2. Literature review

The liver is one of the most important and largest organs in the body, which detoxifies or removes foreign substances or toxins, especially from the gut. It weighs 1 to 1.5 kg and representing 1.5 to 2.5% of the lean body mass (Hyder et al., 2013). It is made up parenchymal cells (hepatocytes) and mainly four different types of nonparenchymal cells namely sinusoid lining endothelial cells, Kupffer cells (liver macrophages), stellate cells (fat storing cells) and pit cells (liver associated natural killer cells) (Blomhoff and Wake, 1991).

Types of liver cells

Hepatocyte- It plays a key role in most metabolic processes, especially detoxification. It detoxifies harmful substances by a series of chemical reactions. The role of these different enzyme activities in the liver is to convert fat-soluble toxins into water-soluble substances that can be excreted in the urine or the bile. These cells are metabolically active and represent the function of liver.

Kupffer cells- These are also called as liver macrophages. They are located in the lining of sinusoids and forms reticuloendothelial system. They removes worn out or old RBCs by phagocytic system and split the hemoglobin molecules. They promote inflammatory reaction in the liver by generating ROS and various cytokines.

Hepatic Stellate cells- They are also called as perisinusoidal cells or Ito cells. These are present between sinusoid and hepatocytes. These are the major cells promoting fibrosis, scar tissue produced due to damage to liver and leading to irreversible stage of liver damage (hepatic cirrhosis) (Braet and Wisse, 2002).

Hepatic NK cells- They are the immune cells present together with Kupffer cells. The number of these cells varies in a ratio of 1:10 of Kupffer cells. Their major functions involve prevention of liver fibrosis and removal of malignant cells together with removal of toxins.
Figure 2.1. Liver lobule, a function unit of liver. It shows functional arrangement of cells. Flood flows through sinusoids, from hepatic artery and portal vein to hepatic venule (central vein). Bile is produced by hepatocyte, flows through canaliculi and collected in bile duct. (Adaptation from http://www.svhrad.com/DigLib/Gastroinestinal/Liver/Segmental%20Anatomy/Liver%20lobule.gif)

Over all the complex action of liver can be summarized as

1. Conversion of food into chemicals necessary for life and growth
2. Manufacturing and export of important substances for rest of the body
3. Processing of absorbed drugs from the gastrointestinal tract in the forms that can be easily used by the body
4. Detoxification and excretion of poisonous substances

The liver is "downstream" from the intestine, which make it most vulnerable to toxic attack (Kidd 2002). These toxicants include wide range food-borne toxins, such as herbicide and pesticide residues, artificial preservatives, and other synthetic food additives. The liver also deals with the toxins that enter to the body through other routes. These include alcohol, cigarette-smoke toxins, street drugs, viral and bacterial antigens, heavy metals, solvent pollutants, and over-the-counter and prescription pharmaceuticals. These toxicants reach to the hepatocytes of
liver following the absorption into the blood and poses threat to the hepatocyte until get detoxified (Kidd, 2002). Increasing concentration of these toxicants in the blood leads to liver disorder.

**Statistics**

Liver disorders are the ninth biggest killer in India and ranks 27th in the list of countries affected by various liver disorders. Approximately, 80% of the hepatocellular carcinoma [HCC] patients had history of progression of liver disorder from fibrosis to cirrhosis and finally to hepatocellular carcinoma (Papatheodoridis et al., 2010, Roncalli et al., 2011). Hepatocellular carcinoma [HCC] is the most common cancer in the world, with 630,000 new cases diagnosed every year (Thyagarajan et al., 2002). The three main causes of HCC are Hepatitis B and Hepatitis C infections and alcohol-induced liver injury. Reports suggest that Hepatitis B virus (HBV) infection is the fourth or fifth most important cause of mortality in the most productive period of life (15-45 years of age). Twenty five percent of all HBsAg positive newborns develop chronic liver disease by third to fourth decade of life. It is estimated that 15-40% of those with chronic HBV infection will eventually form serious complications, such as liver cancer or cirrhosis, and die in later stages. There is a prevalence rate of 26% in South India, 16-20 % in Mumbai and 10-15% in North India. The major way that Hepatitis C presents itself is chronic hepatitis (www.geocite.com). Liver disorder is one of the major causes of drug withdrawal from market by US FDA. Hepatotoxicity is the most common single ADR causing drug withdrawal and refusal for FDA approval. The list includes Lumiracoxib- 2007-2008, Ximelagatran-2006, Pemoline-2005, Nefazodone-2004, and Troglitazone-2000.

**Types of liver diseases**

Liver diseases are clinically classified as hepatocellular, cholestatic (obstructive), or mixed. In hepatocellular diseases like viral hepatitis or alcoholic liver disease, the feature of liver injury predominantly includes inflammation, and necrosis. In cholestatic diseases like gallstone or malignant obstruction, some drug-induced liver diseases, the features of inhibition of bile flow predominate. In a mixed pattern like cholestatic forms of viral hepatitis and many drug-induced liver diseases, the features of both hepatocellular and cholestatic injury are present.
Liver disease progression

Liver disease progression happens in four stages namely, fatty liver, hepatitis, cirrhosis and finally liver carcinoma. The first threat by toxins results in fatty liver of hepatocytes, where the lipids accumulates in hepatocytes. The excess accumulation results in ballooning degeneration. The sinusoids, spaces carrying blood get constricted due to increased size of hepatocyte. The Kupffer cells gets insufficient supply of oxygen which finally results in proliferation and secretion of inflammatory cytokines viz., TNF α, IL-2, IL-6. These cytokines attracts many inflammatory cells viz., lymphocytes, neutrophils, monocytes, eosinophils, which results in progression of liver disorder to second stage, hepatitis. Hepatitis includes a wide range of changes like infiltration of WBCs, congestion of sinusoids, apoptosis or necrosis based on the toxic attack. The Kupffer cells and inflammatory cells play a major role in progression of liver disease to third stage. They secrete various reactive oxygen and nitrogen species (ROS, RNS). These species progress the inflammatory reaction and also decreases antioxidant defense of the cell viz., glutathione level, SOD level, GST level etc. They further promote fibrosis by promoting growth of stellate cells. The fibrotic stage of liver gets divided into segments and results into the next level of liver disorder i.e., cirrhosis. The remaining hepatocytes overgrows to replenish the function of liver and results into hepatic carcinoma.

