



Hepatotoxicity: Its Mechanisms, Experimental Evaluation and Protective Strategies

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Abstract

The liver is a crucial organ for maintenance of gastrointestinal homeostasis and body function in general. In addition to its role in the digestive process, it also serves as a source of nutrients and detoxifier of unwanted substances. Hence, its optimal functioning is crucial for health and disease. An imbalance between aggressive and protective forces results in damage to the liver and complex mechanism are involved in such hepatotoxicity induced by a variety of environmental and chemical agents. It is thus important to understand the mechanisms of hepatotoxicity for devising pharmacological strategies for their prevention. Inflammation, immunomodulation and oxidative stress are amongst the common biological mechanisms contributing to compromised liver function. A variety of drugs and chemicals are used to experimentally induce liver damage, which are assessed in the laboratory by physiological, biochemical and pathological biomarkers. Potential hepatoprotective agents are screened for efficacy and safety by observing their effects on these preclinical models, and their utility in clinical conditions are predicted. Several agents are reported to protect the liver from toxic insults and both synthetic and natural products are known to produce ameliorative effects both prophylactic and therapeutic. Medicinal plants are important alternative/complimentary sources for potential hepatoprotective agents, and their efficacy and safety have been documented in both experimental and clinical studies. The present overview summarizes some of the basic concepts involved in hepatotoxicity, their preclinical evaluation methods and some relevant herbal hepatoprotective strategies.

Introduction

The liver is a vital organ and its strategic location and multidimensional functions support almost every other organ in the body. Liver is also the main organ for metabolism and elimination of drugs [1,2]. At the same time liver is prone to many diseases like allergy to food and involves immune system as well. The bare area of the liver is a site that is vulnerable to the passing of infection from the abdominal cavity to the thorax. Hepatitis is caused due to viruses, poisons, autoimmunity and can also result from non-alcoholic fatty liver disease connected with obesity and steatosis. Hepatic encephalopathy is caused by accumulation of toxins in the bloodstream that are normally removed by the liver.

Liver damage can also be caused by drugs, particularly anti-tubercular drugs, general anesthetics, paracetamol and some anti-cancer drugs. Toxic hepatitis is the most severe adverse reaction to antituberculosis drugs, it usually initiates in the first few weeks of treatment along with liver necrosis, which may evolve to encephalopathy and death. Alcoholic liver diseases with cirrhosis (formation of fibrous tissue in liver) caused by excessive alcohol consumption is a common occurrence. Liver can sometimes be damaged by some chemicals called hepatotoxins, such as galactosamine and chloroform [3]. Moreover, steroids, vaccines and antiviral drugs which are used as therapy for liver diseases, may produce adverse effects especially after chronic administration. There are more than 900 drugs that can lead to hepatotoxicity and is one of the important reasons for some of the drugs withdrawn from market. Liver toxicity not only occurs from direct toxicity of the primary compound but also from reactive metabolite or immunologically-mediated response. This can affect hepatocytes, biliary epithelial cells and liver vasculature [4,5]. Hepatotoxic response generated by chemicals depends upon the concentration of the toxicant, distinctive expression of enzymes and concentration gradient of substance in blood covering the acinus [6].

Traditional healing practices are now wide spread amongst about 80% of the developed countries population and often termed alternative or complementary medicine. In view of the scarcity of reliable liver-protective drugs in modern medicine, hepatoprotective drugs from plant sources seem

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Received Date: 24 Jul 2018

Accepted Date: 16 Aug 2018

Published Date: 20 Aug 2018

Citation:

Gulati K, Reshi MR, Rai N, Ray A. Hepatotoxicity: Its Mechanisms, Experimental Evaluation and Protective Strategies. *Am J Pharmacol.* 2018; 1(1): 1004.

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to have attractive alternatives. In order to validate the potentials of these compounds, their efficacy in experimental models of hepatic dysfunction needs to be investigated. Therefore, an attempt has been made in this review to give an account of some of the *in-vivo* and *in-vitro* experimental models to evaluate new drugs/compounds with potential for hepatoprotective effects. The present review also details about the mechanisms of hepatotoxicity and specific markers of liver function which can be used for the diagnosis of the liver dysfunction as well as outcome of a therapy.

