2. REVIEW OF LITERATURE

2.1. Literature review of Commelina clavata Clarke plant

Botanical Name : Commelina clavata clarke

Synonyms : Commelina salicifolia, Commelina thwaitesii, Commelina diffusa (invalid)

Common Names

Bengali : Panikanchira
Hindi : Jalpipari
Manipur : Wangden
Sanskrit : jalapippali, langulu

Taxonomic classification

Kingdom : Plantage
Order : Commelinales
Family : Commelinaceae (Dayflower family)
Subfamily : Commelinoideae
Genus : Commelina
Species : Clav
Figure: 2.1 Commelina clavata Clarke
Geographical distribution

*Commelina clavata* is a perennial herb with thick, non tuberous roots. Shoots are rising to climbing, rooting on contact with ground. They grow up to 1 meter long. Leaves are narrowly lanceolate shaped to elliptic and 2.5 to 10 cm long and 0.4 to 1.5 cm wide with pointed or long pointed tip. Flowers are borne in 2-flowered clusters. Flowers are sky-blue, about 1.5 cm across, with sepals 3 mm long. Paired petals are 7 mm wide, sky-blue. The third petal is 5 mm wide. The family is important for its ornaments as day flower (*commelina*). In South Africa the young shoots and leaves of *Commelina clavata* are edible. *Commelina* is a genus of approximately 170 species, commonly called dayflowers due to the short lives of their flowers. They are less often known as widow’s tears. It is the largest genus of its family Commelinaceae. The Asiatic dayflower (*Commelina communis*) is probably the best known species in the West. It is a common weed in parts of Europe and throughout eastern North America. Several species, such as *Commelina benghalensis*, are eaten as a leaf vegetable in Southeast Asia and Africa.

There is no scientific evidence on the research work on *Commelina clavata*, for our current research, we have reviewed various research articles related to the similar species of same genus.

The potential of the weed *Commelina diffusa* L., as a fodder crop for ruminants has been studied by (et al., year). This study emphasizes that the species was evaluated in terms of its chemical composition through the *in sacco* technique and its rumen
degradation characteristics. From this study it was concluded that, in nutritional point of view, *C. diffusa* compares well with many commonly used fodder crops and could be used as protein source for ruminants on smallholder farms\textsuperscript{45}.

Antioxidant and α-glucosidase-inhibitory activity of *Commelina communis* has been studied by (Makio Shibano et al.,)\textsuperscript{46}. The extracts and powder of *communis* L *Commelina* herb are important food materials for prophylaxis against type 2 diabetes. This is due to the presence of constituents namely 1-deoxynojirimycin (DNJ) and \((2R, 3R, 4R, 5R)\) 2,5-bis (hydroxymethyl)-3,4-dihydroxypyrrolidine (DMDP), potent α-glucosidase inhibitors. Eleven flavonoid glycosides as antioxidants, isouercitrin, isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-β-D-glucoside, glucoluteolin, chrysoriol-7-O-β-D-glucoside, orientin, vitexin, isoorientin, isovitexin, swertisin, and flavocommelin, were identified from the aerial parts of *C. communis*. Their antioxidant activities were measured using in vitro assays employing the 1,1-diphenyl-2-picrylhydrazyl radical- and superoxide radical-scavenging assays\textsuperscript{47}.

**Uses**

Water accumulated at the base of the bracts collected and administered for eye pain.
2.2. Literature review of Kigelia africana (Lam.) Benth.

**Botanical Name:** *Kigelia africana* (Lam.) Benth.

**Synonym:** *Kigelia pinnata*

**Common Names:** sausage tree (Eng.); worsboom (Afr.); umVunguta, umFongothi (Zulu); Modukguhlu (North Sotho); Muvevha (Venda)

**Taxonomic classification:**

- **Kingdom:** Plantae
- **Phylum:** Angiosperms
- **Class:** Eudicots
- **Order:** Lamiales
- **Tribe:** Coleeeae
- **Subclass:** Asterids
- **Family:** Bignoniaceae
- **Genus:** Kigelia
**Figure: 2.2. Kigelia Africana benth**

**Description**

The short, squat trunk has light brown, sometimes flaky bark and supports a dense rounded to spreading crown (18 m high, 20 m wide) of leathery, slightly glossy foliage (deciduous). The huge, grey-brown fruits, 800 x 120 mm. hang from long stalks, from December (summer) to June (winter) and weigh anything up to 9 kg.

**Natural distribution**

The tree is found on riverbanks, where it may reach 20 m, along streams and on floodplains, also in open woodland, from KwaZulu-Natal to Tanzania.
Chemical Constituents:

The roots, the wood and the leaves have been investigated chemically. They contain naphthoquinones, dihydroisocumarines, flavonoids and aldehydic iridoids. Among the naphthoquinones kigelinole, isokigelinole, pinnatal and isopinnatal were isolated. From the root and its bark the usual plant substances stigmasterol, β-sitisterol, ferulic acid, the naphthoquinones lapachol, 6-methoxymellein and two new phenolic compounds could be isolated.

Kigelin is the main component of the plant having mp 144 degrees C, molecular formula C₁₂H₁₄. 6-methoxymellein is the minor one having mp 76-77 degrees C, molecular formula C₁₁H₁₂O₄.

In a special investigation the attributes of two cyclopenta-c-pyran aldehydes were determined. They are Sonovoburtinal, a yellow compound, subliming at ambient temperature, molecular formula C₉H₆O₂ and Pinnatal. Pinnatal, a phenolic substance with aromatic protons. The molecular formula is C₂₀H₁₈O₅.

Biogenetically pinnatal is probably formed by cyclisation of geranylquinone. The root bark and stem bark from plants collected in Zimbabwe were successively extracted in a soxhlet apparatus with different solvents. The isolation with thick layer chromatography (Kieselgel PF₂₅₄, 0.75mm) afford four naphthoquinones such as Kigelinol, IsoKigelinol, Isopinnatal and 2-(1-hydroxyethyl)-
naphthol (2,3-b)furan-4,9-quinone\textsuperscript{51}. They all were assessed for their biological activity\textsuperscript{55}.

In the polar (methanolic) extract of the fruit from kigelia Africana verminoside (C\textsubscript{24}H\textsubscript{28}O\textsubscript{13}), an iridoid as a major constituent and among a series of polyphenols verbascoside could be isolated\textsuperscript{57}.

**Antimicrobial activity:**

In order to investigate the traditional use of kigelia Africana against venereal diseases and dysentery, ethanolic extracts and substances isolated from the extracts were investigated against microorganisms. It could be shown that ethanolic extracts had a pronounced inhibitory activity against B.Subtilis, E.Coli, Staphylococcus aureus, Ps. Aeruginosa and Candida albicans, against which the strongest activity was noted. A biological monitored fractions of the methanolic extracts of the roots and fruits led to the isolation of the naphthoquinones kigelinone, isopinnatal, dehydro-a-lapachone, lapachol, and the phenylpropanoids p-coumaric acid\textsuperscript{52}, and ferulic acid, as the compounds responsible for an antibacterial and antifungal activity of the root. Kigelinone and caffeic acid\textsuperscript{54} were responcible compounds in the fruits.

**Uses:**

In Malawi, roasted fruits are used to flavour beer and aid fermentation. The tough wood is used for shelving and fruit boxes,
and dugout canoes are made from the tree in Botswana and Zimbabwe. Roots are said to yield a bright yellow dye. Traditional remedies prepared from crushed, dried or fresh fruits are used to deal with ulcers, sores and syphilis - the fruit has antibacterial activity. Today, beauty products and skin ointments are prepared from fruit extracts. Fresh fruit cannot be eaten - it is said to be a strong purgative, and causes blisters in the mouth and on the skin. Green fruits are said to be poisonous. In time of scarcity, seeds are roasted and eaten.

