CHAPTER- 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

Liver is the largest and almost complex organ in the body. It plays an important role in the maintenance of internal environment through its multiple and diverse function. It is involved in the intermediary metabolism of protein, fat and carbohydrates and also it synthesis number of plasma proteins such as albumin, fibrinogen and clotting factors and also produce a number of enzymes and formation and excretion of bile. It act as a storage depot for proteins, glycogen, various vitamins and metals. It also has a role in the regulation of blood volume by transfer the blood from portal to systemic circulation and its reticuloendothelial system and participates in immune mechanism.

Its plays a central role in detoxification and excretion of many endogenous and exogenous compounds. Hence, any injury to it or impairment of its function has grave implication for the health of the affected person. Every year about 18,000 people are reported to die due to liver cirrhosis caused by hepatitis (Handa, 1991). Although viral infection is one of the main causes for hepatic injury.

The human body identifies almost all drugs as foreign substances (i.e. xenobiotics) and subjects them to various chemical processes (i.e. metabolism) to make them suitable for elimination. This involves chemical transformations to (a) reduce fat solubility and (b) to change biological activity. Although almost all tissue in the body have some ability to metabolize chemicals, smooth endoplasmic reticulum in liver is the principal "metabolic clearing house" for both endogenous chemicals (e.g., cholesterol, steroid hormones, fatty acids, and proteins), and exogenous substances (e.g. drugs). The central role played by liver in the clearance and transformation of chemicals also makes it susceptible to drug induced injury.

Drug metabolism is usually divided into two phases: phase 1 and phase 2. Phase 1 reaction is thought to prepare a drug for phase 2. However many compounds can be metabolised by phase 2 directly. Phase 1 reaction involves oxidation, reduction, hydrolysis, hydration and many other rare chemical reactions. These processes tend to
increase water solubility of the drug and can generate metabolites which are more chemically active and potentially toxic. Most of the phase 2 reactions take place in cytosol and involve conjugation with endogenous compounds via transferase enzymes. Chemically active phase 1 products are rendered relatively inert and suitable for elimination by this step.

A group of enzymes located in the endoplasmic reticulum, known as cytochrome P-450. It is the most important family of metabolizing enzymes in the liver. Cytochrome P-450 is the terminal oxidase component of an electron transport chain. It is not a single enzyme, but rather consists of a family closely related to 50 isoforms six of them metabolize 90% of drugs. There is a tremendous diversity of individual P-450 gene products and this heterogeneity allows the liver to perform oxidation on a vast array of chemicals (including almost all drugs) in phase 1. Three important characteristics of the P450 system have role in drug induced toxicity.

1.1.1. Causes

As the major drug metabolizing and detoxifying organ in the body, the liver is subject to potential damage from an enormous array of pharmaceutical and environmental chemical.

1.1.2. Injury may result due to

- Direct toxicity
- Hepatic conversion of xenobiotics to an active toxin
- The immune mechanism usually by a drug or a metabolite acting as hepatic to convert cellular protein into an immunogen

1.1.3. The following drug and toxin induced hepatic injury

Microvascular fatty change is induced by tetracycline, salicylates, yellow phosphorus and ethanol. Macrovascular fatty change is induced by ethanol. Methotrexate, amiodarone. Centriolobular necrosis induced by bromobenzene, CCl4, acetaminophen, halothane and rifampin. Diffuse or massive necrosis are induced by halothane, isoniazid, acetaminophen, methylldopa, trinitrotoluene and amanitaphallodies (mushroom) toxin, Hepatitis, acute and chronic are induced by methylldopa, isoniazid, nitrofurantoin, phenytoin, oxyphenisatin. Fibrosis-cirrhosis is induced by ethanol,
methotrexate and amiodarone, most drugs that cause chronic hepatitis. Granulo formation are induced by methyldopa, quiuidine, phenylebutazone, hydralazine, allopurinol, cholestasis (with or without hepato cellular injury) are induced by chlorpromazine, anabolic steroids, erythromycin estolate, oral contraceptives and organic arsenicals.

1.1.4. Carbon Tetrachloride

Chemical toxicity comprises an important source of reactive oxygen species (ROS), which may occur through processes, such as inhibition of mitochondrial electron transport chain and subsequent accumulation of intermediates inactivation of antioxidant enzymes and deletion of radical scavengers (Mates,2000). Carbon tetrachloride is one of the most commonly used hepatotoxin in the experimental study of liver disease (Johnston et al., 1998).