Mechanism of hepatotoxicity

Liver is the main organ for metabolism and elimination of drugs. Majority of drugs (oral) and xenobiotics are lipophilic which enables their easy absorption across intestinal membranes. They are rendered hydrophilic *via* hepatic metabolism and are easily excreted. Exogenous products are metabolized in the liver mainly through phase I and II reactions [7]. The hepatic metabolism involves Phase I and Phase II reactions. Phase I involves oxidative, reductive, hydroxylation and de-methylation pathways, primarily by way of the cytochrome P-450, the most important family of metabolizing enzymes system which is located in the endoplasmic reticulum in the liver. Phase I reactions mostly produce toxic intermediates which are transformed to non-toxic compounds by phase II reactions, usually considered as detoxification pathways. Phase II reactions involve the conjugation of chemicals with hydrophilic moieties such as glucuronide, sulfate or amino acids and lead to the formation of more water-soluble metabolite which can be excreted easily [6]. Further Phase II reaction implicates glutathione which can covalently bind to toxic intermediates by glutathione-S-transferase [8]. However, this phase can also lead to the formation of unstable precursors of reactive species that can cause hepatotoxicity [9-11]. Figure 1 depicts the flow diagram of metabolism of xenobiotics in liver.

The main role of liver is to metabolize xenobiotics and is the common link with gastrointestinal tract. Liver is highly vulnerable to damage from drugs and some other substances. Nearly 75% blood that reaches liver comes from gastrointestinal organ and spleen *via* portal veins, therefore the drugs and xenobiotic are in concentrated form. The liver injury occurs due to activation of drugs to chemically active metabolites. They have a tendency to react with cellular macromolecules like proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress [12].

Mechanism of liver injury can be classified as pathophysiological or through chemical induced mechanism.

Pathophysiological hepatic damage

Disruption of the hepatocytes: The covalent bonding between chemical/toxicant agent and intracellular proteins can reduce the level of ATP, which leads to actin disruption. Separation of actin fibrils at the exterior of the hepatocyte can lead to blebs and rupture of the membrane [13].

Disruption of the transport proteins: Bile flow is sometimes obstructed by chemical and toxicant agents that affect proteins at the canalicular membrane. Loss of villous processes and disruption of transport pumps like multidrug resistance-associated protein-3 prevents excretion of bilirubin thus causing cholestasis [14].

Cytolytic T-cell activation: Covalent binding between chemical/toxicant agent and the P450 enzyme acts as an immunogen thus

resulting in activation of T cells, cytokines and stimulating a multifaceted immune response which may lead to hepatotoxicity [15].

Apoptosis of hepatocytes: Cell death may occur due to the activation of apoptotic pathway by tumor necrosis factor-alpha (TNF- α) which may trigger the cascade of intercellular caspases in hepatocytes [16].

Mitochondrial disruption: Some agents (chemical) may disrupt function of mitochondria by dual effect on beta-oxidation energy productions by inhibiting the synthesis of nicotinamide adenine dinucleotide and flavum adenine dinucleotide, leading to reduced ATP production [17].

Bile duct injury: The toxic metabolites that are excreted in bile may lead to the injury of the bile duct epithelium.

Chemical induced hepatic damage

The toxic chemicals like antibiotics chemotherapeutic agents, peroxides oil, aflatoxin, CCl₄, chlorinated hydrocarbons etc., may lead to the hepatic disorders. Liver cell damage by these hepatotoxic chemicals occurs mainly by inducing lipid peroxidation and generation of reactive oxidative intermediates in liver [18].

Direct or intrinsic or predictable drug reactions: Predictable reactions are those when a person is exposed for short interval of time and toxicity occurs in a dose related manner e.g., in response to chemicals like carbon tetrachloride, phosphorus, and chloroform which are no longer used as drugs. Acetaminophen is moderately predictable hepatotoxins [19].

Indirect or unpredictable idiosyncratic drug reactions: These unpredictable reactions are unrelated to dose but occur without caution and their time period to produce the response can range from few days to 12 months. Various drugs may produce indirect damage that has characteristic biochemical, clinical, histologic and chronologic features [19].

Models of hepatotoxicity

Galactosamine induced liver necrosis: D-galactosamine leads to acute hepatic necrosis in rats as a single or few repeated doses. For long term administration it leads to cirrhosis. Galactosamine (hepatotoxicant) produces discursive type of injury that imitates viral hepatitis and acute self-limiting hepatitis with necrosis, inflammation and regeneration, resembling a drug-induced disease in humans. Toxicity of D-galactosamine results from reduction of uridine pools that are connected with Ribonucleic Acid (RNA) and protein synthesis, which will in turn affect hepatocellular function [19]. This type of mechanism (toxicity) raises cell membrane porousness and ultimately causes cell death. Cholestasis caused by galactosamine is due to its damaging effect on bile ducts, it reduces bile flow of bile salts, cholic acid and deoxycholic acid [4].