The plant *kigelia africana* will be used as folk medicine in Africa. This is used against dysentery, venereal diseases and as a topical application on wounds and abscesses. In the Nsukka, Nigeria area the bark is used for the treatment of venereal diseases. In addition *kigelia Africana* has a reputation for the treatment of dysentery, and in contradiction to it as a purgative. For these reasons it is sold in market\textsuperscript{52}. Leaves and ground wood soaked with water and pressed through a sieve were mixed with *strophanthus gratus* seeds. The concentrated syrup is then used as a hunting poison by the gbaya in southwest of central African republic\textsuperscript{56}. In Togo the stem bark is the component of a prescription against cancer. So much work has not been done on flowers of *Kigelia africana* that’s why we are taken up this plant for work.
2.3. Literature review of *Spathodea campanulata*.

**Botanical Name:** *Spathodea campanulata*

**Common Names:** Fountain Tree, African Tulip Tree, Flame-of-the-forest, Rudra Palash, Pichkari or Nandi Flame

**Taxonomic classification:**

- **Kingdom:** Plantae
- (unranked) **: Angiosperms**
- (unranked) **: Eudicots**
- (unranked) **: Asterids**
- **Order:** Lamiales
- **Family:** Bignoniaceae
- **Tribe:** Tecomeae
- **Genus:** Spathodea
- **Species:** S. campanulata
Figure: 2.3 Spathodea campanulata
Geographical distribution\textsuperscript{58}:

It is a tree that grows between 7–25 m (23–82 ft) tall and is native to tropical Africa. This tree is planted extensively as an ornamental tree throughout the tropics and is much appreciated for its very showy reddish-orange or crimson (rarely yellow), campanulata flowers. It is an invasive species in many tropical areas, however. The flower bud is ampule-shaped and contains water. These buds are often used by children who play with its ability to squirt the water. The sap sometimes stains yellow on fingers and clothes. The open flowers are cup-shaped and hold rain and dew, making them attractive to many species of birds. In Neo tropical gardens and parks, their nectar is popular with many hummingbirds, such as the Black-throated Mango (\textit{Anthracothorax nigricollis}), the Black Jacobin (\textit{Florisuga fusca}), or the Gilded Hummingbird (\textit{Hylocharis chrysura}). The wood of the tree is soft and is used for nesting by many hole-building birds such as barbets. The generic name comes from the Ancient Greek words σπάθη (\textit{spathe}) and οἶδα (\textit{oida}) referring to the spathe-like calyx.

Chemical investigation:

Chemical investigation of flower of Spathodea campanulata by GC-MS were done by (Kumaresan et., al 2011)\textsuperscript{59}. This study was carried out to analyze the active constituents present in the flower of Spathodea campanulata. Four compounds in ethanolic extract were
identified by GC-MS analysis. Butane 1,1-diethoxy-3-methyl (35.11%) and n-Hexadecanoic acid (30.22%) were the major constituents of ethanolic extract. This is the first report of identification of active constituents from the flower of Spathodea campanulata by GC-MS.

**Antimicrobial activity:**

The antimicrobial activity of ethanol extract of leaf and flower of Spathodea campanulata done by (Mohammed Gulzar Ahmed et., al 2011)\(^6\). In ethanol extract of leaf and flower of Spathodea campanulata was investigated for antimicrobial activity at 10mg/ml concentration by using Kirby-Bauer disc diffusion method against gram positive and gram negative organisms like Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas sps, Salmonella typhimurium, Bacillus subtilis, Staphylococcus aureus, Vibrio cholera. After incubation for 24 hrs, the zone of inhibition was compared with standard antibiotics genatmycin and streptomycin (10μg/disc). From the dose dependent study it was observed that the ethanol flower extract was more potent than leaf extract. Flavonoids and tannins present in the both ethanol extract may be responsible for the antimicrobial activity.

**Antibacterial Activity of Aqueous and Alcoholic Extracts of 34Indian Medicinal Plants against Some Staphylococcus Species** was done by (Jgna , Sumitra, Turk et., al 2007)\(^6\). Thirty-four Indian medicinal plants belonging to 28 different families were screened for
potential antibacterial activity against 3 Staphylococcus species, namely Staphylococcus aureus, Staphylococcus epidermidis, and Staphylococcus subflava. Antibacterial activity of aqueous and alcoholic extracts was performed by agar disc diffusion method and agar well diffusion method. The alcoholic extracts were more active than aqueous extracts for all the plants studied. The most susceptible bacterium was S. aureus. The methanol extract of Woodfordia fruticosa showed the best antibacterial activity. The in vitro susceptibility testing of the studied staphylococcus strains was done against standard antibiotics.

**Anti-oxidant activity:**

Anti-oxidant activity of Spathodea campanulata in prevention of T-BOOH and H$_2$O$_2$ induced DNA damage by (RAJESH KOWTI et. al 2011). Reactive oxygen species (ROS) causes the oxidative damage to biological macromolecules like DNA which leads to the generation of cancer and other chronic diseases. Dietary antioxidants are known to prevent the oxidative damage. In this study, antioxidant rich dietary sources in leaves and flowers of *Spathodea campanulata* are used to prevent the DNA damage induced by t-Butyl hydroperoxide and hydrogen peroxide. The antioxidants present in the ethanol extract of leaves and flowers of *Spathodea campanulata* prevent the DNA damage very effectively. The both ethanol extracts offered protection up to 95% at 50μg concentration against t-BOOH and H$_2$O$_2$ induced DNA fragmentation in agarose gel electrophoresis.
Whereas the standard antioxidants like BHA showed 90% protection at 400µM concentration. Hence the combined effect of antioxidants present in the both ethanol extracts are very potent in prevention of ROS induced DNA damage.

*In vitro* free radical scavenging activity of leaves of *Spathodea campanulata* p. beauv was done by (Mohammed Gulzar Ahmed et.,al 2010). The present study is to investigate the antioxidant activity of ethanol extract of *Spathodea campanulata* leaves in different model systems. Ethanol extract of *Spathodea campanulata* leaves showed significant dose dependent antioxidant activity, with a direct relationship between activity and concentration of extract. The extract showed an effective free radical scavenging activity towards the nitric oxide and superoxide radicals with IC$_{50}$ values of 160 and 198µg/ml respectively. Total antioxidant capacity at 50 and 500 µg/ml of *Spathodea campanulata* extract was equivalent to 29.2 and 199.4 µg/ml of á-Tocopherol. At 500µg/ml, in nitric oxide radical and superoxide radical scavenging assay it showed maximum inhibition of 81 and 79% respectively. These results clearly indicate that leaves of *Spathodea campanulata* is an effective antioxidant and also superoxide and nitric oxide radicals scavenging activity.

*In vitro* Antioxidant and Free Radicals Scavenging Activity of Flower of *Spathodea campanulata* P. Beauv (Hareesh A.R et.,al 2010) was done by. The aim of the study is to investigate the antioxidant activity of ethanol extract of flowers of *Spathodea*
*campanulata* through TBARS, hydroxyl radical, DPPH radical scavenging assay. Ethanol extract of flowers of *Spathodea campanulata* showed significant dose dependent antioxidant activity, with a direct relationship between activity and concentration of extract. The extract showed an important free radical scavenging activity towards the lipid peroxidation inhibition, hydroxyl radical, DPPH radicals, with IC$_{50}$ values of 201, 200 and 225µg/ml respectively. At 500µg/ml lipid peroxidation inhibition, hydroxyl radical, DPPH radical scavenging assay showed maximum inhibition 82, 84 and 68% respectively. The extract showed significant activity in the entire assay when compared to the standard antioxidants. These results clearly indicate that the ethanol extract of *Spathodea campanulata* flowers is a potential antioxidant and free radicals scavenging activity.

**Anti-inflammatory and Analgesic activity:**

The anti-inflammatory and analgesic activity was done by (Emmanuel.E Ilodigwe et., al 2009)\textsuperscript{65} The analgesic and anti-inflammatory potentials of the ethanol leaf extract of *Spathodea campanulata*, a Nigerian traditional medicinal plant was studied using cold, thermal and chemical-induced pain models, and carrageenan-induced acute inflammation in rats. The acute toxicity and the phytochemical constituents of the extract were also determined. The results showed that the extract (250-1000 mg/kg) significantly (P<0.05) and dose-dependently prolonged the pain reaction times in
hot-plate and tail flick pain models, and reduced acetic acid–induced writhing. The extract demonstrated significant anti-inflammatory activity against acute inflammation induced by carrageenan. The estimated $LD_{50}$ of the extract was 4500 mg/kg. Phytochemical analysis revealed the presence of tannins, saponins, anthrax quinone glycosides and flavonoids. These findings indicate that the leaf extract of *Spathodea campanulata* has both analgesic and anti-inflammatory properties and could be beneficial in alleviating painful inflammatory conditions.

