Chapter 2

Review of literature

2.1 The Liver

2.1.1 General structure

structurally and histologically, the liver can be divided into four tissue systems: intrahepatic vascular system, stroma, sinusoidal cells, and hepatocytes (Kuntz & Kuntz 2006). Intrahepatic vascular system consists of hepatic artery, portal vein and hepatic vein that circulates blood within the organ; biliary system and lymph vessels. Stroma of the liver comprises the interstitial connective tissues of the liver. Sinusoidal cells are the mesenchymal cells present in liver, which includes endothelial cells, Kupffer cells, Ito cells, and PIT cells. Sinusoidal cells make up approximately 6.3% of the liver volume and constitute 30-40% of the total cell population. Endothelial cells constitute the greatest proportion (70%) of the sinusoidal cells. They form a continuous lining of the sinusoids with intercellular spaces between them. Endothelial cells are responsible for filtering the blood components and also regulate the exchange of fluid and material between the blood in the sinusoids and the hepatocytes. They also form and secrete cytokines, matrix components, growth factors and vasoactive substances. When these cells get damaged or destroyed due to the effect of toxins, alcohol, hypoxia, viruses or increased pressure in the sinusoids; the hepatocytes are exposed to destructions. Further, Kupffer cells constitute about 25% of the sinusoidal cells and make up 8-12% of the total liver-cell population. Because of the villiform surface and star-shape of Kuffer cells, they
are also referred to as stellate cells. They are mainly involved in the phagocytosis. Other functions include pinocytosis, discharge of signal molecules and clearance of toxins, antigens, antigen-antibody complexes and purines. These functions are subject to disturbances by alcohol and drug abuse. Further, Ito cells are the fat-storing cells of liver and constitute about 3-8% of the total liver cell population. These cells store 75% of the liver retinoids in the form of retinol esters which are also free radical scavengers. Finally, PIT are natural killer cells and destroy tumour cells or foreign cells as well as necrosed cells in the liver (Kuntz & Kuntz 2006). The major portion of rest of the liver is formed of liver parenchyma tissue termed as hepatocytes.

2.1.2 Hepatocytes

Hepatocytes are polygonal epithelial cells with the plate-like, overlapping formations building a 3D-system (Kuntz & Kuntz 2006). The estimated life span of hepatocytes range from 150-200 days, however there is no reliable information available regarding the normal life span of human hepatocytes. It is reported to be determined by genetic and exogenous factors (Kuntz & Kuntz 2006). Structurally, the hepatocytes have a cell membrane divided into 3 compartments based on morphological and functional cellular polarization. About 37% of the external area of the hepatocyte membrane is sinusoidal surface possessing numerous microvilli, 12% consist of canaliculi, with the secretory role; the remaining 50% constitute the smooth intercellular fissure. This fissure is sealed from the canaliculi by tight junctions as well as intermediate junctions and the desmosomes consisting of gap junctions. During apoptosis, these junctions seal, in order to prevent the progression of cell death (Kuntz & Kuntz 2006).

2.1.3 Functions of hepatocytes

The liver constantly maintain the reactivity of numerous biochemical functions and the diverse metabolic processes (Maher 1997; Pandit et al. 2012). A single liver cell accounts for carrying out about 500 different biochemical processes. The morphological and functional integrity of the liver is thus vital to the health of the human. The major
metabolic processes that the liver gets involved in are: bilirubin, porphyrin and bile metabolism; metabolism of biomolecules such as carbohydrates, amino acids, proteins, lipids, hormones and vitamins; homeostasis of trace elements; biotransformation and detoxification; alcohol degradation and maintenance of acid-base balance. The complex network of biochemical functions of hepatocytes require precisely organised metabolic regulatory mechanisms (Kuntz & Kuntz 2006). Within the hepatocytes, the regulations of biochemical process occur at the molecular level, within the organelles, at the cellular level and at the organ level.

Further, biotransformation and detoxifying functions of hepatocytes are of major relevance of all the other metabolic functions of the liver. Since, the disturbance of one or several of the other metabolic functions may be hazardous and associated with complications; any breakdown of the detoxifying function, however, leads to cell necrosis, liver failure and thus death.