During first day of experiment divided doses of 100 mg/kg to 400 mg/kg D galactosamine are injected (i.p. or i.v.) for induction of acute hepatotoxicity. For inducing liver cirrhosis male wistar rats (110g to 180g) are injected D-galactosamine (500 mg/kg, i.p.) three times weekly for a period of one to three months. After that protective substances are given orally with food or by gavages per day followed by sacrifice of rats and livers obtained by autopsy. Light microscopy and immune histology of liver is conducted using antibodies against macrophages, lymphocytes and the extracellular matrix components,

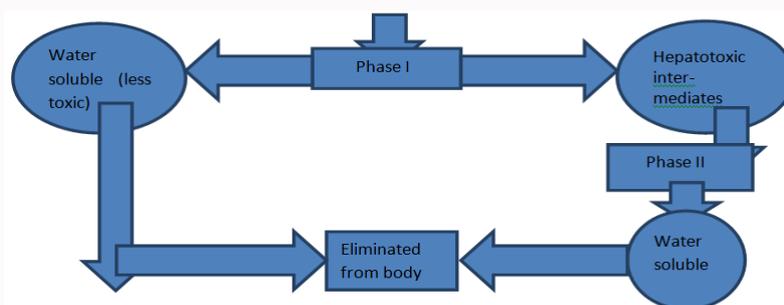


Figure 1: Metabolism of xenobiotics in liver.

for the evaluation of hepatoprotective effect. Then the size of liver cell necrosis and immune reactivity for macrophages, lymphocytes and the extracellular matrix parts is graded semi quantitatively on a 0 to 4+ scale (0= absent, 1+= trace, 2+= weak, 3+= moderate, and 4+= strong) [20].

Some other agents or drugs have been used to induce experimental cirrhosis for e.g., Ethionine, thioacetamide, dialkyl nitrosamines, tannic acid, aflatoxins, pyrrolizidine alkaloids, and hepatotoxic components from mushrooms, such as amatoxins and phallotoxins [21,22].

Paracetamol induced hepatotoxicity: The hepatotoxic effect of paracetamol (acetaminophen) is due to formation of hepatotoxic metabolite [23]. Paracetamol is commonly used as analgesic and antipyretic drug but causes liver damage in high doses [24,25]. Administration of paracetamol produces necrosis of centrilobular hepatocytes characterized by nuclear pyknosis and eosinophilic cytoplasm followed by large excessive hepatic lesions [26]. The paracetamol is broken down to sulphate and glucuronide conjugates after that it's metabolized to reactive intermediate. It's depolluted by conjugation with glutathione [23]. The covalent binding of N-acetyl-P-benzoquinoneimine, an oxidative product of paracetamol to sulphhydryl groups of protein, result in lipid per oxidative degradation of glutathione and causes cell necrosis in the liver [24,25]. In various studies different doses of paracetamol have been used to produce hepatotoxicity. They vary from 1 g/kg to 3 g/kg; p.o. single dose of paracetamol administration on 10th, 5th or 3rd day of experiment [26,27].

Antitubercular drugs induced hepatotoxicity (rifampicin, isoniazid and pyrazinamide): Hepatotoxicity is induced by antitubercular drugs like Isoniazid (INH), pyrazinamide and rifampicin and their combination. Adverse effects of antitubercular drugs are increased by multiple drug regimens. Isoniazid, rifampicin and pyrazinamide are generally hepatotoxic, and when given in combination their toxic effect is further enhanced. INH is metabolized to monoacetyl hydrazine and then it's metabolized in the presence of cytochrome P450 to a toxic product leading to hepatotoxicity. Rifampicin induces hydrolytic pathway of INH and its metabolism into the hepatotoxic metabolite hydrazine. Therefore, when given in combination INH and rifampicin, half-life of INH is reduced and acetyl hydrazine is fastly converted into reactive metabolites and chronic treatment with rifampicin may increase hepatitis in patient. Hepatotoxicity is increased by pyrazinamide when given in combination with INH and rifampicin [28].

Carbontetrachloride induced liver fibrosis in rats: In 1936, it was reported for the first time that carbon tetrachloride produced liver

injury in rats [25]. Since then carbon tetrachloride is being used to induce hepatotoxicity as an experimental model of acute and chronic liver failure. CCl₄ is metabolized by CYP2E1, CYP2B and CYP3A to form Trichloromethyl Radical (CCl₃). This radical (CCl₃) also binds to cellular molecules damaging crucial cellular progressions and also react with oxygen to form trichloromethylperoxy radical CCl₃OO, a highly reactive species. CCl₄ metabolite cause hepatic injury and acute liver injury model [29].