### 2.4. Review on antioxidant activity of plant extracts

#### Introduction

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals.
Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers. The free radical scavenging activity of antioxidants in foods has been substantially investigated and reported in the literature by Miller and Rigelhof et.al 66.

METHOD CONSIDERATIONS

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods. In recent years, oxygen radical absorbance capacity assays and enhanced chemiluminescence assays have been used to evaluate antioxidant activity of foods, serum and other biological fluids.
These methods require special equipment and technical skills for the analysis. The different types of methods published in the literature for the determinations of antioxidant activity of foods involve electron spin resonance (ESR) and chemiluminescence methods. These analytical methods measure the radical-scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O2), the hydroxyl radical (OH), or the peroxyl radical (ROO). The various methods used to measure antioxidant activity of food products can give varying results depending on the specific free radical being used as a reactant. There are other methods which determine the resistance of lipid or lipid emulsions to oxidation in the presence of the antioxidant being tested. The malondialdehyde (MDA) or thiobarbituric acid-reactive-substances (TBARS) \(^{58}\) assays have been used extensively since the 1950’s to estimate the peroxidation of lipids in membrane and biological systems. These methods can be time consuming because they depend on the oxidation of a substrate which is influenced by temperature, pressure, matrix etc. and may not be practical when large numbers of samples are involved. Antioxidant activity methods using free radical traps are relatively straightforward to perform. The ABTS \([2,2’- \text{ azinobis}(3-\text{ethylbenzothiazoline-6-sulfonic acid})]\) radical cation \(^{66}\) has been used to screen the relative radical-scavenging abilities of flavonoids and phenolics through their Prior et al.\(^{68}\) have used the Oxygen Radical Absorbance Capacity (ORAC) procedure to determine antioxidant
capacities of fruits and vegetables. In the ORAC method, a sample is added to the peroxyl radical generator, 2,2'-azobis(2-aminopropane)dihydrochloride (AAPH) and inhibition of the free radical action is measured\(^6\) using the fluorescent compound, B-phycoerythrin, or R phycoerythrin. Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, foods, and beverages and are usually quantified employing Folin’s reagent. Vinson et al. \(^6\) have measured phenolics in fruits and vegetables colorimetrically using the Folin-Ciocalteu reagent and determined the fruit and vegetable’s antioxidant capacity by inhibition of low density lipoprotein oxidation mediated by cupric ions. A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods.

Antioxidant activity has been expressed in various ways including the percentage of the reagent used, the oxidation inhibition rate and so on. An easier way to present antioxidant activity of foods
would be to reference a common reference standard. One common reference standard, (S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, also known as Trolox, serves this purpose.

2.5. Review on liver and its disorders:

The liver is the largest organ in the body weighing 1400-1600 gm in the males and 1200-1400 gm in the females. There are 2 main anatomical lobes—rights and left, the right being about six times the size of the left lobe. The right lobe has quadrate lobe on its inferior surface and a caudate lobe on the posterior surface. The right and left lobes are separated anteriorly by a fold of peritoneum called the falciform ligament, inferiorly by the fissure for the ligamentum teres, and posteriorly by the fissure for the ligamentum venosum.

The porta hepatis is the region on the inferior surface of the right lobe where blood vessels, lymphatics and common hepatic duct form the hilum of the liver. A firm smooth layer of connective tissue called Glisson’s capsule encloses the liver and is continuous with the connective tissue of the porta hepatis forming a sheath around the structures in the porta hepatis. The liver has a double blood supply, the portal vein brings the venous blood from the intestines and spleen and the hepatic artery coming from the coeliac axis supplies arterial blood to the liver. This dual blood supply provides sufficient protection against infarction in the liver. The portal vein and hepatic artery divide into branches to the right and left lobes in the porta. The right and left hepatic ducts also join in the porta to form the common hepatic duct. The venous drainage from the liver is into the
right and left hepatic veins which enter the inferior vena cava. Lymphatics and the nerve fibres accompany the hepatic artery into their branchings and terminate around the porta hepatis.

**Histology:**

The hepatic parenchyma is composed of numerous hexagonal or pyramidal classical lobules; each with a diameter of 0.5 to 2 nm. Each classical lobule has a central tributary from the hepatic vein and at the periphery are 4 to 5 portal tracts or triads containing branches of bile duct, portal vein and hepatic artery. Cords of hepatocytes and blood-containing sinusoids radiate from the central vein to the peripheral portal triads. The functioning lobule or liver acinus as described by Rappaport has a portal triad in the centre and is surrounded at the periphery by portions of several classical lobules. However, in most descriptions on pathology of the liver, the term lobule is used in its classical form.

The blood supply to the liver parenchyma flows from the portal triads to the central veins. Accordingly, the hepatic parenchyma of liver lobule is divided into 3 zones.

- Zone 1 or the periportal (peripheral) area is closest to the arterial and portal blood supply and hence bears the brunt of all forms of toxic injury.
- Zone 3 or the centrilobular area surrounds the central vein and is most remote from the blood supply and thus suffers from the effects of hypoxic injury.
- Zone 2 is the intermediate midzonal area.
The hepatocytes are polygonal cells with a round single nucleus and a prominent nucleolus. The liver cells have a remarkable capability to undergo mitosis and regeneration. Thus it is not uncommon to find liver cells containing more than one nuclei and having polyploidy up to octoploidy. A hepatocyte has 3 surfaces: one facing the sinusoid and space of disse, the second facing the canaliculus and the third facing neighbouring hepatocytes.

The blood-containing sinusoids between cords of hepatocytes are lined by discontinuous endothelial cells and scattered flat Kupffer cells belonging to the reticuloendothelial system.

The space of disse is the space between hepatocytes and sinusoidal lining endothelial cells. A few scattered fat storing Ito cells lie within the space of disse.

The portal triad or tract besides containing portal vein radicle, the hepatic arteriole and bile duct, has a few mononuclear cells and a little connective tissue considered to be extension of Glisson’s capsule. The portal triads are surrounded by a limiting plate of hepatocytes.

![Figure: 2.4 Anatomy of Liver](image)
The intrahepatic biliary system begins with the bile canaliculi interposed between the adjacent hepatocytes. The bile canaliculi are simply grooves between the contact surfaces of the liver cells and are covered by microvilli. These canaliculi join at the periphery of the lobule to drain eventually into terminal bile ducts or ductules (canal of Hering) which are lined by cuboidal epithelium.

**Functions of Hepatocytes**:

The liver serves both as an exocrine and an endocrine gland. The exocrine secretion of the liver is bile. The critical factor in bile formation is the secretion of bile acids and bile salts, detergents important for the emulsification of fat in the intestine. Bile also has an excretory function. Conjugates, both of endogenous materials such as bilirubin and some of xenobiotics, are secreted into bile.

In its second role, as an endocrine gland, the liver secretes almost all the major proteins of plasma with the exception of the immunoglobulins. In addition the liver plays a central role in lipid metabolism, taking up chylomicrons arriving in the blood from the intestine and re-packaging their lipid with a new group of proteins to form very low density lipoprotein (VLDL) particles which are then exported from the liver. The transport of proteins through the exocytic pathway is especially sensitive to changes in intracellular ATP. This is reflected in the accumulation of fat in the damaged liver and fall in plasma proteins and disturbances in blood clotting that follow long standing liver damage.
In addition to forming the bulk of the plasma proteins, the liver is also responsible for their recycling. The liver is also responsible for recycling old red blood cells. This task is carried out principally by Kupffer cells.

In addition to these roles the liver acts as the centre of intermediary metabolism in the body and the liver stores of sugar, in the form of glycogen. Further it is also a major site for the conversion of sugars to lipids and for conversion of amino acids to sugars and lipids. This may have marked consequences. For example in rats the metabolic demands of lactation result in a 30 percent increase in liver weight, exacerbating the toxicity of liver enlarging agents such as BHT. In addition the liver is the main, or in the case of humans almost the sole, site of de novo cholesterol synthesis. The result is that liver damage causes marked disturbances in endocrine function, for example liver cirrhosis results in feminization in men and masculinization in women.