Mechanisms of hepatotoxicity

- Hepatocyte Disruption: Drugs bind covalently with the critical intracellular proteins. It includes disruption in enzymatic functions and disassembly of actin fibrils which results in blebs formation and rupture of the membrane (Pumford et al., 1989).
- Disruption of the transport proteins: Drugs (ex. Rifampin, cyclosporine A etc.) that affect transport proteins at the canalicular membrane can interrupt bile flow (Keitel et al., 2005).
- Kupffer cell activation and neutrophil infiltration: Covalent binding of a drug to the P-450 enzyme acts as an immunogen, activating Kupffer cells and T cells. Cytokines released in that response stimulating a multifaceted immune response (e.g. paracetamol) (Jaeschke et al., 2002b).
- Apoptosis of hepatocytes: Activation of the apoptotic pathways by the tumor necrosis factor-alpha receptor of Fas may trigger the cascade of intercellular caspases, which results in programmed cell death (Jaeschke et al., 2002b).
Mitochondrial disruption: Certain drugs inhibit mitochondrial function by affecting beta-oxidation and energy production by inhibiting the synthesis of nicotinamide adenine dinucleotide and flavin adenine dinucleotide, which results in decreased ATP production (Raj et al., 2011).

Bile duct injury: Toxic metabolites excreted in bile may cause injury to the bile duct epithelium (Jaeschke et al., 2002b).

**Drug induced liver injury (DILI)**

It can be summarized in two categories:

- Intrinsic or predictable drug reactions: Drugs of this category cause a reproducible injury in animals, and the injuries are dose related. (e.g. Paracetamol at high dose)
- Idiosyncratic drug reactions: this type of reaction may be with low incidence with variable days of administration from weeks to years and may or may not be dose related. It can be further classified based on latency period to hypersensitivity or immunoallergic and metabolic-idiosyncratic.
  - Hypersensitivity or immune mediated: It is characterized by fever, rash, and eosinophilia and is an immune-related response. It takes typically a short latency period of 1-6 weeks. Upon further exposure, the liver injury reappears (e.g. Phenytoin).
  - Metabolic-idiosyncratic or non-immune mediated: It takes month to years for appearance of disease. This type of reaction is mediated by an indirect metabolite of the drug and happens in minority of patients (e.g. INH, Halothane) (Saukkonen et al., 2006).

**Types of hepatotoxic drugs**

It can be generally classified as-

a. Natural origin- e.g. Tannic acid, pyrrolidizone alkaloids, gyrometrin etc.

b. Synthetic origin:
   - Therapeutically important hepatotoxin:
     - e.g. Paracetamol, isoniazide, sulphonamides, para- amino salicylic acid, ethanol, rifampicin etc.
   - Hepatotoxin used for experimental purpose:
     - e.g. Carbon tetrachloride, chloroform, thioacetamide etc.
Hepatotoxins used in this study

1. **Paracetamol**
Paracetamol is an effective analgesic & antipyretic drug, classified in the category of OTC drugs. It is highly misused and one of the major causes of hepatotoxicity (Obu et al., 2012). It is safe in therapeutic doses, but in over doses it is toxic to liver. It is metabolized by cytochrome P-450 (CYP) to a toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). The isoforms of CYP found to be responsible for metabolism were CYP2E1, CYP1A2, CYP3A4, and CYP2D6 (Dong et al., 2000, Raucy et al., 1989, Snawder et al., 1994, Thummel et al., 1993). NAPQI formed by these enzymes is detoxified by glutathione conjugate to 3-glutathion-S-ylacetaminophen (Jollow et al., 1973, Mitchell et al., 1973a, Mitchell et al., 1973b). At over dose of paracetamol, the remaining unconjugated toxic metabolite covalently binds with proteins and causes damage to cells (necrosis) (Cohen and Khairallah, 1997). It is mainly represented by centrilobular necrosis and fatty accumulation in hepatocyte histologically and with elevation in AST, ALT and mild elevation in bilirubin in serum parameters. N-acetyl cysteine, a glutathione precursor, can limit the severity of the liver damage by supplying the glutathione and by capturing the toxic acetaminophen metabolite (Lauterburg et al., 1983).

2. **D-galactosamine**
It leads to induction of hepatic injury (extensive necrosis) in experimental animals. The metabolism of D-galactosamine (UDP-Galactosamine) interferes with nucleic acid metabolism (Decker and Keppler, 1974). UDP-Galactosamine is produced (Zimmerman, 1978) by the metabolism of galactose and resembles with UDP-glucose. UDP-glucose is a normal metabolic product that serves as an uridylate donor in the uridyl transferase reaction in RNA synthesis. Thus, it interferes uridylate biosynthesis and results in failure of RNA synthesis. (Zimmerman, 1978)

3. **Alcohol induced toxicity**
Alcohol is metabolized by three enzymes namely catalase, CYP2E1 and alcohol dehydrogenase. The end product of the metabolism is acetaldehyde, which gets oxidized to acetate by aldehyde dehydrogenases. The raised level of acetaldehyde shows the deleterious outcomes of alcoholic liver disease. It reacts with integral components of cell membrane like cysteine and other proteins resulting in damage to liver. Alteration in these molecules is associated with immune
response like autoimmune disorder. It affects both innate and acquired immunity. Increased gut permeability of endotoxin (lipopolysaccharide) and raised level of aldehyde result in activation of innate immunity mediator cells viz., Kupffer cells and stellate cells that further results in rise in levels of cytokines and causes necrosis and inflammatory changes (TSUKAMOTO and LU, 2001, Lieber, 2000, Ramaiah et al., 2004).