Wistar rats are administered carbon tetrachloride 1 mg/kg (dissolved in olive oil 1:1) orally, twice weekly, for a period of 8 weeks. Animals are kept under the standard conditions. Controls can receive olive oil only, and the test compound in different doses can be given as per schedule. The animals are weighed weekly. After end of experiment (8weeks), animals are anesthetized and exsanguinated [20]. The parameters of hepatic functions viz. total bilirubin, total bile acids, 7S fragment of type IV collagen, procollagen III N-peptide can be determined in the serum.

For histological examination 3 to 5 pieces of liver weighing about 1 g fixed in formalin and Carnoy solution. 3 to 5 parts of each liver are fixed, cut and stained with Azocarmine Aniline Blue (AZAN) and evaluated for the development of fibrosis using a score of 0 to IV [20].

Grade 0: Normal liver histology.

Grade I: Tiny and short septa of connective tissue without influence on the structure of the hepatic lobules.

Grade II: Large septa of connective tissues flowing together and penetrating into the parenchyma and tendency to develop nodules.

Grade III: Nodular transformation of the liver architecture with loss of the structure of the hepatic lobules.

Grade IV: Excessive formation and deposition of connective tissue with subdivision of the regenerating lobules with development of scars.

Alkyl alcohol induced liver necrosis in rats: Liver is much vulnerable to the toxic effect of ethanol. Alcohol is commonly used to induce fatty infiltration, hepatitis and cirrhosis. Fat infiltration (reversible phenomenon) occurs at that time when alcohol replaces fatty acids in mitochondria. Both hepatitis and cirrhosis occurs due to higher lipid per oxidative reaction during the microsomal metabolism of ethanol [30]. Alcohol can usually cause *in vivo*.

Changes in membrane lipid composition and fluidity that may affect cellular function [31]. Alcohol can produce changes in membrane phospholipids and raise lipid peroxidation. During oxidation of ethanol in liver its effect magnifies generation of oxy

Table 1: List of hepatoprotective plants.

Botanical name	Family	Parts used	Chemical constituents	Ref.
<i>Acalypha racemosa</i>	<i>Euphorbiaceae</i>	Leaves	Flavonoids	[62]
<i>Asparagus racemosus</i> Linn	<i>Asparagaceae</i>	Roots	Phenols, coumarins	[63]
<i>Azadirachta indica</i>	<i>Meliaceae</i>	Aerial parts	Azadirachtin, margolone	[61]
<i>Carissa carindas</i> Linn	<i>Apocyanaceae</i>	Root	Alkaloids, tannins, steroids	[64]
<i>Diospyros malabarica</i>	<i>Ebenaceae</i>	Bark	β -sitosterol, Gallic acid, betulin	[62]
<i>Ficus religiosa</i> Linn	<i>Moraceae</i>	Stem bark	Glycosides, steroids, tannins	[65]
<i>Morinda citrifolia</i> Linn	<i>Rubiaceae</i>	Fruit	Saponins, triterpins, steroids	[66]
<i>Picrorhiza kurroa</i>	<i>Scrophulariaceae</i>	Rhizome	kutkin	[67]
<i>Silybium marianum</i>	<i>Asteraceae</i>	Seeds	silybin, silidianin and silychristine	[61]
<i>Solanum nigrum</i> Linn	<i>Combretaceae</i>	Fruits	Flavonoids, terpenoids	[68]
<i>Terminalia belerica</i>	<i>Terminalia</i>	Whole plant	Flavonoids	[62]
<i>Urtica parviflora</i>	<i>Urticaceae</i>	Leaves	Flavonoids, alkaloids, , sterols, tannins	[69]
<i>Vicia calcarata</i>	<i>Fabaceae</i>	Aerial parts	Quercetin, kaempferol	[70]
<i>Xylopiya phloiodora</i>	<i>Annonaceae</i>	Stem bark & leaves	Flavonoids	[71]

free radicals. The effect of free radical is due to reduction in catalase, superoxide dismutase and glutathione peroxidase.

For inducing the experimental model of alcohol Induced Liver Necrosis in rats, food but not water is withdrawn on first day of experiment and the drug to be tested for protective activity is administered i.p. or orally after about 8 hours. After one hour 0.4 ml/kg (1.25% solution of alkyl alcohol in water) is given to animals orally. Till third day of experiment food but not water is withheld. On third day animals are sacrificed and liver is removed. Then the parietal sides of liver are examined by using stereomicroscope (25 magnification). So the focal necrosis is perceived as white-green or yellowish hemorrhagic areas clearly separated from unaffected tissue, ocular-micrometer is used for insistent diameter of necrotic areas. Result values are added up for every animal to obtain an index for necrosis [20].