One more important function of the liver is the metabolism and excretion of variety of hydrophobic compounds. The liver metabolises and excretes both endogenous body constituents, such as heam or steroids, nutrients and exogenously administered xenobiotics, toxins, etc. In general the physiological role of the metabolism is to prepare the compounds for excretion. The metabolic reactions are conveniently divided into two phases. Phase 1 reactions involve chemical modification of the reactant normally by oxidation whereas phase 2 reactions are biosynthetic, generally involving conjugation.
with a hydrophilic moiety such as glucuronic acid or glutathione. Under most circumstances these biphasic metabolic reactions are protective and results in the removal of potentially harmful materials from the body. However, under some circumstances, the metabolites are markedly more toxic / potent than the parent compound.

Hepatic tissue or liver is involved in the metabolic degradation of various endogenous and exogenous substances, resulting in the generation of various intermediary highly reactive species. Hence it is susceptible to the attack by highly reactive species generated during the metabolic functioning of the liver, resulting in the hepatic damage. Various types of hepatic diseases induced by xenobiotics, toxins, etc are given as below.

**Diseases of liver**\(^{72,73}\):

The liver injury may take several forms and involve the hepatocytes, vascular cells or bile ducts. The most important disease are:

- Biliary obstruction
- Metabolic lesions caused by genetic disease or exogenous substance, such as alcohol.
- Inflammation, especially caused by hepatitis virus.
- Cirrhosis
- Neoplasia (Liver tumor)

The response of the liver injury may take several forms and involve the hepatocytes, vascular cells or bile ducts. The most important diseases are:
**Biliary obstruction:**

Bile flow obstruction results in the jaundice. Lesions in the main extra hepatic bile duct, such as carcinoma, impacted bile stones, or sclerosing cholangitis typically cause obstructive jaundice. Prolonged bile duct obstruction may cause secondary biliary cirrhosis.

**Metabolic disorders:**

Metabolic disorder of the liver may be hereditary (genetic) or acquired. Representative hereditary hyperbolic rubinemias and disorders involving intermediate metabolism of lipids, carbohydrates, proteins and heavy metals.

**Congenital Metabolic Disorder:**

Congenital hyperbilirubinemia occurs in several forms. The best known congenital jaundice syndromes are Gilbert syndrome, Rotor syndrome, and Dubin – Johnson syndrome. Genetic enzyme deficiencies such as alpha–1–antitrypsin may also results in liver injury, which ultimately lead to cirrhosis.

**Acquired Metabolic Disorders:**

Metabolic disorder can be induced in liver cells by a variety of ingested substances such as toxins, drugs, foods and beverages. Alcohol produces three types of liver disease such as hepatomegaly, alcoholic hepatitis and cirrhosis.

**Viral Hepatitis:**

Acute viral hepatitis is a systemic infection manifested primarily by an acute attack on the hepatocytes. Five hepatotropic
viruses have been identified (HAV, HBV, HCV, HDV, HEV). Hepatitis A (HAV) causes acute, self-limited disease that is transmitted orally. Hepatitis B Viruses (HBV) and Hepatitis C Viruses (HCV) are transmitted by exchange of body fluids such as through blood transfusion or sexual contacts. Hepatitis D virus (HDV) is a viroid that causes inflammation only in concrete with HBV. Hepatitis E (HEV) virus is transmitted by enteric route and cause self-limited diseases.

Chronic hepatitis is an uncommon, but important, complication of HBV and combined HBV – HDV infection. The liver injury results from an inflammatory immune attack against hepatocytes.

In drug induced hepatitis, a number of drugs have been reported, with hepatotoxicity including methyldopa, nitrofurantoin, isoniazid, ketoconazole and acetaminophen.

**Cirrhosis:**

Cirrhosis is a chronic liver disease characterized by wide spread fibrosis and regenerative nodules, which diffusely replace the normal liver parenchyma. The major causes of cirrhosis are alcoholism and viral hepatitis (HBV, HCV and HDV).

**Neoplasia (Liver Tumor):**

Primarily liver tumors may originate from liver cells, from bile ductules and less often from kupffer cells and connective tissue cells of hepatic capsule and portal tracts. Hepatocellular carcinoma (malignant hepatoma) is the most common primary malignant liver
tumor. Cholangio cellular carcinoma is a malignant tumor of bile ducts.

A few compounds produce metabolites that cause liver injury in a uniform, dose dependent fashion. Injury to hepatocytes results in either directly from the disruption of intracellular functions or membrane integrity or indirectly from immune-mediated membrane damage\textsuperscript{74}.

Though modern allopathic system of medicine has evolved phenomenally, the remedy for hepatic disease still depends on herbs or herbal medicines. Many herbs and herbal medicines are being used since ages to treat hepatic diseases. However the scientific basis for such is not completely established. Therefore, it is the used to screen the various herbs and herbal products for their claimed hepatoprotective potential. Several chemical / drug induced hepatotoxicity in animals have been developed as experimental models to screen hepatoprotective potential of various drugs/chemicals/herbs.

The clinical consequences of liver disease, laboratory evaluation of liver disease, types of hepatotoxic agents, classification of hepatotoxins and mechanism of each group presented in Table: 2.1, 2.2, 2.3 and 2.4.
Table: 2.1. Clinical consequences of liver disease

<table>
<thead>
<tr>
<th>Characteristic signs</th>
<th>Hepatic dysfunction:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jaundice and cholestasis</td>
</tr>
<tr>
<td></td>
<td>Hypoalbuminemia</td>
</tr>
<tr>
<td></td>
<td>Hyperammonemia</td>
</tr>
<tr>
<td></td>
<td>Hypoglycemia</td>
</tr>
<tr>
<td></td>
<td>Fetor hepaticus</td>
</tr>
<tr>
<td></td>
<td>Palmar erythema</td>
</tr>
<tr>
<td></td>
<td>Spider angiomas</td>
</tr>
<tr>
<td></td>
<td>Hypogonadism</td>
</tr>
<tr>
<td></td>
<td>Gynecomastia</td>
</tr>
<tr>
<td></td>
<td>Weight loss</td>
</tr>
<tr>
<td></td>
<td>Muscle wasting</td>
</tr>
<tr>
<td></td>
<td>Portal hypertension from cirrhosis:</td>
</tr>
<tr>
<td></td>
<td>Ascites</td>
</tr>
<tr>
<td></td>
<td>Splenomegaly</td>
</tr>
<tr>
<td></td>
<td>Hemorrhoids</td>
</tr>
<tr>
<td></td>
<td>Caput medusae-abdominal skin.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Life-threatening complications</th>
<th>Hepatic failure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td></td>
<td>Coagulopathy</td>
</tr>
<tr>
<td></td>
<td>Hepatic encephalopathy</td>
</tr>
<tr>
<td></td>
<td>Hepatorenal syndrome</td>
</tr>
<tr>
<td></td>
<td>Portal hypertension from cirrhosis</td>
</tr>
<tr>
<td></td>
<td>Malignancy with chronic disease</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma</td>
</tr>
</tbody>
</table>

Table: 2.2. Laboratory evaluation of liver disease

<table>
<thead>
<tr>
<th>Test category</th>
<th>Serum measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte integrity</td>
<td>Cytosolic hepatocellular enzymes</td>
</tr>
<tr>
<td></td>
<td><em>Serum aspartate aminotransferase (AST)</em></td>
</tr>
<tr>
<td></td>
<td><em>Serum alanine aminotransferase (ALT)</em></td>
</tr>
<tr>
<td></td>
<td><em>Serum lactate dehydrogenase (LDH)</em></td>
</tr>
<tr>
<td>Biliary excretory function</td>
<td>Substances normally secreted in the <em>Serum bilirubin</em></td>
</tr>
<tr>
<td></td>
<td><em>Total</em>: unconjugated plus conjugated</td>
</tr>
<tr>
<td></td>
<td><em>Direct</em>: conjugated only</td>
</tr>
<tr>
<td></td>
<td>Delta: covalently linked to albumin</td>
</tr>
<tr>
<td></td>
<td><em>Urine bilirubin</em></td>
</tr>
<tr>
<td></td>
<td>Serum bile acids*</td>
</tr>
<tr>
<td></td>
<td>Plasma membrane enzymes</td>
</tr>
<tr>
<td></td>
<td><em>(from damage to bile canaliculus)</em></td>
</tr>
</tbody>
</table>
Review of Literature

| Hepatocyte function | Proteins secreted in to the blood Serums albumin+ Prothrombin time* (factors V, VII, X, prothrombin, fibrinogen) Hepatocyte metabolism Serum ammonia* Aminopyrine breath test (hepatic demethylation)+ Galactose elimination (intravenous injection)+ |

The most common tests are in italics.  
*An elevation implicated liver disease.  
+A decrease implicates liver disease.