Current treatment for Liver diseases

Current treatment of liver diseases includes various strategies. It can be summarized as follows:

1. **Removal of underlying causes of hepatotoxicity**

Various types of liver disorders have various causes. Thus, strategy of elimination varies accordingly.

1.1. Viral hepatitis- Interferon based therapy is considered the first line treatment for removal of virus. However, the outcome of interferon-based therapy is heterogeneous, some people respond well, but others do not. The other limitations of interferon therapy include the side effects, which ranges from fatigue to mood disorder. Some other anti-viral therapy includes lamivudine and adefovir.

1.2. Autoimmune hepatitis- Glucocorticoid treatment for immune suppression is considered to be most important in this disorder.

1.3. Alcoholic hepatitis- Alcoholic intoxication resulting into inflammatory changes, which include increase in immune response. For this condition, also immunosuppressant therapy is included.

1.4. Paracetamol acute intoxication induced hepatitis- N-acetyl cysteine is an antidote given in the early stages of paracetamol intoxication. It replenishes glutathione stores.

6. **Supportive / Non- Pharmacological measures** (such as modification of diet which contain less fat)

Tailoring diet to a patient’s lifestyle is more important than a universal specific dietary program. This type of management requires personalized therapy depending upon the condition of the liver (Jun, 2013).
7. **Hepatoprotective agents from herbal sources**

A variety of herbal hepatoprotective agents are available in the Indian market since many years. One such is Liv.52, a herbal formulation based on Ayurvedic principles, which is known to protect the liver from damage produced by toxic substances (Chauhan and Kulkarni, 1991, Narayanasamy and Selvi, 2005).

8. **Liver transplantation**

 Despite improvements in immunosuppressive therapy, acute cellular rejection remains an important cause of mortality in patients undergoing liver transplantation (Kamei et al., 2013). Adding to this, are the regulatory issues and problems of unavailability of the donor (Gramenzi et al., 2011).

9. **Regeneration of the liver cells (Miyaoka and Miyajima, 2013)**

An alternative safe measure of liver regeneration is always welcome which has been in practice for a long time, starting with Ayurveda and spreading to Chinese and European traditional medicine (Zheng et al., 2013).

Even after extensive use in clinical practice, it has not been listed in treatment algorithm in any standard reference manual. However, the 21st century has seen a paradigm shift towards the evaluation of herbal products by strengthening of traditional systems of medicine with modern scientific approaches including standardization of herbal products and randomization in liver diseases with placebo controlled clinical trials to establish their clinical efficacy. In India, more than 300 preparations made from 87 herbal plants are available for treatment of jaundice and other chronic liver disorders (Thyagarajan et al., 2002). These plants have been studied scientifically while adhering with the internationally acceptable scientific protocols including chemo-biological fingerprinting, methodology for standardization, bioactive marker characterization, and preparation of most active fraction with maximum amount of bioactive component. Based on these studies, many herbal drugs have come into picture, which include silymarin, phyllanthin, lecithin, catechin, glycyrrhizin, picroside, baicalein, daphnoretin, L-ornithin-L-aspartate etc.
Drugs for hepatoprotection

A few drugs selected in our study were based on their wide usability pattern. They are discussed in detail for their available study reports.

Silymarin

Silymarin, a hepatoprotective agent, obtained from single herb *Silybum marianum*, is widely used in the treatment of liver diseases. It contains a mixture of five flavolignan isomers, namely silybin, isosilybin, silydianin, silychristin and taxifolin. The combination of these compounds is collectively expressed as silymarin (Campodonico et al., 2001). Among these isomers, the most active component is silybin, which accounts for 60 to 70 per cent of the total content of silymarin and is considered as the marker of silymarin (Pade, 2007). Clinical trial showed safety and efficacy of silymarin from 1200mg/day to 1500mg/day. The dose of silymarin is 240-800 mg per day in two to three divided doses. Physical property indicates low solubility in water but no lipophilic property. Bioavailability study of silymarin referred to quantification of silybin, as silybin is a chief active ingredient. Orally administered silymarin (silybin) shows maximum absorption in 2-4h with a half-life of 6h. The oral absorption of silymarin is only about 23-47% (Morazzoni et al., 1993, Blumenthal et al., 2000), leading to low oral bioavailability to 0.73% (Wu et al., 2007). The tissue distribution of this drug is very good in the various tissues as demonstrated in mice (Zhao and Agarwal, 1999). It is metabolized in phase I and phase II biotransformation reactions in the liver. Silymarin components rapidly eliminate in free form (half-life 1-3h) and conjugated form (3-8h). Thus, the limiting factors for bioavailability of silymarin can be summarized as extensive phase II metabolism, low permeability across intestinal epithelial cells, low aqueous solubility and rapid excretion in bile and urine (Javed et al., 2011). Researchers have adopted a number of strategies for improving the bioavailability of silymarin. Accordingly success in terms of bioavailability enhancement has been achieved with varieties of formulations viz., salt forms of silymarin (2 fold) (Madaus et al., 1976) self-microemulsifying drug-delivery system of silymarin (4 fold) (Woo et al., 2007), solid dispersions (5 fold) (Chen et al., 2005), inclusion complexes of silybin with cyclodextrin (6 fold) (Valcavi et al., 1993) and complexation with phospholipids (10 fold) (Vailati et al., 1993). Besides this, a significant improvement of bioavailability has been achieved with proliposomes (Yan-yu et al., 2006) and hybrid liposomes of silymarin (El-Samaligy et al., 2006).
Many experimental studies have proved the hepatoprotective activity of silymarin in different models of hepatotoxicity [CCL₄ model (Achliya et al., 2004); paracetamol model (Ramellini and Meldolesi, 1976); alcohol model (Wang et al., 1996, Pradhan and Girish, 2006), thioacetamide (Madani et al., 2008), D-galactosamine (Chungoo et al., 1997), erythromycin estolate (Davila et al., 1989), microcystine (Dixit et al., 2007), amanita phalloid toxins (Dixit et al., 2007)]. Together with hepatoprotective effect, it is also known to have anti-inflammatory activity (Dixit et al., 2007). Various mechanisms have been proposed for hepatoprotective action of silymarin namely antioxidation (Miguez et al., 1994), stimulation of ribosomal RNA polymerase (Sonnenbichler and Zetl, 1986), inhibition of leukotriene and prostaglandin synthesis (anti-inflammatory effect), inhibition and reversal of fibrosis process, anticarcinogenesis and immunomodulatory (Dixit et al., 2007) etc.