2.1.4 Biotransformation and detoxification

If an endogenous or an exogenous substance do not serve the production of energy, nor needed for the maintenance of structure, nor be stored without causing harm, then it becomes necessary to eliminate these substances from the body. Of these, gaseous substances are excreted via lungs and water-soluble substances are excreted via kidney or through bile in the faeces. However, fat soluble foreign substances cannot be excreted in an unchanged state, and they must be broken down, detoxified and made water-soluble to be excreted.

Compared to consumption, only a small fraction of xenobiotic compounds, with strongly polar functional groups, are sufficiently water-soluble to be excreted unchanged; and undergo biotransformation by phase I & II metabolism in liver (Gunaratna 2000). These biotransformation processes take place principally in the liver smooth endoplasmic reticulum, and partly in the mitochondria. The enzyme systems involved in biotransformation are largely substrate-nonspecific. It is present either structure-bound in the biomembranes or found as soluble enzymes. Phase I of the biotransformation process
consists of functionalization reactions where, reactive polar groups such as -OH, -COOH, -SH, -NH₂ are either activated or inserted into the substance by means of either oxidation, reduction, hydrolysis, or hydration; thus providing the lipophilic molecule with a functional hydrophilic group (Kuntz & Kuntz 2006). Oxidation reactions by oxidases and dioxygenases as well as by monooxygenases are of great significance in biotransformation. They contain the haem protein CYP450 enzyme system consisting of a complex of various isoenzymes. In phase II of biotransformation process, the foreign substance molecule or a metabolite formed in phase I, conjugates with an endogenous substance by means of specific transferases. The resulting conjugation products are highly hydrophilic and are therefore readily excreted.

Hepatic damage is often associated with distortion of these functions (Russmann et al. 2009). The recognition that the framework that metabolize drug can likewise generate exceedingly reactive toxic metabolites is important. Since, liver is the hub of biotransformation reaction systems, reactive metabolites are also generated making it susceptible to drug-induced toxicity. Thus, to maintain a healthy liver is crucial for the overall health and wellbeing. Drugs must therefore be viewed as potential hepatotoxicants. Foreign substances, including medicinal products, are classified as obligate or as facultative hepatotoxins, depending on their degree of hepatic toxicity and the pathogenetic mechanisms of liver damage.

2.2 Hepatotoxicity

2.2.1 Hepatotoxicants

Hepatotoxicity is the liver dysfunction or damage associated with excess drugs or xenobiotics (Navarro, Victor and Senior 2006) and the chemicals responsible are called hepatotoxicants. These are exogenous compounds of clinical relevance which include overdoses of certain medicinal drugs, industrial chemicals, natural chemicals like microcystins, herbal remedies and dietary supplements (Papay et al. 2009). Certain drugs may cause liver injury when introduced even within the therapeutic ranges. Hepatotoxicity may result not only from direct toxicity of the primary compound but also
from a reactive metabolite or from an immunologically-mediated response affecting hepatocytes, biliary epithelial cells and/or liver vasculature (Deng et al. 2009). The hepatotoxic response elicited by a chemical agent depends on the concentration of the toxicant which may be either parent compound or toxic metabolite and differential expression of enzymes (Kedderis 1996). Main patterns of liver injury during hepatotoxicity may include zonal necrosis, hepatitis, cholestasis, steatosis, granuloma, vascular lesions, neoplasm and veno-occlusive diseases (Kedderis 1996).