Table: 2.3. Types of hepatotoxic agents

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INORGANIC AGENTS</strong></td>
<td>Metals and metalloids: antimony, arsenic, beryllium, bismuth, boron, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, gold, phosphorous, selenium, tellurium, thallium, zinc, hydrazine derivatives, iodides.</td>
</tr>
<tr>
<td><strong>ORGANIC AGENTS</strong></td>
<td></td>
</tr>
<tr>
<td>Natural: Plant toxins</td>
<td>Albitocin, cycasin, nutmeg, tannic acid, icterogenin, pyrrolidizines, saferole, indospicine.</td>
</tr>
<tr>
<td>Mycotoxins</td>
<td>Aflatoxins, cyclochlorotine, ethanol, luteoskyrin, griseofulvin, sporidesmin, tetracycline, and other antibiotics.</td>
</tr>
<tr>
<td>Bacterial toxins</td>
<td>Exotoxins (C. diphteria, Clostridium botulinus), endotoxins, ethionine.</td>
</tr>
<tr>
<td>Synthetic: Non-medicinal</td>
<td>Haloalkanes and haloolephins, Nitroalkanes, Chloroaromatic compounds, Nitroaromatic compounds, organic amines, Azo compounds. Phenol and derivatives, various other organic compounds.</td>
</tr>
<tr>
<td><strong>MEDICINAL AGENTS</strong></td>
<td></td>
</tr>
<tr>
<td>Category of drugs</td>
<td>Examples</td>
</tr>
<tr>
<td>Neuro</td>
<td>Hydrazine, tranylcypromine</td>
</tr>
</tbody>
</table>
Table: 2.4. Classification of hepatotoxins and mechanism of action of each Group\textsuperscript{76, 77}

<table>
<thead>
<tr>
<th>Category of agents</th>
<th>Mechanism of action</th>
<th>Histologic lesion</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrinsic Toxicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct</td>
<td>Direct Physicochemical destruction by peroxidation of hepatocytes.</td>
<td>Necrosis and/or steatosis</td>
<td>CCl\textsubscript{4}, phosphorus</td>
</tr>
<tr>
<td>Indirect cytotoxic</td>
<td>Interference with hepatocellular metabolic pathways</td>
<td>Steatosis or necrosis</td>
<td>Ethionine, ethyl alcohol, tetracycline,</td>
</tr>
<tr>
<td>Cholestatic</td>
<td>Interference with bile excretory pathways</td>
<td>Cholestasis due destruction</td>
<td>Methylene dianiline, anabolic and contraceptive steroids</td>
</tr>
<tr>
<td><strong>Host Idiosyncracy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypersensitivity</td>
<td>Drug allergy</td>
<td>Necrosis or cholestasis</td>
<td>Chlorpromazine, phenytoin, sulfonamides.</td>
</tr>
<tr>
<td>Metabolic</td>
<td>Production of hepatotoxic metabolites</td>
<td>Necrosis or cholestasis</td>
<td>Isoniazid, valproic acid</td>
</tr>
</tbody>
</table>
2.6. Methods of pharmacological evaluation of hepatoprotective plants:

In vivo Models:

A toxic dose or repeated doses of a known hepatotoxin (paracetamol, carbon tetrachloride, thioacetamide, alcohol, D-Galactosamine/lipopolysaccharide, azathioprine, tert – butyl hydroperoxide, allylalcohol, etc) are administrated, to induce liver damage in experimental animals. The test substance is administered along with, prior to and/or after the toxin treatment. If the hepatotoxicity is prevented or reduced by the pre-treatment or after toxin challenge then it is inferred that the test substance is effective\textsuperscript{21, 78-84}.

Liver damage and recovery from damage are assessed by measuring serum marker enzymes, bilirubin, histopathological changes in the liver, biochemical changes in liver (e.g.: hydroxyproline, lipid etc) and bile flow. When the liver is damaged, liver-enzymes such as glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT) and alkaline phosphatase enter into the circulation. An increase in the levels of these marker enzymes in the serum is an indication of liver damage. Other effects of induced liver damage such as reduction of prothrombin synthesis giving an extended prothrombin time and reduction in clearance of certain substances such as bromosulphthalein can be used in the evaluation of hepatoprotective plants.
The hepatoprotective effect of a drug against different hepatotoxins differs especially when the mechanism of action of toxins are different. Therefore, the efficacy of each drug has to be tested against hepatotoxins, which act by different mechanisms.

2.6.1. Mechanism of carbon tetrachloride (CCl₄) induced hepatotoxicity:

CCl₄ is a potent hepatotoxin producing centrilobular hepatic necrosis, which causes liver injury⁸⁵.

CCl₄ induces fatty liver and cell necrosis and play a significant role in inducing triacylglycerol accumulation, depletion of GSH, increased lipid peroxidation, membrane damage, and depression of protein synthesis and loss of enzyme activity. Being cytoplasmic in location the damage marker enzymes GOT, GPT and HDL are released in the serum⁸⁶.

It is now generally accepted that the hepatotoxicity of CCl₄ is the result of reductive dehalogenation, which is catalyzed by cytochrome P450 enzyme and forms the highly reactive trichloromethyl free radical. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical. The free radical can form covalent bond with sulfahydryl group, such as glutathione (GSH), protein thiol and lipids or abstracting a hydrogen atom from an unsaturated lipid. This covalent binding of free radical to cell macromolecules is considered the initial step in a chain of events,
which eventually leads to membrane lipid peroxidation, liver damage and finally cell necrosis\textsuperscript{87-91}.

CCl\textsubscript{4} is reductively converted by P450 to the trichloromethyl radical the fate of this radical is of interest. First the radical add covalently to unsaturated fatty acids, trichloromethyl fatty acids, particularly of membrane phospholipids.

\textbf{Figure: 2.5. Schematic representation of reactive mechanism of CCl\textsubscript{4} induced hepatic injury}

Recently these substituted fatty acids have been noted to be partially resistant to replace from endoplasmic reticular phospholipase A\textsubscript{2}. This seems to be result of cross linking of trichloromethyl fatty acid radical, which adds to double bond of another adjacent fatty acids (link).

\textbf{Figure: 2.6. Covalent binding to lipids}
The physiologic significance of this cross-linking on membrane structure and function may be of great importance, particularly if these phospholipids are transformed to other critical sites in the cell. Besides covalent binding to lipid, the cells can abstract an electron from unsaturated fatty acids, yielding CHCl₃ and or fatty acid radical. Either the trichloromethyl fatty acid radical or the fatty acid radical can react with oxygen to form peroxy radical, which initiates the lipid peroxidation chain reaction⁹².

2.6.2. Mechanism of Paracetamol (PCM) induced hepatotoxicity:

Paracetamol (N-acetyl-p-aminophenol) is a widely used analgesic and antipyretic drug and is safe when used in therapeutic doses. However, over dosage of Paracetamol is known to be hepatotoxic and nephrotoxic in man and in experimental animals⁹³. Paracetamol is a direct hepatotoxin i.e. intoxication is dose dependent and reproducible⁹⁴. Exposure of animals to higher doses produces centrilobular or massive hepatic necrosis followed by congestion and failure. The hepatic necrosis is associated with damage to sub cellular organelle including mitochondria. Thus the drug is used as a typical hepatotoxin to produce hepatic failure experimentally⁹⁵.