Ranitidine induced hepatotoxicity: The injury produced by ranitidine occurs due to the presence of its metabolites that cause oxidative damage in liver or initiate immune allergic reaction. It also creates a reaction as reflected by infiltration of hepatocytes with ranitidine (dose of either 50 mg/kg or 30 mg/kg). After prominent centrilobular and bridging necrosis drastic inflammatory changes with formation of collagenous septa. In parenchyma there occurs focal liver cell necrosis with some accumulation of histocytic elements and slight steatosis and cholestasis. It leads to fibrosis, bile duct proliferation, and infiltration of lymphocytes, plasma cells, polymorphs, and eosinophils. Hepatic damage is classified in terms of rise in serum aminotransferases, modest hepatic infiltration by both lymphocytes and eosinophils and slight focal hepatocellular necrosis. Ranitidine also induces liver cholestasis associated with raise in plasma bilirubin and alkaline phosphatase [32].

Thioacetamide induced hepatotoxicity: After the administration of drug (thioacetamide), a selective hepatotoxicity appears within a short period of time [30]. Thioacetamide also produces membrane injury, due to inhibition of the movement of RNA from the nucleus to cytoplasm. For hepatic injury thioacetamide (s-oxide) metabolite is responsible. Thioacetamide decreases the number of liver cells and rate of oxygen consumption more over reduces the volume of bile and its content [4]. Thioacetamide undergoes immense metabolism

to acetamide and thioacetamide S-dioxide. Chronic exposure to thioacetamide causes cirrhosis in rats [33]. The thioacetamide toxicity occurs due to thioacetamide S-oxide which is responsible for the alteration in cell permeability and concentration of intracellular Ca^{2+} rises and also obstructs mitochondrial activity which leads to cell death [34]. Generally a dose of 100 mg/kg s.c. of thioacetamide is used to induce the model of hepatotoxicity [4].

Disruption of collagen synthesis in human skin fibroblasts culture: Secretion of collagen by fibroblast and further ability of cells to synthesize extracellular matrix is dependent on the hydroxylation of proline residues by prolyl 4-hydroxylase. This enzyme is located in the cisternae of the endoplasmic reticulum. Any agent required to inhibit this enzyme should pass both the external cell membrane and the endoplasmic reticular membrane. This prolyl 4-hydroxylase inhibitor can be reached by applying a prodrug which can be converted to the active agent only in cells of specialized tissues, e. g., in the liver, but not generally in fibroblasts [20].

The hydroxyproline synthesis can be evaluated in cultures of human skin fibroblasts preincubated at 37°C for 24 hours then cells are exposed to the potential inhibitor at various concentrations for 20 minutes, followed by the addition of 2 mCi [¹⁴C] proline/ml. The incubation is continued for 5 hours at 37°C. After that cells are separated from the medium and non-incorporated [¹⁴C] proline is removed, and the hydroxyproline content is determined by amino acid analysis. The total incorporation of radioactivity serves as marker for protein synthesis. Proline incorporation is expressed as % of control radioactivity [20]. Hydroxyproline synthesis is expressed as relative hydroxyproline/Proline ratio according to the formula:

$$\frac{\text{Hydroxyproline/Proline sample}}{\text{Hydroxyproline/Proline control}} \times 100$$

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2D culture models of hepatocytes: This model has been used from decades for *in vitro* divination of *in vivo* metabolic pathways and hepatotoxicity of drugs. This type of model offers the advantages of being relatively affordable, reproducible, strapping and convenient. Experimental species mostly rat and mouse cultured hepatocytes have been used. For the prognosis of drug metabolism and the estimation of hepatotoxicity human hepatocytes have been the gold standard *in*

vitro model, due to qualitative and quantitative interspecies differences in drug metabolizing enzymes, the extrapolation of drug metabolism and hepatotoxic effects from animal hepatocytes to man is difficult [35-39]. One main drawback of monolayer cultures is that they lose hepatic functionality over a period of time [35,37-41]. A sandwich design was developed for an attempt to maintain liver functionality over a longer period in a culture (Table 1). In between two matrix layers hepatocytes are placed, conventionally (collagen or Matrigel). So for retaining hepatocytes in a sandwich culture arrests cell viability loss, intensify secretion of organic compounds, including urea and albumin, increases basal and induced drug-metabolizing enzyme activities, and mimics *in vivo* biliary excretion rates [36,38,42,43]. Hence it has been recommended that the sandwich culture model is most useful for mechanistic studies of hepatobiliary toxicity [42,44-46].