The hepatotoxic effect of CCl₄ are largely due to its active metabolites CCl₃⁻ (trichloro methyl radicals), CCl₅⁻ [tri chloro methyl peroxy radicals (Flow chart 1)] CCl₃⁻ is most reactive species and causes damage to biological macromolecules by combining with them there by causing covalent modification and setting the chain reactions of lipidperoxidation and ultimately cell death (Benedetti et al.,1987 ). Among various mechanism involved in hepatotoxic effect of carbon tetrachloride, one is oxidative damage through free radicals generation (Deleve et al., 1995)
Flow chart: 1

CCl₄
↓ sER
CCl₃*
↓
Lipid radicals
↓
Lipid peroxidation
(Autocatalytic spread along microsomal membrane)
↓ Membrane damage to rER
↓ Release of products of lipid peroxidation
↓ Polysome Detachment
↓ Damage to plasma membrane
↓ Permeability to Na⁺, H₂O, Ca²⁺
↓ Apo protein synthesis
↓ Cell swelling
↓ Fatty liver
↓ Massive influx of Ca²⁺
↓ Inactivation of Mitochondria, cell enzymes, Denaturation of proteins
1.1.5. Free Radical

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. A free radical can be defined as any species, which is capable of independent existence and contains one or more unpaired electrons (Halliwell and Gutteridge, 1989). Free radicals promote beneficial oxidation that generates energy and kills bacterial invaders. In excess, they can damage cell membranes and cause cell necrosis. Free radicals are highly toxic in nature and if allowed to accumulate they can cause damage to all macromolecules, lipids, proteins mitochondrial and nuclear DNA molecules of the cells causing severe oxidative stress (Halliwell and Gutteridge, 1984). It is increasingly being realized that majority of disorders/diseases is mainly due to the imbalance between pro-oxidant and antioxidant homeostatic phenomenon in the body. Pro-oxidants conditions either dominate due to increase in the generation of free radicals or and their inadequate quenching or scavenging in the body (Tiwari, 2001).

It is commonly accepted that, in a situation of oxidative stress, reactive oxygen species, such as superoxide (O$_2^-$), hydroxyl (OH$^-$) and peroxy (OOH$^-$, ROO$^-$) radicals, are generated (Chang et al., 2007). The reactive oxygen species play an important role related to the degenerative or pathological processes of various serious diseases, such as aging (Burns et al., 2001), cancer, coronary heart disease, Alzheimer’s disease (Smith et al., 1996; Diaz et al., 1997), neurodegenerative disorders, atherosclerosis, cataracts and inflammation (Aruoma, 1998).

Major reactions of the free radicals include initiation of auto oxidation chain process by hydroxyl and hydroperoxyl radicals. Addition of hydroxyl radicals and singlet oxygen to double bonds, hydrogen abstraction from allylic carbon atoms by hydroxyl radicals and oxidation of sulphhydryl thioether of biomolecules. Lipid peroxidation products and oxidized forms of LDL accumulate in the atherosclerotic lesions .The numerous modified DNA bases formed under the conditions of oxidative stress are highly mutagenic such as 8- oxy guanine (Balzfrei,1994). The biological consequences are mutations, sister chromatid exchanges, chromosomal aberrations, cytotoxicity leading to cellular degeneration and carcinogenesis. Oxygen free radicals have been shown to stimulate cancer development at all three stages of carcinogenesis namely initiation, promotion and progression (Cerutti, 1994).
Flow chart: 2

Oxidative Stress

Excess ROS/RNS of low antioxidant defense

Damage to biomolecules (lipid, DNA, Protein)

Lipid peroxidation damage (damage to membrane ion channel, transports)

DNA damage (strand breakage, base modification)

protein receptor, enzyme, ion Channel, Raised Intracellular Ca$^{2+}$

Cellular damage with release of more radicals

Cell death and tissue damage

Carcinogenesis, atherosclerosis, ageing, etc,

1.1.6. Free radicals and oxidative damage

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko et al., 2001; Maritim et al., 2003). Free radicals, ROS and RNS are molecules with one or more unpaired electrons. An important feature of free radical reactions with non-radicals is that they result in new radicals, which leads to chain reactions. Electron acceptors such as molecular oxygen react easily with free radicals, to become radicals themselves called ROS (Flow chart 2).
Fig. 1.1. Formation of free radicals