Exogenous substances like APAP (Boyd & Bereczky 1966) and carbon tetrachloride (CCl₄) (Singh et al. 2011; Klassen 2001) cause zonal necrosis. Amatoxins produced by toxic mushrooms cause necrosis by cessation of protein synthesis as a result of inhibition of RNA synthesis. Manifestation of hepatocellular necrosis with infiltration of inflammatory cells is referred to as hepatitis. Viral hepatitis with features like acute hepatitis is caused due to halothane, isoniazid, APAP, bromfenac, ritonavir; whereas aspirin cause focal hepatitis in which the foci of scattered cell necrosis may accompany lymphocytic infiltration. On the other hand chemicals like methyldopa, diclofenac, dantrolene, minocycline and nitrofurantoin cause chronic hepatitis which is similar to autoimmune hepatitis clinically, serologically and histologically (Murray et al. 2008). Angiotensin-converting enzyme inhibitors, amoxicillin, chlorpromazine, erythromycins and sulindac lead to the impairment of bile flow, itching and jaundice leading to the condition termed as cholestasis (Kaplowitz 2004). Steatosis is accumulation of triglycerides leading to fatty liver diseases, caused due to aspirin, synthetic estrogens, corticosteroids, amiodarone and methotrexate (Yu & Keffe 2002; Chang & Schiano 2007; Kaplowitz 2004). Hepatic granulomas caused due to allopurinol, sulphonamides, isoniazide, penicillin are associated with granulomas located in periportal or portal areas (Chang & Schiano 2007). Chemotherapeutic agents and anabolic steroids lead to what is termed as vascular lesions due to injury to the vascular endothelium (King & Perry 2001; Ishak & Zimmerman 1995). Prolonged exposure to some medications and toxins like vinyl chloride, anabolic steroids, arsenic may cause neoplasms like hepatocellular carcinoma, angiosarcoma and liver adenomas (Brind 2007). The clogging of the hepatic vein blocking the blood supply due to destruction of small intrahepatic veins caused due to pyrrolizidine alkaloids, susulfan and cyclophosphamide is referred to as veno-occlusive diseases (Rollins 1986; Kaplowitz 2004; King & Perry 2001).
2.2.2 APAP-induced hepatotoxicity

APAP is a drug consumed as an analgesic, and it was not fully recognized that consumption of APAP could lead to hepatotoxicity causing acute liver failure (ALF) in the United States until the mid-1980s (Marzilawati et al. 2012). However, it has been observed that since then, APAP-induced toxicity constitutes majority of cases of drug-induced ALFs in the United States and the United Kingdom (Russmann et al. 2009). The metabolism of APAP in Asians is thought to differ from Caucasians. Detailed clinical features of APAP-induced hepatotoxicity among this population remains largely unreported (Marzilawati et al. 2012). Typically, greater than 90% of APAP is metabolized to phenolic glucuronide and sulfate in the liver by glucuronyltransferases and sulfotransferases and excreted in the urine; about 2% is excreted in the urine unchanged and approximately 5% to 10% is metabolized by CYP450, mainly the enzyme CYP2E1, CYP1A2 and CYP3A4 to NAPQI (Jaeschke, McGill, et al. 2011). NAPQI is a highly reactive, electrophilic molecule which damages the cell by forming covalent bonds with other macromolecules in cells (James et al. 2003). The diagrammatic representation of APAP-induced hepatotoxicity is depicted in Figure 2.1 (Jaeschke et al. 2012). In therapeutic doses, these deleterious effects are prevented by conjugation with GSH and consequent reactions to generate water-soluble products that can be excreted into bile. However, in APAP overdoses, glucuronyltransferase and sulfotransferase enzymes saturate, and divert the drug to be metabolized by CYP450 generating NAPQI in amounts that can deplete GSH and accumulate within the cells (Sumioka et al. 2004). Under the conditions of GSH depletion, NAPQI binds covalently with cellular proteins altering their structures and functions. These cellular disturbance lead to a decrease in calcium ATPase activities with an increase in levels of cytosolic calcium that results in alterations of cell permeability causing the formation of blebs in the cell membrane and loss of membrane integrity (Jaeschke et al. 2012). These turbulences dampen mitochondrial respiration, adenosine triphosphate (ATP) synthesis further inducing mitochondrial oxidant stress (Marzilawati et al. 2012). Oxidative stress eventually results in increased production of peroxynitrite, which is a potent oxidant and nitrating agent and generates additional covalent bonds with cellular proteins, causing further mitochondrial dysfunction (Ni et al. 2012). Ultimately this brings about the
Figure 2.1 Mechanism of APAP-induced hepatotoxicity