3D culture models of hepatocytes: Due to failure of detection of hepatotoxic drugs in preclinical testing using conventional hepatocyte cultures, other models with phenol typically stabilized liver cell functions over a long period of time have been developed. They are based on recreating micro-environmental signal *in-vivo*, like 3D architecture of multiple cell types, cell-cell and cell-matrix interactions, soluble factors, and dynamic nutrient flow for screening of drugs and predicting drug efficacy and toxicity in humans. 3D liver cell models are mostly useful for industrial drug discovery to allow the practical assessment of drug metabolism and adverse effects, which are receptive for normal use and high-throughput adaptation [40,47]. They are described as four based system viz. Scaffold-based systems, Multicellular spheroids, 3D co-cultures of hepatocytes and non-parenchymal cells and Microfluidic devices [48].

Common liver function and diagnostic tests

There are different types of test/markers which can be used to diagnose hepatotoxicity, assess the prognosis of disease and differentiate between acute and chronic hepatic disorders. Most commonly used blood tests are as under:

Serum glutamate oxaloacetate transaminase (SGOT) test: SGOT enzyme is released from liver, heart, kidney, muscle or brain cells when these are damaged. Its normal serum value is up to 46 IU/L at 37°C [17]. The value of this enzyme is increased 10 to 200 fold in patient with acute hepatic necrosis, viral hepatitis, CCl₄ and drug induced poisoning. Also increased 10 fold in patients of post hepatic jaundice, intra hepatic cholestasis and in alcoholic and hepatic steatosis less than 10 fold [49].

Serum glutamate pyruvate transaminase (SGPT) test: SGPT enzyme is released from damaged liver cells. Normal serum value of SGPT is up to 49 IU/L at 37°C. The level of SGPT is very high in patients of viral hepatitis and hepatic necrosis. Also higher in patients of post hepatic jaundice, intrahepatic cholestasis 10 to 200 fold and below 10 in patients of metastatic carcinoma, cirrhosis and alcoholic hepatitis [50].

Serum alkaline phosphates test: Alkaline phosphatase enzyme is found in liver and obstruction of bile flow, liver injury, or certain cancers results in increased level of this enzyme [50]. Normal serum values of 25 IU/dl to 85 IU/dl of serum alkaline phosphatase are found to be increased in diseases of bone, hepatobiliary disease and in pregnancy. The levels are 3 to 10 times of normal in biliary tract obstruction and slight to moderate increase is seen in Parenchymal liver diseases (hepatitis, cirrhosis and metastatic) liver disease [3].

Serum total protein and albumin test: Total protein is in the normal range of 5.5 g/dl to 8 g/dl when frequently estimated. In extensive liver damage level of blood plasma protein is reduced. Normal range of albumin synthesized in the liver is 3.5 g/dl to 5.0 g/dl. In chronic liver disease the level of serum albumin is low [51]. Hypoalbuminaemia may occur in liver diseases due to remarkable demolition of hepatocytes. Hyperglobulinaemia may be present in chronic inflammatory disorders (cirrhosis and in chronic hepatitis).

Serum total and direct bilirubin test: Every day about 7.5 g of hemoglobin is catabolized with corresponding production of 250 mg of bilirubin. In the blood normally 0.25 mg/dl of conjugated bilirubin is present in adult. Normal value of total bilirubin is 0.2 mg/dl to 1.2 mg/dl. Level of bilirubin increases in diseases such as obstruction of biliary excretion (into duodenum), hemolysis and in defects of hepatic uptake and conjugation of bilirubin such as in Gilberts disease [17]. If the value of conjugated or direct bilirubin is low and that of total bilirubin is high this indicates hepatic cell damage or bile duct damage [52].

Urine bilirubin: Presence of bilirubin in the urine is an indication of hepatobiliary disease. The unconjugated bilirubin is tightly bound to albumin, hence not filtered by the glomerulus and is not seen in urine. So the measurable amount of conjugated bilirubin in serum is found only in hepatobiliary disease [53]. The threshold of renal conjugated bilirubin is low and laboratory test can diagnose low levels of urine bilirubin. So when the level of serum bilirubin is normal conjugated bilirubin may be found in urine for e.g., during early acute viral hepatitis [53].

Urobilinogen: As rise in the urobilinogen level in urine is a sensitive sign of hepatocellular dysfunction, it is a good marker of alcoholic liver damage, well compensated cirrhosis or malignant disease of the liver. As a viral hepatitis it may come early in urine and markedly rises in hemolysis. Also in cholestatic jaundice urobilinogen vanish from urine but may be present intermittently in case of gallstones. After that Urobilinogen gives a purple reaction to Ehrlich's aldehyde reagent and a dipstick containing this reagent allows rough and ready quantification in freshly voided urine [53].