**Catechin**

Catechins are richly present flavonoids in green tea, black tea, wine and other plant foods, such as fruits and cocoa products. Green tea contains six primary catechin compounds: catechin, epicatechin, epicatechin gallate, epigallocatechin, gallocatechin, and epigallocatechin gallate (EGCG) (Graham, 1992). Catechins are reported to have antioxidative ability. The orally ingested catechin is absorbed from small intestine and metabolized to a conjugated and/or methylated form excreted in urine in rats and humans. Studies have explored various biological activities of catechins namely ROS scavenging activities (Graham, 1992), preventive effect on experimental tumor metastasis (Shimizu et al., 2010), protection against ethanol induced lipid peroxidation (Ostrowska et al., 2004) and protection in tamoxifen-induced liver injury (El-Beshbishy, 2005) etc. Various reports support the hepatoprotective effects of green tea and catechins against ethanol intoxication (Bharrhan et al., 2011, Yuan et al., 2006, Baltaziak et al., 2004, Arteel et al., 2002, Skrzydlewska et al., 2002). Effect of green tea and epigallocatechin gallate on ethanol-induced toxicity in HepG2 Cells was also reported (Lee et al., 2008). Proposed mechanism of catechin includes a powerful antioxidant and free radical scavenging action. Catechins have oral bioavailability less than 5% (Baba et al., 2001, Catterall et al., 2003) and a short half-life due to fast systemic clearance (Cai et al., 2002).
Phyllanthin from *Phyllanthus amarus* extract

Phyllanthin is a lignan obtained from Phyllanthus species namely, *P. amarus* Schumach, *P. polyphyllus*, *P. emblica* and *P. indofischeri* etc. Phyllanthus species have long traditional history. It is listed in Ayurveda for liver disorder. In current scenario many preclinical studies proves its hepatoprotective action. *Phyllanthus amarus* extract has shown HBsAG negative in clinical trial. Phyllanthin has very less oral absorption with an absolute oral bioavailability of 0.62 % (Murugaiyah and Chan, 2007).

**Lecithin**

Lecithin or phosphatidyl choline is an important phospholipid and found in the cell membrane of every cell. It is an important component of cell membrane of every cell of our body such as heart, liver, and kidneys (Iwata et al., 1993, Jimenez et al., 1990). It helps in signaling pathway in cell membrane. Studies have shown its potential benefit in liver repair. Phosphatidyl choline in mice has shown healing effect in Hepatitis A (Glück et al., 1992), B and C (Niederau et al., 1998). It has shown protection of liver against D-galactosamine induced toxicity in Wistar rats (Rah et al., 2011).

**Parameters to Evaluate Effect of Drugs on Liver**

The function of liver has been evaluated based on various parameters. These parameters are categorized as follows (Sumanth, 2007):

1. **Non-invasive functional methods**:
   1.1. Ascorbic acid content in urine
   1.2. Pentobarbitone induced sleeping time
   1.3. Bromosulphthaline clearance test

   1.1. Ascorbic acid content in urine- It is reported as a non-invasive test for screening hepatoprotective drugs against CCl4-induced hepatotoxicity in rats (Prakash et al., 2008).
   1.2. Pentobarbitone induced sleeping time- Pentobarbitone is metabolized by liver. When liver architecture is disturbed, the pentobarbitone does not get metabolized and sleeping time increases in mice, and rats (Janbaz et al., 2004).
1.3. Bromosulphthaline clearance test- Liver clears bromosulphthalein (BSP) dye from the blood. The abnormal function of liver increases the retention of BSP in blood (Woodman and Evants, 1996).

2. Biochemical analysis of blood plasma and serum (Reichling and Kaplan, 1988, Sallie et al., 1991)

   a. Aspartate transaminase
   b. Alanine transaminase
   c. Alkaline phosphatase
   d. Serum bilirubin
   e. Total proteins

Aspartate transaminase (AST or SGOT) and alanine transaminase (ALT or SGPT) are sensitive markers of hepatocellular injury. Injury to liver cells results in leakage of these enzymes into the circulation and serum levels of these enzymes increases.

   a. AST is a mitochondrial enzyme present in the tissues of liver, kidney, heart, and skeletal muscles. It reversibly catalyses transfer of amino group from aspartate to α-ketoglutarate. Its normal serum level is 5-40 IU/L.

   \[ \text{L-Aspartate} + \text{L-} \alpha \text{-oxoglutarate} \xrightarrow{\text{AST (GOT)}} \text{L-oxaloacetate} + \text{L-glutamate} \]

   Levels of AST increases 10 to 200-fold in patients with acute hepatic necrosis, CCl₄, viral hepatitis, and drug induced poisoning.

   b. ALT is a cytosolic enzyme primarily present in the liver and reversibly catalyses amino group transfer from alanine to α-ketoglutarate. Its normal serum level is 7-56 IU/L in human.

   \[ \text{L-Alanine} + \alpha \text{-ketoglutarate} \xrightarrow{\text{ALT (GPT)}} \text{Pyruvate} + \text{Glutamic acid} \]