Free radicals are generated in normal pathological cell metabolism from external factors such as foreign materials or UV radiation. When human use oxygen for respiration and combustion, molecular oxygen reacts easily with free radicals to form ROS including superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$), singlet oxygen, free radicals of lipids such as alkoxy radical (RO'), peroxyl radical (ROO') and peroxynitrite (ONOO$^-$) as the reactive nitrogen species (RNS) formed by in vitro reaction of nitric oxide (NO) and O$_2$ (Fig. 1.1).

At a high concentration, free radicals and radical-derived species are hazardous for living organisms and damage all major cellular components. At moderate concentrations, however, nitric oxide, superoxide anion and related reactive oxygen species, play an important role as regulatory mediators in signalling processes. Maintaining the redox homeostasis is therefore crucial for cellular wellbeing. These agents damage several components in the body, such as lipids, proteins, nucleic acid, and DNA causing inflammation or lesion on various organs (Beckman et al., 1990). Also, these reactive species are likely to be involved in the pathogenesis of many human degenerative diseases as represented by cancer aging, atherosclerosis, rheumatoid arthritis and allergy.

An imbalance of the homeostasis with elevated ROS is the basis of ageing and diseases such as atherosclerosis, autoimmune disorders, neuronal degeneration, and especially cancer. An over production of these reactive species can occur, due to oxidative stress brought about by the imbalance of the bodily antioxidant defence system and free-radical formation (Wong et al., 2000). ROS such as superoxide radical...
Oxidative stress results due to the cytotoxic consequence of reactive oxygen byproducts: superoxide anions and hydroxyl radicals, which are generated as metabolites of normal and aberrant metabolic processes that utilize molecular oxygen. (Pourmorad et al., 2006). The products of lipid peroxidation such as MDA and 4-hydroxynonenal are toxic to cells (Dinis et al., 1994). Lipid peroxidation within the membrane has a devastating effect on the functional state of the membrane because it alters membrane fluidity, typically decreasing it and thereby allowing ions such as Ca$^{2+}$ to leak into the cell. The peroxyl radical formed from lipid peroxidation attacks membrane protein, enzymes and reinitiates lipid peroxidation. The preservation of cellular membrane integrity depends on protection or repair mechanisms capable of neutralizing oxidative reactions. (Fig. 1.2)

![Molecular mechanism of free radicals](image)

**Fig. 1.2.** Molecular mechanism of free radicals
1.1.7. Antioxidant

Almost all organisms are well protected against free radical damage by antioxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT), or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione. When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, deterioration of physiological functions may occur, resulting in diseases and accelerated ageing. However, antioxidant supplements or antioxidant containing foods may be used to help the human body to reduce oxidative damage (Mau et al., 2001 and Gulcin et al., 2002).

All living organisms have endogenous defense systems against oxidative damage, such as lipid peroxidation, DNA damage (Lee et al., 2005) and inhibition of cell communication due to reactive oxygen species (ROS). Antioxidants protect against chemotherapy toxicity and local toxic effects of tumors or surrounding tissues. The protection of cells against damage from oxygen and its metabolites can be accomplished through enzymatic and non-enzymatic means. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are considered to be the primary antioxidant enzymes, since they are involved in the direct elimination of reactive oxygen species. Glutathione-S-transferase (GST), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) are secondary antioxidant enzymes which help in the detoxification of reactive oxygen species by decreasing peroxide levels by GST or by maintaining a steady supply of metabolic intermediates like glutathione as by GR and NADPH by, G6PD for the primary antioxidant enzymes. The non-enzymatic small molecular antioxidants include sulfhydryl compounds such as glutathione (GSH) and thiols; NADPH; ascorbate, α-tocopherol etc., (Halliwell and Gutteridge 1984). Phytochemical components, such as polyphenols, ascorbic acid, and carotenoids also serve as antioxidants (Rice-Evans et al., 1997) Oxygen are essential for aerobic life process. However, cells under aerobic condition are threatened with the insult of ROMs that are efficiently taken care of by the powerful antioxidant system in human body.