Serum Lipid Profile Test: Hepatic toxicants may lead to disruption in the synthesis and metabolism of triglycerides, cholesterol and lipoproteins, thus damaging the basic function of living cells. Liver cells synthesize cholesterol and bile salts, so hepatic intoxication reduces their levels. The normal value of cholesterol is up to 200 mg/dl. But it is important to know the HDL and LDL value for analysis. Fatty degeneration of the liver raises triglyceride (normal range >150 mg/dl) content in the blood [54].

Lactic dehydrogenase (LDH) test: Total LDH reference value varies from one laboratory to another laboratory. Also normal value is higher in childhood. In most laboratories, the value can be up to approximately 200 U/L for adults. But usually it is found within 45 U/L to 90 U/L. When LDH containing tissues are damaged, higher than normal of its levels are released into the bloodstream. The level of LDH is also raised in the presence of liver diseases, heart attack and certain types of anemia and in cases of excessive destruction of cells, in fractures, trauma, muscle damage and shock [54].

Ascorbic acid content in urine: Ascorbic acid content in urine has been used for screening of hepatoprotective agents against CCl₄ induced hepatotoxicity in rats and is considered as a non-invasive test. During CCl₄ induced liver damage the excretion of ascorbic acid

in urine is decreased in rats [2].

Bromosulphthaline clearance test: A dye Bromosulphthaline (BSP) is clarified from the blood by the liver by the same mechanisms of binding, conjugation and excretion as bilirubin. After the intravenous injection of BSP, percentage of injected dye remaining in the blood (taken 45 minutes later) is tested. The test is mostly performed nowadays due to availability of enzyme estimation and the value of BSP excretion is used for the diagnosis of Dubin-Johnson's syndrome [2,3].

Superoxide dismutase (SOD): Superoxide dismutase is an enzyme that converts superoxide radical to hydrogen peroxide and oxygen [55]. Dismutation means a single reactant is converted into two separate products. Superoxide dismutase is one of the most important mechanisms to scavenge superoxide radicals by catalyzing the conversion of these two radicals into (hydrogen peroxide and molecular oxygen).

Glutathione (GSH): Glutathione is a main naturally occurring antioxidant. It's a peptide of glycine, glutamic acid and cysteine. Also it can prevent the hydrogen of sulfhydryl group to be distracted as an alternative of methylene hydrogen of unsaturated lipids. So the level of glutathione has a crucial importance in tissue injury produced by toxic substances. Both SOD and glutathione (antioxidant enzyme) are first line of defense against free radical induced damage. GSH offers protection against free radicals and also maintain low level of lipid peroxides [27]. Glutathione has a primary function as a non-enzymatic reducing agent, help to keep cysteine thiol side chains in a reduced state at the surface of proteins. It's also used to prevent oxidative stress in most cells.

Lipid peroxidation: Lipid peroxidation is defined as an oxidative deterioration of lipids. Malondialdehyde (MDA) is a marker of lipid peroxidation following damage by reactive oxygen species. MDA is a major reactive aldehyde resulting from the peroxidation of biological membranes. These are formed by enzymatic or nonenzymatic reactions involving free radical [56].

Glutathione peroxidase: Glutathione Peroxidase (GPx) induces reduction of hydro peroxides and blocks the cell from peroxidative damage. It is a selenium containing enzyme which can reduce H_2O_2 to H_2O by oxidizing glutathione (GSH). Rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase. The function of GPx can be calculated incidentally by a coupled reaction with glutathione reductase. The oxidized glutathione (GSSG) is created upon reduction of an organic hydro peroxide by GPx. Then it is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the absorbance is directly proportional to the GPx activity in the sample [2].

Prothrombin time (PT): The blood coagulation proteins are synthesized by the liver like fibrinogen, prothrombin, prekallikrein, high molecular weight kininogen etc. Most of these are available in excess and the deformity of coagulation only occurs due to substantial impairment in the capability of the liver to synthesize these factors. The standard method to estimate is the one stage prothrombin time, as it can evaluate the extrinsic coagulation pathway. The test results are indicated in sec or as a ratio of the plasma prothrombin time to control plasma time. Normal value is usually 9 seconds to 11 seconds, if it is further prolonged by more than 2 seconds represent

abnormality [57].

In hepatocellular disease (acute or chronic) PT serves as a prognostic indicator. In acute disease rise of PT indicates acute liver failure. However, prolonged PT is not specific for liver diseases and can be seen in deficiencies of various coagulation factors [57].