At lower doses, about 80% of ingested paracetamol is eliminated mainly as sulfate and glucoronide conjugates before oxidation and only 5% is oxidized by hepatic cytochrome P450 (CYP2E₁) to a highly reactive and toxic electrophile i.e. N-acetyl-p-benzoquinoneimine (NAPQI). After over dosage of paracetamol the
glucoronidation and sulfation routes become saturated and as a consequence, paracetamol is increasingly metabolized into NAPQI\textsuperscript{96}. Semiquinone radical, one-electron reduction metabolite of NAPQI mediates the cytotoxic effects of NAPQI. Production of these toxic semiquinone radicals is catalyzed by the microsomal cytochrome P450 reductase. These semiquinone radicals, in turn, can bind directly with cellular macromolecules to produce toxicity or alternatively, the radical can be reoxidized back to their original quinones by donating one electron to molecular oxygen under aerobic conditions. This donation of one electron then generates reduced oxygen radical species and hydroxyl radical. Both semiquinone and oxygen radical are known to be responsible for cytotoxic effects observed with quinones.

Alternatively to this toxic one-electron reduction pathway quinone compounds also can be reduced by a direct two-electron reduction pathway to non-toxic hydroquinones, either enzymatically or by quinone reduction of two molecules of GSH. Both of these direct two-electron reductions will occur without any production of the toxic semiquinone or oxygen radicals and therefore, may provide a competitive protection pathway against the toxicity caused by one-electron reduction of NAPQI\textsuperscript{97}. Also NAPQI is detoxified by glutathione (GSH) to form 3-(GSH-S-yl) acetaminophen. Paracetamol overdose saturates the nontoxic metabolic pathway, i.e. sulfation, glucuronidation, and detoxification of NAPQI by glutathione. The
reactive NAPQI may oxidize and arylate cysteiny1 thiol group, forming adducts which inhibit the function of cellular proteins. Adducts formation has been demonstrated for a selenium-binding protein, for microsomal subunit of glutamine. Other mechanism, such as oxidation of pyridine nucleotides and lipid peroxidation, may contribute to cell damage by Paracetamol overdose.96

Nevertheless at high doses of Paracetamol NAPQI can alkylate and oxidize intracellular GSH and protein thiol group, which result in the liver GSH pool depletion and the reactive intermediate reacts with other nucleophilic centers of vital molecules in liver cells leading subsequently to hepatotoxicity. Besides, Paracetamol is also shown to directly inhibit cellular proliferation, induce oxidative stress, resulting in lipid peroxidation, deplete ATP levels and alter Ca++ homeostasis; all of these changes are considered potentially fatal to the cell.95, 96
2.6.3. Mechanism of Thioacetamide Induced Hepatotoxicity:

Thioacetamide was originally used as a fungicide to protect against decay of organs\textsuperscript{98}.

It was soon recognized as a potent hepatotoxin and carcinogen in rat\textsuperscript{99}. The compound has also been reported to be toxic to kidney and thymus\textsuperscript{100}. It is also reported that chronic thioacetamide exposure produces cirrhosis in rat\textsuperscript{101}. Its long term administration causes the development of cirrhosis associated with an increased extent of lipid peroxidation. The toxicity experienced by the liver during thioacetamide poisoning results from the production of its metabolite, namely thioacetamide-5-oxide, which is a direct hepatotoxin\textsuperscript{102}. Thioacetamide is metabolised by liver CYP 450.
Review of Literature

2E, enzymes, rendering sulfone and sulfoxide derivatives which are apparently responsible for structural proteins and enzyme inactivation.

The thioacetamide-5-oxide is responsible for the change in cell permeability, increased intracellular concentration of calcium, increase in nuclear volume and enlargement of nucleoli and also inhibits mitochondrial activity which leads to cell death.

Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. Damage to liver cell causes leakage of cellular enzyme into serum.

In-vivo administration of thioacetamide to rodents results in cell death in centrilobular zones both by apoptosis and necrosis. The cellular changes induced by apoptosis occur after a cascade of cell signaling and cascade mediated events and is triggered by two major pathways: extrinsic and intrinsic pathway.

The extrinsic pathway implicates death ligands such as Fas ligand, TNFα, TRAIL and their receptors. The intrinsic pathway includes apoptotic stimuli induced by cytotoxic drugs or oxidative stress which target mitochondria. This pathway involves the release of cytochrome C from mitochondria to the cytosol, which induces apoptosome complex formation and results in protease pro-caspase-9 activation and subsequent activation of pro-caspase-3 through proteolytic cleavage visualized by the decrease of pro-form level and appearance of cleavage products. Both the pathway leads to
caspase-3 activation and cleavage of limited set of essential cellular protein, leading to cell dismantlement. In the liver the apoptosis induced by thioacetamide could result from a combination of both pathways: intrinsic apoptosis pathway by generation of oxidative stress and the extrinsic apoptosis pathway by activation of kupffer cells which can secrete TNFα.

2.6.4 Mechanism of D-Galactosamine (D-GalN) / Lipopolysaccharide (LPS) induced hepatotoxicity:

D-GalN/LPS induced hepatocellular damage, a well-established model of hepatitis takes advantage of the ability of D-GalN to potentiate the toxic effects of LPS producing fulminant hepatitis within a few hours of administration. A high dose of D-GalN causes necrosis of the liver by UTP depletion and inhibition of protein synthesis, although D-GalN is often used in combination with lipopolysaccharide or tumor necrosis factor. Accumulation of UDP-sugar nucleotides may contribute to the changes in the rough endoplasmic reticulum and to the disturbance in the protein metabolism. Further, intense galactosamination of membrane structure is thought to be responsible for loss in the activity of ionic pumps. The impairment in the calcium pump, with consequent increase in the intracellular calcium is considered to be responsible for cell death. In recent years, apart from the well documented inhibition of protein synthesis, it has been suggested that reactive oxygen species produced by activated macrophages might be the primary cause in D-GalN-induced liver damage. Liver damage
induced by D-GalN/LPS generally reflects disturbances of liver cell metabolism which lead to characteristic changes in the activities of serum enzymes. The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of membranes. In this context, we can observe a significant increase in the serum activities of AST, ALT, ALP, LDH and γ-GT which is in accordance with the earlier findings. Because the levels of these marker enzymes are proportional to the extent of damage, the activity of these enzymes can be used for diagnosis as indicators of prognosis of the disease.

The rise in serum levels of AST and ALT has been attributed to the damaged structural integrity of the liver because these are cytoplasmic in location and are released into circulation after cellular damage.\textsuperscript{81}

\textbf{2.6.5. Mechanism of azathioprine (AZP) induced hepatotoxicity:}

The AZP-induced hepatotoxicity observed 24 hours post treatment is documented by significant increments in the activities of both serum ALT and AST and confirmed by histological changes in liver of male albino rats. Most of the hepatocytes in AZP-treated rats displayed mild cellular degeneration and loss of their characteristic configuration. Moreover, the marked necrobiotic changes in the liver were mainly in the form of degenerated, vacuolated cells and karyolysis or pyknosis of nuclei. Histopathological changes in the liver included also dilatation of blood vessels, congestion in the
lobules, some hemorrhagic coagulative foci in hepatic parenchyma and infiltration of mixed inflammatory cells around the necrotic hepatocytes. In this report, the AZP-induced hepatotoxicity. Mammalian model was successfully established as reflected in the dramatic increase of the liver function indictors (ALT, AST). A single dose of AZP has been shown to increase the serum ALT and AST activities 24 hours post treatment. Interestingly, levels of the endogenous liver antioxidants such as GSH, CAT and SOD enzymes as well as the MDA showed clear association with the developed AZP-induced heptotoxicity. In hepatocytes GSH is consumed during metabolism of AZP to 6-mercaptopurine (6-MP). The mechanism of AZP toxicity to hepatocytes involves depletion of GSH leading to mitochondrial injury with profound depletion of ATP and cell death by necrosis. Lipid peroxidation is a free radical-inducible process in which membrane polyunsaturated fatty acids are oxidative degraded into a variety of products including MDA. Therefore it is conceivable that AZP-induced depletion of hepatic GSH and its associated increase of MDA originated as a result of the AZP-induced elevation of free radicals which in turn speed up lipid peroxidation and cause irreversible cell damage.