1.1.8. Role of herbal drug against liver disease

Traditional medicine is widespread and plants still presents a large source of natural antioxidants that might serve as leads for the development of novel drugs.
Several anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activity (Repetto and Llesuy, 2002). In search of sources for natural antioxidants and compounds with radical scavenging activity during recent years, some have been found such as echinacoside in *Echinacea* root (Hu and Kitts, 2000), anthocyanin phenolic compounds (Rice-Evans *et al*., 1997) and thioredoxin protein from sweet potato (Huang *et al*., 2004). Resveratrol in grapes and other food products has been shown to protect cells from oxidative damage and cell death (Jang *et al*., 1997; Chanvitayapongs *et al*., 1997). Herbal-based therapeutics for liver disorders has been in use in India for a long time and has been popularized world over by leading pharmaceuticals. Despite the significant popularity of several herbal medicines in general and for liver diseases in particular, they are still unacceptable treatment modalities for liver diseases. The limiting factors that contribute to this eventuality are (i) lack of standardization of the herbal drugs (ii) lack of identification of active ingredient(s) principles(s) (iii) lack of randomized controlled clinical trials (RCTs) and (iv) lack of toxicological evaluation. The use of natural remedies for the treatment of liver diseases have a long history, starting with the ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver disease models by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence-based medicinal evaluation, standardization and randomized place to controlled clinical trials to support clinical efficacy.

A large number of plants and formulations have been claimed to have hepatoprotective activity (Shiha *et al*., 2005, Agarwal *et al*., 2006, Krishna *et al*., 2007, Murugaian *et al*., 2008, Arulkumaran *et al*., 2009, Takate *et al*., 2010). Nearly 160 phytoconstituents from 101 plants have been claimed to possess liver protecting activity. In India, more than 87 plants are used in 33 patented and proprietary multi-ingredient plant formulations. In spite of the tremendous advances made, no significant and safe hepatoprotective agents are available in modern therapeutics. Therefore, due importance have been given globally to develop plant-based hepatoprotective drugs effective against a variety of liver disorders.
1.1.9. *Crinum asiaticum*

The *Crinum asiaticum* is a bulbous herb (Fig. 1.3.). The leaves do not possess a petiole and are green, feathery and narrow. The leaves can grow between 0.5-1.5 m long. Its flower stalk grows from the middle of the plant and can grow between 1-1.2 m. The flowers of *C. asiaticum* are fragrant and do not possess crowns. The flowers have red filaments, inferior ovaries, 6 stamens, brown anthers and are red at the upper part while white below. The fruits are almost spherical with a few big green seeds and can span 4-5 cm wide.

**Order:** Asparagales

**Family:** Amaryllidaceae

**Tribe:** Amaryllideae

**Sub tribe:** Crininae

**Genus:** Crinum

**Species:** asiaticum

**Common names:** *Crinum Lily*, Seashore *Crinum*, Spider Lily, Bakung, bawang tanah, bawang hutan, poison bulb, pindar, nagadamani, vishamandala, vishamungali, barakanur, nagduan, nagdowan, vishamangil, Asian poison bulb.
**Parts used**- Leaves and root

**Plant**: In Southeast Asian countries, *C. asiaticum* has a considerable medicinal reputation as a potent folk medicine in the treatment of injury and inflamed joints.

**Leaves**: In Indonesia, the oiled and heated leaves are useful for strangury wounds by poisoned arrows, bites and stings. In Malaysia, poultice of the leaves are applied to swellings, swollen joints, lumbago, pains and in case of headache and fever. The leaves are also an emollient. In Northwest Solomon Islands, the leaves make a topical anti-inflammatory remedy (Wiart, 2000). In Malaysia, the leaves are used as a rheumatic remedy and to relief local pain (Awatef et al., 1999). On Karkar Island and in Simbu, Papua New Guinea the latex from the leaves is applied to cuts. In India, the leaves are used for skin diseases and inflammation (Goeltenboth et al., 1991). The crushed leaves are used to wash piles or mixed with honey and applied to wounds and abscesses (Wee, 1992)

