Liver section of rat intoxicated with CCl_4, (M×400), haematoxylin–eosin stain. Liver section of the rat showing disarrangement and degeneration of normal hepatic cells with centrilobular necrosis extending to midzone and sinusoidal haemorrhages and dilation.

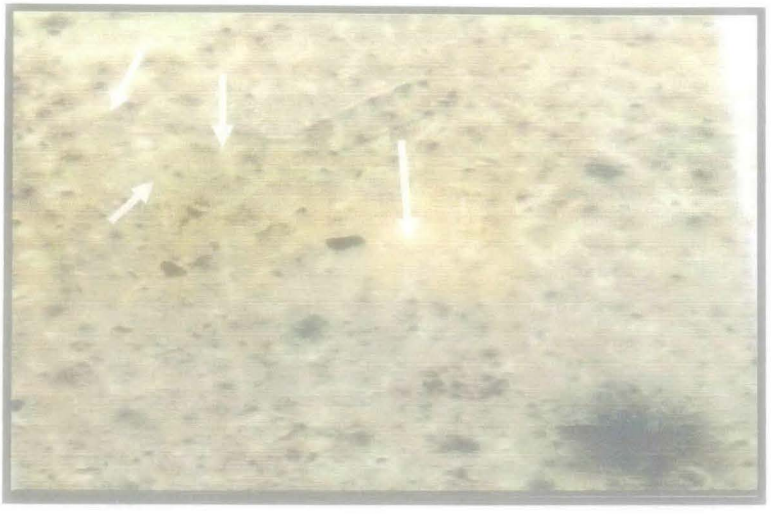


Fig 5.3 Liver section of rat treated with ethanolic extract and intoxicated with CCl_4. (M×400), haematoxylin–eosin stain. Liver section of the rat showing less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes.

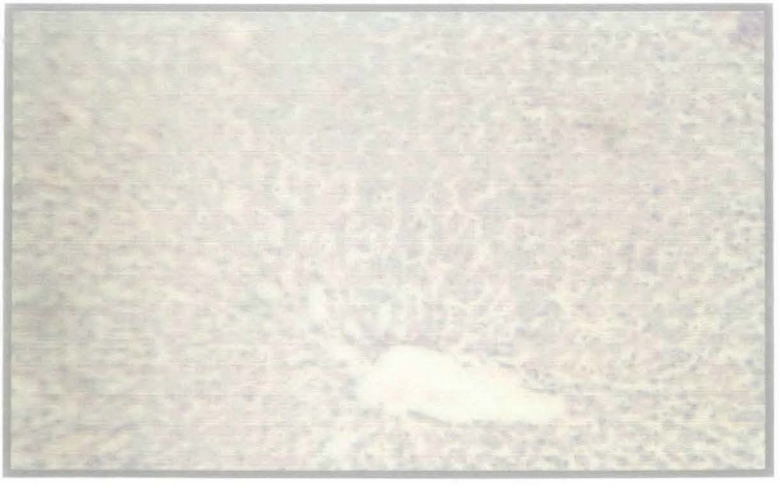
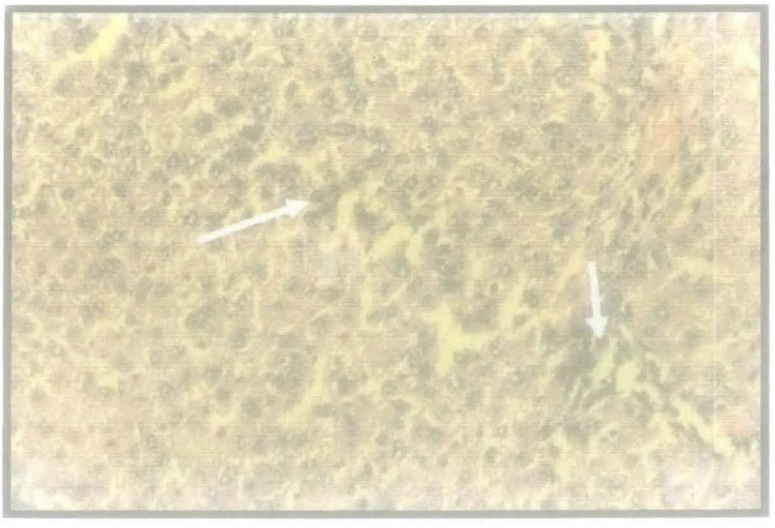


Fig 5.4 Liver section of rat treated with silymarin and intoxicated with CCl_4, (M×400), haematoxylin–eosin stain. Liver section of the rat showing less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes.



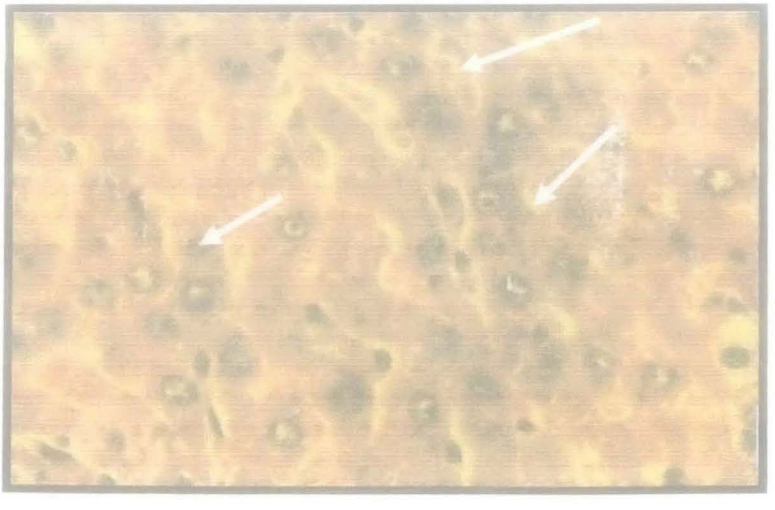


Fig: 5.5 and 5.6. Liver section of rat treated with aqueous extract and intoxicated with CCl_4, (M×400), haematoxylin–eosin stain. Liver section of the rat shows less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes.

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