2.6.6. Mechanism of tert-Butyl alcohol (t-BHP) induced hepatotoxicity:

t-BHP is metabolized in hepatocytes by two distinct pathways. One involves cytochrome P450, leading to the formationof toxic
peroxyl and alkoxyl radicals that initiate lipid peroxidation, affect cell integrity and form covalent bonds with cellular molecules, resulting in cell death. The second toxicological pathway of t-BHP is a detoxification reaction involving glutathione peroxidase, which gives rise to t-butanol and GSSG that in turn alters Ca2+ homeostasis and increases physiological formation of ROS. GSH is widely distributed among living cells and is involved in many biological functions. It is well-established that GSH acts as an essential intracellular reducing agent for maintenance of antioxidant molecules and the thiol groups on intracellular proteins, namely de Ca2+ATPase transporter of endoplasmic reticulum. GSH is also the most important biomolecule protecting against chemically induced cytotoxicity, by participating in the elimination of reactive intermediates by conjugation and hydroperoxide reduction, or of free radicals by direct quenching. t-BHP caused a significant depletion in total glutathione and GSH contents. An important aspect of t-BHP hepatotoxicity is related with its reduction by glutathione peroxidase to the corresponding alcohol, at the expense of GSH which is converted to GSSG. Under severe exposure to t-BHP, the reduction of GSSG by glutathione reductase or the regeneration of NADPH may be insufficient, leading to GSSG accumulation. Metabolisation of t-BHP mediated by cytochrome P450 is an additional factor for the depletion of GSH, since it scavenges the resulting peroxyl and alkoxyl radicals being oxidised to GSSG. GSH depletion was not proportionally correlated with GSSG increase and these results can be due to covalent binding of GSH to some
electrophilic species generated from t-BHP metabolism or to GSH reaction with protein thiols, protecting them from oxidation. Lipid peroxidation has been recognized as a potential mechanism of cell injury83.

2.6.7. Ethyl alcohol induced hepatotoxicity:

Alcoholic liver disease continues to the most serious liver disorder throughout the world, including India, where alcohol is mainly consumed in the form of country made liquor (CML). Alteration in liver functions due to alcohol range from fatty liver to cirrhosis. After its ingestion, ethanol is readily absorbed from the gastrointestinal tract. Only 2-10% of that absorbed is eliminated through the kidneys and lungs; the rest is oxidized in the body, mainly in the liver106.

The hepatocytes contains three main pathways for ethanol metabolism, each located in a different sub cellular compartment: the alcohol dehydrogenase (ADH) pathway of the cytosol, the microsomal ethanol oxidizing system (MEOS) located in the endoplasmic reticulum and catalase located in the peroxisomes.
Alcohol dehydrogenase pathway:

A major pathway for ethanol disposition involves ADH, an enzyme that catalyzes the conversion of ethanol to acetaldehyde. In ADH–mediated oxidation of ethanol, hydrogen is transferred from the substrate to the cofactor nicotinamide adenine dinucleotide (NAD), converting it to its reduced form (NADH) and acetaldehyde is produced. The dissociation of the NADH-enzyme complex has been shown to be a rate limiting step in this reaction. As a net result, the first step in the oxidation of ethanol generates an excess of reducing equivalents in the cytosol, primarily as NADH. In normal rates when ethanol is given there is marked shift redox potential of the cytosol as measured by changes in the lactate: pyruvate ratio that leads to hyperlactacidemia because of both decreased utilization and enhanced production of lactate by the liver.
The altered redox state also impairs gluconeogenesis from amino acids and favors hypoglycemia. The increased NADH/NAD ratio raises the concentration of glycerophosphate, which favors hepatic triglyceride accumulation by trapping fatty acids. A major interaction site of ethanol in the citric acid cycle (in the mitochondria) is found to be with ketoglutarate oxidation. Moreover, the redox change associated with ethanol oxidation decreases the hepatic concentration of oxaloacetate, the availability of which controls the activity of citrate synthetase. The mitochondria will therefore use the hydrogen equivalents originating from ethanol, rather than from oxidation through the citric acid cycle of two carbon fragments derived from fatty acids. Thus, fatty acids that normally serve as the main energy source for the liver are supplanted by ethanol. Depressed fatty acid oxidation by ethanol has been demonstrated in liver slices, isolated hepatocytes, human liver biopsy tissue and \textit{in-vivo}. This change results in the deposition of fat in the liver, the first stage of alcoholic liver injury. In other experimental model, chronic alcohol consumption is associated with the progression of alcoholic liver injury beyond the fatty liver stage, affecting even protein metabolism\textsuperscript{108}. 

The capacity of acetaldehyde to cause lipid peroxidation has been demonstrated in isolated perfused livers and has been linked to acetaldehyde oxidation. In addition, the binding of acetaldehyde with cysteine, cysteine containing glutathione or both may contribute to the depression of liver glutathione, thereby reducing the scavenging of
toxic free radicals by this tripeptide. Rats given ethanol for long periods have significantly increased rates of glutathione turnover in association with increased activity of hepatic gamma-glutamyl transpeptidase. Severe glutathione reduction favours lipid peroxidation which can be prevented or impaired in vivo by the administration of methionine, a precursor of cysteine and glutathione. The increased activity of microsomal NADPH oxidase after ethanol consumption may result in enhanced superoxide and hydrogen peroxide production, thereby theoretically favouring lipid peroxidation.

In addition, it has been postulated that purine metabolism by means of xanthine oxidase may lead to the production of oxygen radicals. Another potential mechanism of cellular injury in acute alcoholic liver disease is generation of free-radicals by neutrophils. Although the pathogenesis of early alcoholic liver disease is still largely unknown, accumulating evidence suggests that endotoxins (lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α) and free radicals are involved. Ethanol increases permeability of the isolated small bowel to endotoxin and elevates circulating endotoxin. This is most likely the starting point of a pathophysiologic cascade leading to liver injury. Circulating LPS associates with LPS-binding protein (LBP) and the LPS-LBP complex binds to the CD14 receptor of Kupffer cell, the resident liver macrophages are the major population of the monocyte macrophage lineage. Recently, early alcohol induced liver injury was blocked in CD14 and TLR-4 knock out mice. The
interaction of LPC with CD14 triggers a signaling cascade and activates kuffer cells that release many potent effectors cytokines. Furthermore, in humans, it was recently shown that promoter polymorphism of the CD14 receptor gene is a risk factor for alcoholic liver disease.

Recently, a study with the continuous intragastric feeding model in mice showed that early alcohol-induced liver injury was blocked in animals lacking TNF-α in bile from rat exposed to ethanol in the Tsukamoto-French model using the spin-trapping technique and Electron Spin Resonance (ESR) spectroscopy. Free radical signals were diminished over 50% when kupffer cells were destroyed by treatment with gadolinium chloride (GdCl₃)\textsuperscript{110}.

**Microsomal ethanol oxidizing system (MEOS):**

The first indication of an interaction of ethanol with the microsomal fraction of the hepatocyte was provided by the morphologic observation that in rats, ethanol feeding results in a proliferation of the smooth endoplasmic reticulum (SER), this resembles the change seen after the administration of many xenobiotics compounds including known hepatotoxins, numerous therapeutic agents and food additives. Most of the substances that stimulate proliferation of the SER are metabolized, at least in part, in the microsomal fraction of the hepatocyte. This observation raised the possibility that, in addition to its oxidation by ADH in the cytosol, ethanol may be metabolized by the microsomes. The system required NADPH & O\textsubscript{2} and was relatively insensitive to catalase inhibition.
Furthermore, the MEOS was differentiated from catalase by its oxidase long-chain aliphatic alcohols, which are not substrate for catalase\textsuperscript{108}.

**Role of catalase:**

Catalase is a haemoprotein located in the peroxisomes of most tissues. Small amounts are also found in isolated hepatocyte microsomes. As early as 1936 Keilin and Hartee suggested that catalase may play a role in alcohol metabolism. In 1955 this was confirmed by Laser who showed that ethanol could be effectively oxidized in the presence of hydrogen peroxide and catalase.