**Seeds**: The seeds are considered purgative and emmenagogue (Duke and Ayensu, 1985)

**Stem**: In the Trobriands, the stem fibers are used to stop bleeding. In New Ireland, the milky sap from the stem is used for stonefish wounds (Goeltenboth et al., 1991). The bulb is an emetic and counter-irritant. In Papua New Guinea, the juice obtained from bulb is ingested regularly during 2 months for gonorrhoea. In the Philippines, the bulbs are crushed and applied as an ointment (Wiart, 2000). Juice from the fresh bulbs, taken several times per month induces vomiting. It is also instilled in the ear to treat otitis (Nguyen and Doan, 1989). Root The root is also an emetic, diaphoretic and nauseant when fresh (Jain and Defilipps, 1991). In Finschhafen village, Papua New Guinea, the cut root is cooked in banana leaf, then cooled and placed on the aching tooth. Roots are used in New Caledonia, Indonesia and Malaysia in a poultice for wounds, ulcers and swellings (Goeltenboth et al., 1991).

**1.1.10. Scientific information about *C. asiaticum***

**1.1.10.1. Analgesic**

Lycorine was more active than aspirin in the analgesic modified Koster’s test. The vasorelaxing and analgesic activities of lycorine are due to Amaryllidaceae alkaloids being structurally related to the isoquinoline alkaloids which are known to provide numerous smooth muscle relaxant and anaesthetic molecules such as papaverine (Wiart, 2000).
1.1.10.2. **Anti-bacterial**

Crinamine was shown to be active against *Bacillus cereus* and *Pseudomonas aeruginosa* (Wiart, 2000). Ethanolic extract shows the inhibitory activity against human pathogenic bacteria (Ilavenil *et al*., 2010).

1.1.10.3. **Anti-inflammatory**

A study indicated that the anti-inflammatory activity of methanol extract of the plant (50 mg/kg) was stronger than indomethacin in acute paw oedema induced by carrageenan in mice (Awatef *et al*., 1999).

1.1.10.4. **Antiviral**

Lycoricidine negated in vitro the development of RNA containing flaviviruses and bunyaviruses (Wiart, 2000). The roots of the plant also showed significant activity against HIV-1 virus (Byung *et al*., 2001). Inhibitors of hypoxia inducible factor-1 (HIF-1) Crinamine showed dose-dependent inhibition (IC50=2.7 µM) of HIF-1alpha in a cell-based reporter gene assay.

1.1.10.5. **Mast cells degranulation**

Lower *in vitro* concentrations (1-20 µg/ml) of lycoriside protected the peritoneal mast cells of albino rats against Tween 80-induced degranulation. It also provided the protection against degranulation of mast cells when administered in vivo (1-5 mg/kg, orally). At higher concentrations (100 µg/ml) *in vitro*, it had a mast cell degranulation promoting effect (Ghosal *et al*., 1986).

1.1.10.6. **Antioxidant**

The ethanolic extract of *Crinum asiaticum* possessed the free radical scavenging activity in *in-vitro* analysis (Ilavenil *et al*., 2010)

1.1.11. **Scientific information about Lycorine**

Lycorine is the major leaf and root bulb alkaloid of the amaryllidaceae plants (Fig. 1.4.). This alkaloid has been shown to behave as potent therapeutic agent in numerous experimental models. Pharmacological activities such as antiviral (Ieven *et al*.,1983), anticancer (Wagner *et al*., 1988), anti-inflammatory (Wiart, 2000), antioxidant (Ilavenil *et al*., 2010) antibacterial and analgesics agents (Lewis, 1990) have been associated with the ability of lycorine to inhibit the in-vivo growth of a murine ascite tumour and reduced the viability of in vitro grown tumour cells.
(Ghosal et al., 1985). However, no such a study has been investigated the hepatocytes ultra structure protective nature of *C. asiaticum* and lycorine in CCl₄ induced mice.

![Fig. 1.4. Structure of Lycorine](image)

Hence, the present study is aimed to investigate the antioxidant potential of *C. asiaticum* (L) and lycorine on pathophysiological marker enzyme, enzymic and non enzymic antioxidants, protein alteration, CCl₄ mediated oxidative stress and hepatocytes dysfunction in CCl₄ induced mice and the efficacy of *C. asiaticum* (L) and lycorine were compared with silymarin is a standard well known positive hepatoprotective compound.