APAP-induced toxicity occurs by a complex sequence of events. (1) CYP450 metabolism of APAP to a reactive metabolite NAPQI which depletes GSH and covalently binds to proteins; (2) loss of GSH with an increased formation of ROS and RNS in hepatocytes undergoing necrotic changes; (3) increased oxidative stress, associated with alterations in calcium homeostasis and initiation of signal transduction responses, causing MPT; (4) Bcl-2 family members Bax and Bid then form pores in the outer mitochondrial membrane and release intermembrane proteins, e.g., apoptosis-inducing factor (AIF), second mitochondrial activator of caspases (smac) and endonuclease G, which then translocate to the nucleus and initiate chromatin condensation and DNA fragmentation, respectively; (5) multiple events including massive mitochondrial dysfunction and ATP depletion, extensive DNA fragmentation, and modification of intracellular proteins contribute to the development of oncosic necrotic cell death. Associated with these essential events, a number of inflammatory mediators such as certain cytokines and chemokines are released that can modify the toxicity.
changes in membrane permeability collapsing mitochondrial membrane potential termed as mitochondrial permeability transition (MPT), cessation of ATP synthesis, release of mitochondrial proteins into the cell cytoplasm, and oncotic necrosis of hepatocytes (Marzilawati et al. 2012).

Hepatic non-parenchymal cells such as the Kupffer, sinusoidal endothelial, stellate and newly recruited leukocytes, also contribute to the progression of pathogenesis of APAP-induced toxicity (Jaeschke et al. 2002). Kupffer cells and neutrophils are a source of proinflammatory cytokines and chemokines, reactive oxygen and nitrogen species, which promote oxidative stress. Cell death caused by the toxic APAP metabolites first activate Kupffer cells, to release cytokines including interleukin-12, interleukin-18, and tumor necrosis factor-α that may activate natural killer and natural killer thymus lymphocytes (Bessems & Vermeulen 2001). When activated, these cells cause liver damage by cytotoxic activity, promoting further activation of Kupffer cells and stimulating local production of chemokines. Inflammatory mediators, cytokines, and chemokines, recruit and accumulate neutrophils in the liver and exacerbate the hepatic injury (Jaeschke & Bajt 2006).

The manifestations of APAP overdose depend on the interval after ingestion and are defined in phases (Chun et al. 2009). In the first 24 h, the patient suffer from anorexia, abdominal pain, nausea, vomiting, lethargy, malaise, and diaphoresis. Within the period of 24 to 48 h, the condition may improve or even disappear; whereas biochemical abnormalities such as elevated transaminases, bilirubin and prolonged prothrombin time, become evident. From 72 to 96 h post the over-ingestion episode, nausea and vomiting reappear and are accompanied by malaise, jaundice, and central nervous system symptoms including confusion, somnolence, or coma (Chun et al. 2009). Hepatocellular injury and death most commonly occur in this stage. From 4 to 14 days after the overdose, the damage starts subsiding, with the return of normal hepatic architecture within 3 months.

Interventions for APAP overdose include inhibition of absorption, removal of APAP from the blood, prevention of the conversion of APAP into the toxic metabolite NAPQI, detoxification of NAPQI and liver transplantation. However, the choice of
therapies depends upon the timing of presentation and the degree of hepatic decompensation (Chun et al. 2009).

2.2.3 In vitro toxicity

With notable growth in pharmaceutical industry, certain issues associated with it principally includes knowledge on potential drug-induced toxicity. In an effort to address these needs, various models are constantly developed for this purpose. The key issues that needs to be addressed for the development of models are, retention of tissue forms and function, structural and mechanical properties, biochemical microenvironment, closeness to human species, extrapolation of dose-response relationships and feasibility. Preclinical animal studies are insufficient to evaluate toxicity because of its limitation in addressing species specific variations, largely reducing its relevance to toxicity and disease in man (Neuwelt et al. 2009). The differences in enzyme expression and substrate specificity in species, strain or gender can produce qualitative differences or quantitative differences in the metabolic pathways involved in the bioactivation or detoxification of hepatotoxicanst. Moreover, the classic thalidomide distaster caused in 1990, substantiates the use of models derived from human origin (Kim & Scialli 2011). Further, growing animal welfare issues, time and cost constraints are also forcing scientists globally to avoid in vivo studies and go for in vitro studies. Due to the lack of sufficient data to reliably assess the value of preclinical animal studies to predict hepatotoxicity in humans, the preclinical animal toxicity studies may not be sufficient as the only modelling systems to predict hepatotoxicity. Moreover, considering the ever increasing number of phytochemicals that need testing, establishing practical in vitro culture systems has become a priority for the toxicological evaluation.