Catalase of oxidizing ethanol *in-vitro* only in the presence of a hydrogen peroxide generating system. The reaction is limited by the rate of hydrogen peroxide generation rather than by the amount of catalase itself. The physiological rate of hydrogen peroxide production is less, suggesting that catalase could account for only 2\% of the *in-vivo* rate of ethanol oxidation\textsuperscript{111}.

**In vitro studies:**

Fresh hepatocyte preparations and primary cultured hepatocytes are used to study direct anti-hepatotoxic activity of drugs. Hepatocytes are treated with hepatotoxin and the effect of the plant drug on the same is evaluated. The activities of the transaminases released into the medium are determined. An increase in the activities in the medium indicated liver damage. Parameters
such as hepatocyte multiplication, morphology, macromolecular synthesis and oxygen consumption are determined.

**Biochemical Assays:**

Since, many toxic chemicals induce liver damage by inducing lipid peroxidation and/or oxidative damage to DNA and reduction in the levels of glutathione, assessment of antioxidant property is useful. Antioxidant property of plant drugs is studied using liver homogenates, isolated liver cell membranes, DNA etc. In the process leading to cirrhosis, accumulation of connective tissue and parenchymal regeneration are competing events. Therefore, the search for agents to prevent liver cirrhosis is also focused on inhibitors of excessive connective tissue formation in the liver. Fibro-suppressive effects by inhibitors of protein hydroxylation can be screened\textsuperscript{112}.

**2.7. Free radical generated radical hepatotoxicity:**

The presence of free radicals in biological materials was discovered less than 50 years ago. Denham Harman hypothesized that oxygen radical may be formed as by-products of enzymatic reaction in vivo. He described that free radical may account for gross cellular damage, mutagenesis, and cancer and last but not least, the degenerative process of biological aging.

**2.7.1. Major Types of Free Radicals:**

**Reactive oxygen species (ROS):**
The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen (O₂).

This process is medicated by enzymes such as NAD (P) H oxidases xanthine oxidase. Superoxide dismutase (SOD) converts superoxide enzymatically into hydrogen peroxide. In the presence of reduced transition metals (ex. Ferrous or cuprous ions), hydrogen peroxide can be converted into highly reactive hydroxyl radical (OH⁻). Alternatively hydrogen peroxide may be converted into water by the enzyme catalase or glutathione peroxidase. In glutathione peroxidase reaction glutathione is oxidized to glutathione by glutathione reductase in an NADPH-consuming process.

**Figure: 2.9. Pathway of reactive oxygen species (ROS) production and clearance**

**Reactive nitrogen species (RNS):**
The nitrogen radical (NO•) is produced in higher organisms by the oxidation of one of the terminal guanidonitrogen atoms of L-arginine. This process is catalysed by the enzyme NOS. Depending on the microenvironment, NO can be converted to various other reactive nitrogen species (RNS) such as nitrogonium cation (NO⁺), nitroxyl anion (NO⁻) or peroxynitrite (ONOO⁻)\textsuperscript{113}.

**Oxygen free radical (OFR):**

These are not the only important free radicals in biochemistry, although they are often the initial species formed. Other free radicals of importance are a wide range of carbon centered radicals that arise from the attack of an oxidizing radical on a biomolecule such as lipid, nucleic acid, proteins, carbohydrates. These react with oxygen very rapidly to form the corresponding peroxy radicals. Sulphur containing free radicals such as “Thiol radicals” formed during the oxidation of glutathione\textsuperscript{32}.

**2.7.2. Free Radical Induced Cell Injury:**

ODFR (oxygen derived free radicals) are highly reactive species with, OH as one of the most reactive free radicals. In-vitro and In-vivo studies have shown that ODFR can produce chemical modification proteins, lipids, carbohydrates and nucleotides with varying cytotoxic effects\textsuperscript{114}.

Addition of electrons to molecular oxygen leads to the genesis to a series of reactive molecules collectively called reactive oxygen intermediates (ROI). These are capable of injuring tissue and be responsible for necrosis. The most significant ROI are superoxide
anion (O$_{2}^-$), perhydroxyl radical (HO$_2^{++}$), the peroxide ion (O$_2$), hydroxyl radical (OH$^+$) and the hydroxyl anion (OH$^-$).

ROI react with themselves and other molecules available within tissues to make further molecular species. These are capable of inflicting cell injury in their own right, for example,

$$\text{O}_2^{.-} + \text{H}_2\text{O}_2 \rightarrow \text{H}_2 \text{O}^+ \text{O}_2$$

This is Haber-Weiss reaction, and is accelerated by metal ions such as Fe$^{2+}$ in metalloproteins.

$$2\text{O}_2^{.-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2^+ \text{O}_2$$

This is dismutation reaction, and is accelerated by enzymed superoxide dismutase. As we have discussed earlier that ROI may cause cell injury and responsible for cell necrosis, in can be depicted form the figure below.

![Diagram of the effects of ROI on cellular processes](image-url)
2.7.3. Cell Injury at Molecular Level:

Among all major class of biomolecules like protein, lipids, carbohydrates, nucleotides etc, lipids are most susceptible towards free radical attack and this leads to lipid peroxidation.

2.7.4. Lipid Peroxidation:

Free radical specially acts on polyunsaturated fatty acids (PUFA), either directly or through the formation of reactive oxygen species.

Figure: 2.11. Formation and propagation of lipid radicals leading to lipid peroxidation

Peroxidation of membrane lipid results in the formation lipid peroxy radical (ROO•) which then produce lipid hydroperoxide (ROOH). The peroxy radical is unstable and readily undergoes decomposition, catalyzed by transition metals ions, particularly Fe^{3+},
to form additional radical products. Further, lipid peroxy radical (ROO\(^-\)), lipid radicals (R\(^-\)), lipid peroxidation may be mediated by OH\(^-\), or though the formation of an ADP-perfferly ion complex (ADP-Fe\(^{2+}\)-O\(_2\), ADP- Fe\(^{3+}\)-O\(_2\)). Superoxide (O\(^{2-}\)) may also initiate lipid peroxidation through the intermediate formation of singlet oxygen. The breakdown of lipid hydroperoxidases (ROOH) may also liberate singlet oxygen, which will react with other unsaturated lipids to form additional lipid hydroperoxides.

**Protein Oxidation:**

Thiol containing proteins are particularly susceptible to peroxidation damage. This may have special relevance in the genesis of disturbed cellular in homeostasis, since the Ca-ATPase and Na-ATPase of plasma membranes are both thiol containing proteins. Further, binding of transition metal ions at specific sites in protein result in specific damage. This type of specific metal binding is called as “site specific damage”.

Moreover, proteins and nucleic acid appear less susceptible than PUFA to free radical attack. Random attack of radicals on proteins is unlikely to very damaging unless very extensive\(^{115}\).

**Carbohydrate Oxidation:**

Free radical induced oxidation of carbohydrate result in the formation of oxaldehyde from monosaccharide sugar. These oxaldehyde have been implicated in proteins aggregation. ODFR may also depolymerize carbohydrate polymer such as hyaluronic acid,
which is responsible for maintenance of high viscosity of synovial fluid and thus may have role in rheumatoid arthritis\textsuperscript{114}.

**DNA Oxidation:**

ROI causes strand breaks and has the secondary effect of inducing the enzyme poy (ADP-ribose) polymerase. It has been suggested that the resulting depletion of cellular ADP may be sufficiently sever to reduce total cellular adenine nucleotide (including ATP) to critical levels.

Table No. 2.5 shows examples of injurious stimuli producing cell injury\textsuperscript{106}

**Table: 2.5.Noxious stimuli producing cell injury**

<table>
<thead>
<tr>
<th>Noxious stimulus</th>
<th>Cell types affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrimycin</td>
<td>Cardiac</td>
</tr>
<tr>
<td>Ischemia/reperfusion</td>
<td>Neuronal/cardiac/endothelial</td>
</tr>
<tr>
<td>Mercury</td>
<td>Renal/neuronal</td>
</tr>
<tr>
<td>Menadione</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>Hepatic</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Hepatic / renal</td>
</tr>
<tr>
<td>Cepaloridine</td>
<td>Renal</td>
</tr>
<tr>
<td>Alloxan</td>
<td>Pancreatic</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Epithelial</td>
</tr>
</tbody>
</table>