   ALT levels increases to very high levels in patients of viral hepatitis and hepatic necrosis. The increase varies from 10 to 200 fold higher in patients of post hepatic jaundice, intrahepatic cholestasis while below 10 fold in patients of alcoholic hepatitis, metastatic carcinoma and cirrhosis.
c. Alkaline phosphatase- Serum alkaline phosphatase is produced in many tissues, especially in bone, liver, intestine, and placenta. It is excreted in the bile. In the absence of pregnancy and bone disease, an elevated serum alkaline phosphatase level mostly reflects hepatobiliary disease. The elevation ALP levels in hepatic disorders may be due to abnormal hepatic excretion or increased production by hepatic parenchymal cells. Its normal value in adult is 30-120 IU/L. Principle involved in estimation of alkaline phosphatase:

\[
P\text{-nitrophenylphosphate} + \text{H}_2\text{O} \xrightarrow{\text{Alkaline medium}} \text{Phosphate ion} + P\text{-nitrophenol}
\]

ALP hydrolyses substrate \( P\text{-nitrophenylphosphate} \) with the formation of \( P\text{-nitrophenol} \) and liberation of phosphate ion. (Kind and King, 1954)

d. Serum Bilirubin- Bilirubin is a metabolic product of heme (porphyrin ring). Normal value of conjugated bilirubin is 0.25 mg/dl in the blood of an adult. Its level rises in diseases of hepatocytes, obstruction to biliary excretion, increase in hemolysis and defects of hepatic uptake.

e. Serum Protein- Liver cells is the main site for synthesis of plasma proteins namely albumin, fibrinogen, prothrombin, alpha-L-antitrypsin, transferrin, alpha foetoproteins, acute phase reactant proteins etc. The blood levels of these plasma proteins are decreased in extensive liver damage (Harsh, 2000).

**Antioxidant levels in liver**

Our body is protected by various endogenous antioxidants, which acts and scavenges free radicals generated in various biological processes. These free radicals include lipid peroxides, reactive oxygen species (ROS), reactive nitrogen species (RNS). These free radicals are generated by the biochemical process inside mitochondria and cytosols. In certain condition like in toxicities of xenobiotics or in disease state, the formation of free radicals increases and starts damaging cellular and cytosolic membranes.

1. Glutathione
2. Lipid peroxidation
3. Superoxide dismutase
4. Catalase
1. **Glutathione (GSH)**

GSH, a tripeptide of glycine, glutamic acid, and cysteine is a naturally occurring antioxidant. It neutralizes free radicals and ROS, and maintains exogenous antioxidants such as vitamins C and E reduced (active) forms. It is a substrate in both conjugation reactions and reduction reactions that are catalyze by glutathione S-transferase enzymes in cytosol, microsomes, and mitochondria. It is also capable of non-enzymatic conjugation with some chemicals like paracetamol. Glutathione prevents oxidative stress in most cells by trapping free radicals that can damage DNA and RNA. Therefore, its level is critically importance in tissue injury caused by toxic substances.

2. **Lipid peroxidation**

Lipid peroxidation is one of the most widely used indicators of free radical formation. Oxidative deterioration of lipids such as various unsaturated fatty acids, cholesterol, phospholipids, glycolipids of membrane, results in formation of lipid hydroperoxides. These are formed in enzymatic or non-enzymatic reactions involving free radical. Lipid peroxides are unstable and decompose to form a complex series of compounds, like reactive carbonyl compounds, such as malondialdehyde (MDA) and 4-hydroxynonenal. MDA is a key marker for estimation of lipid peroxidation.

3. **Catalase**

Catalase catalyses hydrogen peroxide formed by superoxide dismutase and other processes into water and molecular oxygen.

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2 \]

4. **Superoxide dismutase (SOD)**

SOD is a chief cellular defense enzyme present in most of the cell, which dismutates superoxide radical to hydrogen peroxide and oxygen. Dismutation is a reaction in which a single reactant, converted into two different products.

\[ 2\text{O}_2 + 2\text{H}^+ \xrightarrow{\text{Superoxide dismutase}} \text{O}_2 + \text{H}_2\text{O}_2 \]
Carrier systems for liver regenerator

Liposome

Liposomes are micro-particulate or colloidal carriers system, usually 0.025-5.0 µm in diameter. It is composed of biodegradable, biocompatible components and provides a unique opportunity to deliver pharmaceuticals into the cells or even inside individual cellular compartments. It forms spontaneously when the lipids are hydrated in aqueous media (Bangham and Horne, 1964) at transition temperature. They are composed of natural and/or synthetic lipids (phospholipids and sphingolipids), and may contain other bilayer constituents such as cholesterol and hydrophilic polymer lipids.

**Structural components:**

1) Phospholipids- Phospholipid forms a membrane in reversible manner at phase transition temperature. The hydrocarbon chain of the phospholipid undergoes a transition from gel form to liquid crystalline state.

   E.g. of Glycerol containing phospholipids
   - Phosphatidyl choline (Lecithin) - PC
   - Phosphatidyl serine (PS)
   - Phosphatidyl ethanolamine (cephalin) (PE)
   - Phosphatidyl inositol (PI)

2) Sphingolipids: sphingosine or a related base.
   E.g.- Glycosphingolipids, Sphingomyelin etc.

3) Cholesterol- included in liposomes for
   - Decreasing the fluidity of the bilayer and stabilizing the membrane
   - Decreasing permeability of bilayer

4) Synthetic Phospholipids- Includes saturated and unsaturated phospholipids
   - Saturated phospholipids E.g. Distearoyl phosphatidyl choline (DSPC), Dipalmitoyl phosphatidyl choline (DPPC), Dipalmitoyl phosphatidyl ethanolamine (DPPE) etc.
     - Unsaturated phospholipids- E.g. Dioleoyl phosphatidyl glycerol (DOPG), Dioleoyl phophatidyl choline (DOPC) etc.