Imaging examination: In acute drug induced liver injury condition, there are usually no significant changes in liver on imaging or only hepatomegaly. Patients with drug-induced ALF (Acute Liver Failure) may have reduced liver volumes as the disease improves. Condition in patients with chronic liver disease generally do not have appreciably dilated intra- and extra-hepatic bile ducts, but in some cases changes consistent with cirrhosis, such as splenomegaly and enlarged internal diameter of portal vein. For the diagnose of SOS/VOD (Sinusoidal Obstruction Syndrome/Hepatic Venous Occlusive Disease) imaging diagnosis become helpful, a plain Computed Tomography (CT) scan may show a swollen liver, and an intensify CT scan during portal venous phase may show uneven or patchy change of the liver images, blurred hepatic veins, and ascites [54]. Continuously imaging examinations such as ultrasound, CT, or MRI scan, as well as retrograde cholangiopancreatography have good importance for distinguishing cholestatic liver disease from biliary obstruction caused by gall stones biliary or pancreatic malignancies [58].

Ultrasonography: This test is ancillary diagnostic test. Examination of liver by ultrasound is indicated in the following situations:

- Cholestasis of numerous etiologies to see the dilated intra- and extrahepatic canalicular tree.
- Within the liver Space-Occupying Lesions (SOLs) to decide whether they are neoplasms or non-neoplastic cysts.
- To allocate US-guidance for FNAC or liver biopsy [3].

FNAC and/or percutaneous liver biopsy: This ancillary diagnostic test FNAC and percutaneous liver biopsy are used to examine the microscopic changes of liver morphology in numerous hepatic diseases [3]. Their main indications are as follows:

- Hepatocellular disease of unknown cause;
- Suspected cases of chronic hepatitis;
- Hepatomegaly of various etiologies;
- Splenomegaly of unknown cause; and
- Fever of unknown cause.

Histopathological examination: When we are taking a series of clinical examination and laboratory tests but these tests do not yield assured diagnosis of drug induced liver injury. So a liver biopsy may be helpful for the diagnosis and assessment of the severity of liver injury [58].

Hepatoprotective medicinal plants

There are lots of advances in modern medicine, but unfortunately there are very few drugs that protect the damaged liver and help in regeneration of hepatic cells. Traditional uses of herbal medicines have been documented since long historical period and they are widely acknowledged to be safe and effective and recognized as a form of alternative medicine in conventional scientific based health care system. In recent decade, complementary and alternative medicine

approach using medicinal plants for prevention and treatment of diseases have been gaining importance. Medicinal plants exhibit efficacy in treatment of a number of diseases which are not otherwise cured by synthetic drugs. Herbal drugs are rapidly emerging as safer alternatives/adjuncts in several chronic diseases and this has been shown in some inflammatory disorders following modern scientific methodology [59,60]. The classical texts on traditional system of medicines viz. Ayurvedic, Unani etc. can provide us with valuable guidelines to the selection, preparation and application of herbal formulation. A large number of medicinal plants have been used traditionally for immunomodulation and hepatoprotection and can be an important source of hepatoprotective drugs. Various Indian medicinal plants are being widely used in the Indian traditional system of medicine for the management of liver disorders. Herbal drugs are inexpensive, possess minimal adverse effects and people have faith in them, therefore plant based hepatoprotective drugs are getting importance and being promoted in the global market. Nearly about 170 phytoconstituents isolated from 110 plants belonging to 55 families do hold hepatoprotective activity [61]. Different types of chemical ingredients are present in hepatoprotective plants such as phenols, coumarins, lignans, essential oils, monoterpenes, carotenoids, glycosides, flavonoids, etc., which may contribute to their hepatoprotective effects. Some of the important hepatoprotective plants with their part used and chemical constituents have been listed in Table 1.

Conclusions

Liver diseases are among the top ten killer diseases in India, causing lakhs of deaths every year. Besides, there are those who suffer from chronic liver problems, needing recurrent hospitalization and prolonged medical attention, which leaves them physically, mentally, emotionally and financially devastated. On the other hand there are millions of cases of hepatic diseases, which result from exposure to various xenobiotics/drugs. The traditional system of medicines viz. Ayurvedic, Unani etc. can provide us with valuable guidelines to the selection, preparation and application of herbal formulation for hepatic dysfunction. A large number of medicinal plants have been used traditionally for immunomodulation and hepatoprotection. In order to validate the effects of these compounds, their efficacy in experimental models of hepatic dysfunction needs to be investigated. Therefore, an attempt has been made in this review to give details about the mechanisms of hepatotoxicity, specific markers of liver function which can be used for the diagnosis of the liver dysfunction and to devise strategies for its protection. The present review also gives an account of some of the *in vivo* and *in vitro* experimental models to evaluate new drugs/compounds and tabulate some of the important hepatoprotective plants with their parts used and chemical constituents which can be further validated using the modern scientific methodology.

Acknowledgement

The research on hepatotoxicity and protective strategies was supported by grants from the CCRUM, Ministry of AYUSH, New Delhi, which is duly acknowledged.

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