![Figure 2.2. Structure of Phosphatidyl choline (Süleymanoglu)](image-url)
5) Cationic lipids- E.g. DODAB/C-Dioctadecyl dimethyl ammonium bromide or chloride

Other substances: Stearyl amine and dicetyl phosphate as charge imparting agent

Compositions of liposomes determine interaction with blood and tissues. They decide the net physicochemical properties of liposomes namely membrane fluidity, charge density, steric hindrance permeability. They are found to be useful carriers for both hydrophilic and hydrophobic drugs. These drug delivery systems employed for the delivery of drugs with varying lipophilicities like water-soluble drug will be encapsulated within the aqueous compartment; lipophilic drug is usually bound to the lipid bi-layer or dissolved in the lipid phase (Fig. 1).

Figure 2.3. Distribution of drug in the liposome (adapted from image by Dr. Kosi Gramatikoff from Wikipedia "liposome")
Types of liposomes

Liposomes can be classified in various types based on their composition, delivery of drug to cell, size, and number of bilayer (Sharma and Sharma, 1997).

Based on composition of bilayer and drug delivery of drug to cell, liposomes are classified into five types namely:

a) Conventional liposomes (CL)
b) Cationic liposomes (Highly interactive)
c) pH-sensitive liposomes
d) Long-circulating (non-reactive stearically stabilized)
e) Immunoliposome

Based on size and number of bilayers, liposomes are classified into three types namely:

a. Small unilamellar vesicles (SUV) - size ≤0.1µm; single bilayer; prepared by size reduction of MUV, or LUV; less targeted to RES.
b. Large unilamellar vesicles (LUV) - size>0.1µm; single bilayer; prepared by detergent dialysis, reverse phase evaporation or active loading method, ether injection; targeted to RES.
c. Multilamellar vesicles (MLV) - size>0.1µm; multilayer; prepared by thin film hydration; targeted to RES.

Figure 2.4. Types of liposomes (Sharma and Sharma, 1997)
Conventional liposomes possess low stability against shear forces, osmotic stress, and extreme pHs. They are degraded due to opsonisation followed by uptake from mononuclear phagocytic system mainly in liver (Needham et al., 1992, Sadzuka et al., 2003). Research on clodronate liposome suggest that liposomal carrier systems selectively target macrophages (Rooijen and Sanders, 1994). Thus, liposomal formulation of hepatoprotectant can be beneficial in preventing and treatment of the inflammatory status of liver in liver disorders.

![Opsonization of liposomes by the RES](image)

**Figure 2.5. Opsonization of liposomes by the RES**

Long circulation of liposomes in *in vivo* system has been achieved by surface modification of liposomes with the inert, biocompatible polymers like polyethylene glycol (PEG) (Momekova et al., 2007). Thus, prolonged circulation of liposomes can be obtained by repulsive barrier around the liposomes, which reduces the interaction of liposomes with blood components. It increases circulation time of liposomes in the blood. These steric stabilizers have molecular weights ranging from 350 to 5000 da.

Charged liposome showed a difference in distribution pattern in plasma and liver. Positive charged liposomes showed preferably higher concentration in plasma compared to negative charged liposomes that maintain higher concentration in liver (Yu and Lin, 1997).
Many anticancer drugs are available in market as liposomes e.g. Doxil™- Doxorubicin Liposomes (LCL) from Sequus Pharmaceuticals for Kaposi sarcoma, Ambisome™- Amphotericin B Liposomes (CL) from NeXstar Pharmaceutical Inc., DaunoX™- Daunorubicin liposome (LCL) NeXstar Pharmaceutical Inc. and Amphocil™- Amphotericin B lipid complex from Sequus Pharmaceutical Inc. etc. (Sharma and Sharma, 1997).

Different methods of preparation (Mozafari, 2005):
The methods of liposomal preparation can be classified into three main groups

1. Mechanical methods

A. Film method

B. Ultrasonication method


A. Reverse-phase evaporation

B. Ether vaporisation method

3. Methods based on size transformation or fusion of preformed vesicles.

A. Freeze-thaw extrusion method

B. Thin film hydration method (dehydration-rehydration method)

Thin film hydration method (dehydration-rehydration method)

In the present study, thin film hydration method was followed which is an advantageous method as it includes solubilisation of lipids in organic phase. The organic phase is removed in such a way that a thin lipid film forms. The film is then hydrated and the hydrated lipid sheets detach during agitation and self-close to form large, multilamellar vesicles (LMV). Size reduction of these particles requires energy input, which is provided in the form of sonic energy (sonication) or mechanical energy (extrusion). Drugs can be added either during film formation or during hydration step.
Phytosomes (Choubey, 2011, Patel et al., 2009)

Phytosomes refers to advanced forms of herbal formulations containing the bioactive phytoconstituents of herbal extracts in complex with lipid. The term “phyto” stands for plant while “some” means cell-like. Bioactive constituents of phytomedicines such as flavonoids, glycosides, terpenoids are water-soluble compounds. Because of lipophilic outer layer of phytosomes, these phyto-constituent shows better absorption and results in better bioavailability than the conventional dosage form. A patented process produced this carrier system where they found the standardized plant extract or its constituents are bound to phospholipids, mainly phosphatidylcholine. The flavonoid of plant extracts anchored through chemical bonds to the polar choline head of the phospholipids. These phyto-phospholipid complex (phytosome) exhibited better pharmacokinetic and pharmacodynamic profile than conventional herbal extracts. Since then this technology has been effectively used to enhance the bioavailability of many popular herbal extracts namely milk thistle (Kidd, 2009), grape seed (Kidd, 2009), green tea (Kidd, 2009), ginkgo biloba (Semalty et al., 2010), ginseng (Sharma and Roy, 2010) etc.

Differences between phytosome and liposome (Choubey, 2011, Patel et al., 2009)

Phytosomes and liposomes are formed by mixing a drug with phosphatidyl choline in definite ratio under specific conditions.

Table 2.1. Difference between liposome, phytosome and current formulation

<table>
<thead>
<tr>
<th>Liposome</th>
<th>Phytosome</th>
<th>Current formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl choline and cholesterol forms a membranous structure enclosing the drug</td>
<td>Polar head of Phosphatidylcholine forms chemical bond with drug</td>
<td>Phosphatidyl choline and drug form chemical bond supported by cholesterol in making a vesicle like structure.</td>
</tr>
<tr>
<td>Like a physical mixture of excipient and drug</td>
<td>Like chemical mixture of excipient and drug</td>
<td>It is chemical mixture like phytosome.</td>
</tr>
</tbody>
</table>
Vesicle like appearance | After hydration form vesicle | Vesicle like appearance
---|---|---
More than 1:1 or 2:1 mixture of phosphatidyl choline and drug. Ratio depends on entrapment efficiency | 1:1 or 2:1 mixture of phosphatidyl choline and drug | More than 1:1 or 2:1 mixture of phosphatidyl choline and drug. Ratio depends on entrapment efficiency

**Method development for analysis**

A precise and specific reverse phase-high performance liquid chromatographic method is required for development of the quantification of silymarin. Silybin is used as marker for this purpose. Below a list of methods are mentioned which are used for estimation used for silymarin in various studies. In the present study, a minor modification has been applied in the chromatographic separation. It is carried out in RP-C18 column and mobile phase was Methanol:Water (50:50), pH 3.5 adjusted with glacial acetic acid at flow rate 1mL/min. λmax was 286 nm. The calibration curve was linear over the concentration range of 2µg to 50µg/mL with coefficient of determination (r² >0.999). The lower limit of detection was 0.05 µg/mL and lower limit of quantification was 0.2 µg/mL.

**Table 2.2. Review of chromatographic conditions of silymarin**

<table>
<thead>
<tr>
<th>Author</th>
<th>Method</th>
<th>Column</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Kvasnička et al., 2003 (Kvasnička et al., 2003)</td>
<td>Analytical method</td>
<td>Purospher RP18 (150×4 mm, 5 μm)</td>
<td>Isocratic elution- 85% phosphoric acid–methanol–water:: 0.5:46:64, v/v ;flow-rate 1 ml/min</td>
</tr>
<tr>
<td>2 Ding et al., 2001 (Ding et al., 2001)</td>
<td>Analytical method</td>
<td>Shim-pack VP-ODS (150×4.6 mm, 5μm) and guard column</td>
<td>Gradient flow-methanol (A) and aqueous dioxane (B) (90% water+10% dioxane) with gradient elution, flow rate 1.5 ml/min</td>
</tr>
<tr>
<td>3 Yan-yu et al., 2006 (Yan-yu et al., 2006)</td>
<td>Bioanalytical, beagle dog</td>
<td>µBondapak C 18 column (150 mm × 4.6 mm, 5 μm)</td>
<td>methanol:double distilled water:0.05M KH₂PO₄:: 60:40:5, pH 4 flow rate 1 ml/min</td>
</tr>
<tr>
<td></td>
<td>Authors, Year</td>
<td>Type</td>
<td>Column Details</td>
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<tr>
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</tr>
<tr>
<td>4</td>
<td>Li et al., 2006</td>
<td>Bioanalytical, Human Volunteer</td>
<td>C 18 column (250 mm × 4.6 mm, 5 μm) with guard column</td>
</tr>
<tr>
<td>5</td>
<td>Wu et al., 2006</td>
<td>Bioanalytical, Rabbit Plasma</td>
<td>C18 column (250 mm×4.6 m, 5 μm)</td>
</tr>
<tr>
<td>6</td>
<td>Usman et al., 2012</td>
<td>Bioanalytical, Human Plasma</td>
<td>C18 column (200 mm×4.6 mm, 5 μm)</td>
</tr>
<tr>
<td>7</td>
<td>Jia et al., 2010</td>
<td>Bioanalytical, Rabbit Plasma and Tissue</td>
<td>C18 column (150 mm×4.60 mm, 5 μm)</td>
</tr>
<tr>
<td>8</td>
<td>Wei et al., 2012</td>
<td>Bioanalytical, Rat</td>
<td>C18 column (250mm×4.6mm, 5 μm)</td>
</tr>
<tr>
<td>9</td>
<td>Campodonico et al., 2001</td>
<td>Analytical</td>
<td>C-18 Cartridge (250 ×4 mm, 5 μm)</td>
</tr>
<tr>
<td>10</td>
<td>Liu et al., 2007</td>
<td>Analytical</td>
<td>C18 column (200 mm × 4.6 mm, 5 μm)</td>
</tr>
</tbody>
</table>


Bioanalysis is a method of the quantitative determination of drugs and their metabolites in biological fluids. It plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies (Shah et al., 2000).
Chapter II - Literature Review

The process of specific bioanalytical method development can be divided into (1) reference standard selection and initial method development, (2) bioanalytical method development and establishment of assay procedure, and (3) application of validated bioanalytical method to routine drug analysis using acceptance criteria for the analytical run and/or batch. The most widely employed bioanalytical techniques include conventional chromatographic-based method (such as GC and HPLC), mass spectrometry-based method (such as GC-MS and LC-MS, LC-MS/MS), and ligand-based assays (such as radioimmunoassay [RIA] and enzyme-linked immunosorbent assay [ELISA]). Most of the part of these techniques viz., principles, procedures, and requirements for quantitative bioanalytical method validation are almost common. The parameters essential for acceptability of the performance of a bioanalytical method are accuracy, precision, selectivity, sensitivity, reproducibility, and stability (Shah et al., 2000).

Bioanalytical method validation is a process which includes procedures that ensure reliability and reproducibility of a particular method used for quantitative measurement of analyte in a given biological matrix, such as blood, plasma, serum, or urine, depending on its intended use (Reid and Wilson, 1990).

**Scheme for the development and validation of new HPLC method for bioanalysis:**

Stage I - Initial method development
   
   A. HPLC method development
   B. Sample preparation

Stage II - Validation

Stage III - Use of the validation method in pharmacokinetic and other studies namely bioequivalence studies, toxicokinetic, drug interaction.

**Stage I- Initial method development**

**A. HPLC method development**

It involves optimization of chromatographic separation using the knowledge of physicochemical properties of drug substance (structure, solubility, pKa, logP, logD etc) and involves the
selection of column, ionic strength and pH of buffer, flow rate, column oven temperature, injection volume, auto sampler temperature. The selection of suitable internal standard (IS) is one of the important steps.

IS selection requiring consideration of certain point, which include:

- Resolved peak with no interference with analyte and its metabolite
- Elute closer to the compound of interest
- Equivalent properties to the analyte of interest in processes like pretreatment, derivatisation etc.
- Produce a peak area or peak height in a ratio of about unity with the analyte of interest
- Absent in the tested sample.
- Stable and inert with sample components, column packing and mobile phase and,
- Available in high purity

B. **Bioanalytical sample preparation**

Sample preparation involves cleaning of sample before analysis and/or to concentrate analyte of interest for detection. Biological samples like plasma cannot be injected directly into HPLC column without sample preparation. Sample pretreatment is required for achieving sufficient sensitivity and selectivity, whereas the time should be kept minimum during the process.

**Importance of sample preparation**

1. Remove unwanted matrix components, which interfere in analysis process
2. Concentrate an analyte to improve limit of detection.
3. Change the environment of analyte from aqueous solvent to organic solvent
4. Selective isolation analyte for better resolution in chromatogram
5. Removal of material which can interfere in column condition or detector sensitivity
6. To achieve proper dilution
7. Solubilization of compounds for injection
8. Stabilization of analyte to avoid hydrolytic or enzymatic degradation
Removal of matrix components
Matrix component includes various proteins, endogenous macromolecules, small molecules, salts, and metabolic byproducts. Injection of these materials causes rapid deterioration in the separation performance of the chromatographic column, clogged frits or lines, impaired selectivity of the particles of column due to irreversible adsorption of proteins and detector fouling resulting in reduced system performance. These conditions may require system maintenance for further study.

Extraction of analyte
Wide ranges of techniques are available for extraction analyte from the matrix.

1. Protein precipitation
2. Liquid–liquid extraction
3. Solid phase extraction
4. Filtration
5. Dilution followed by injection
6. Protein removal by equilibrium dialysis or ultra-filtration
7. Turbulent flow chromatography
8. Monolithic columns
9. Immunoaffinity extraction
10. Combination of the above

Commonly used techniques

1. Protein precipitation

It is often used as the initial sample preparation scheme. one part of sample matrix volume is vortexes with 3-4 parts (volume) of organic solvent or other precipitating agent, followed by centrifugation, and/or filtration to isolate or remove the precipitated protein mass.

1.1. Acids used as precipitating agents
A protein has minimum viscosity at its isoelectric point and can be coagulated more easily at this point. Thus, acids such as trichloroacetic acid (TCA; 10%, w/v), perchloric acid (6%, w/v),
metaphosphoric acid (5%, w/v) etc. are used to protonate the basic sites on the protein, which changes its conformation by forming insoluble salts at a pH below their isoelectric point.

1.2. Organic solvents used as precipitating agents

Organic solvents lower the dielectric constant of protein solution and increase the protein – protein interaction. Solvents such as methanol, ethanol, acetonitrile and acetone are reported to be slightly less effective than acids due its mild protein precipitation. However, organic solvents are preferred in bioanalysis over acids because they avoid analyte degradation.

1.3. Other protein precipitating agents

Hydrated salts ions (e.g. saturated aqueous ammonium sulfate), heavy metal salts of zinc and copper are other precipitating agent.

2. Liquid-liquid extraction

It is also known as solvent extraction and partitioning. In this method, two different immiscible liquids are used based on the relative solubility of analyte (usually water and an organic solvent). Analyte is extracted from one liquid phase into another liquid phase. Aqueous sample solution (e.g., plasma) containing analytes is mixed with an internal standard. To this solution, buffer of known pH is added (a strongly acidic or basic solution for adjusting the pH) to maintain the analytes in unionized (unchanged) state. The resulting solution is vigorously mixed with several ratios (volumes) of a water immiscible organic solvent or mixtures of two or more solvents [e.g., n-hexane, dichloromethane, methyl tert-butyl ether (MTBE) or ethyl acetate]. Initially analytes distribute between two liquid phases (aqueous and organic) and later partition preferentially into the organic phase due to unionized nature.

3. Solid-phase extraction

In this method, an analyte present in a liquid phase selectively adsorbed onto the surface of that solid phase containing sorbent particles within a packed bed or a disk by chemical attraction. Entire analyte does not adsorb and pass through the sorbent particle bed to waste. The wash solution is passed through the sorbent bed to remove adsorbed endogenous contaminants from the sample matrix. A selective elution step is performed in which the analytes partition away from the solid support into another solvent in which there is a greater affinity than for the sorbent bed.
Stage II - Validation (Shah et al., 2000)

Validation demonstrates the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix (blood, plasma, urine, saliva, tissue).

Types of Method Validation

1. Full Validation

A full validation is required when a method is developed and implemented for the first time or in case of new drug, or when metabolites are added to an existing assay for quantification.

2. Partial Validation

Partial validations are modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation.

3. Cross-Validation

Validation parameters of two or more bioanalytical methods used within same study or different studies are compared in cross validation. For the cross validation, the same set of QC sample should be analysed by both analytical method. The difference between the two measurements should not exceed 15%.

Parameters required for the bioanalytical method validation as per the FDA guidelines, 2001 are system suitability, selectivity, sensitivity, linearity, carry over check, accuracy, precision, extraction efficiency (recovery), dilution integrity, re-injection reproducibility, ruggedness, stability.