2.2.4 In vitro toxicity models

Several in vitro human liver models currently used are precision cut liver slices (PCLS), microsomes, cell lines, primary hepatocytes and of further interest are hepatocytes derived from stem cells to mimic the properties of hepatocytes in vivo. PCLS
retains three-dimensional native liver architecture (Szalowska et al. 2014; de Graaf et al. 2010) with maintained cell diversity possessing parenchymal and non-parenchymal cellular components and cell–matrix interactions (Hassan 2012). It mimics in vivo systems for assessing liver viability and functions for examining hepatotoxicants making it a well-accepted in vitro system to study the hepatotoxicity and biotransformation (Gandolfi et al. 1996; Boess et al. 2003). A major limitation of this model is that, it is labour intensive. Also, the slices remain viable only for limited duration under optimal incubation conditions. Maintenance of optimal tissue slice thickness is also very critical because of oxygen and nutrient deprivation of the inner layers. Further, it is also difficult to procure normal human liver tissues (Bach et al. 1996; Somers et al. 2004) making this model largely unsuitable. Hence cultures of primary hepatocytes became an inherent part of the preclinical pharmaco-toxicological testing battery. Their primary utility is for assessing the toxicity-induction potential of new chemical entities and appeared as the closest model for the liver in vivo, thus serving as the gold standard for hepatotoxicity testing (LeCluyse 2001). However, there are few confinements to the use of the model system. There are no clearance and no extrahepatic metabolism occurring in the model system, the levels of drug metabolizing enzymes differently decrease during culture and phase I and II enzymes remain inducible but the extent of induction is variable. Also it has been described, that hepatotoxicity takes several hours to establish, but the viability of the culture for the long experimental conditions is uncertain as they are phenotypically unstable, have a limited life span. In addition, the culture system exhibits large inter-donor variability when of human origin which demands short-term and long-term storages.

Further, it is of importance to maintain liver tissue organization and cell contacts to retain their specific functions. In vivo, cells function at a high density within a 3D structure of various liver cell populations as discussed in section 2.1.1, which promote cell-cell interactions, and play role in the modulation of xenobiotic metabolism in liver, influencing its pharmacological and toxicological consequences. Therefore, the role of cell-to-cell interactions in herbal induced liver toxicity are investigated in co-cultures systems of cells.

However, to understand fully how tissues form and function, as well as their pathophysiology, it is crucial to study how cells and tissues behave as parts of whole
living organs that are composed of multiple, tightly opposed tissue types that are highly
dynamic and variable in terms of their 3D structure, mechanical properties and
biochemical microenvironment (Huh et al. 2011). Efforts to address these limitations led
to the development of 3D cell-culture models in which cells are grown within
extracellular matrix gels (Godoy et al. 2013). This approach enhances expression of
differentiated functions and improves tissue organization (Lee et al. 2008). A lot of
contemporary research work now focus on the next wave of 3D cell culture models that
better mimic the microstructure, dynamic mechanical properties and biochemical
functionalities of whole living organs (Gunness et al. 2013). Sandwich cultures, gravity-
enforced hanging drop spheroids to polystyrene or PEG derived models look promising.
The most recent innovations and developments include scaffold-free and scaffold-based
3D culture systems employing microengineering technolgies of microfabrication and
microfluidics (Evenou et al. 2007). Each model offers a few major strengths and
weakness for strategies in drug toxicity assessment, predicting impending hazards to
humans. However, this research is still in its infancy and needs to be explored in greater
details with respect to the stability, viability, reproducibility and reliability.

2.2.5 Hepatocellular carcinoma (HepG2) cell line as an in vitro hepatotoxicity model

HepG2 cell line was derived from the liver tissue of fifteen year old Caucasian
male with differentiated hepatocellular carcinoma. HepG2 cell line is adherent with an
epitheliod morphology, which grow as monolayers, and has been proposed a good
alternative model to study hepatotoxicity (Dehn et al. 2004).

HepG2 cell line secrete various proteins, such as albumin, transferrin, fibrinogen,
α-2-macroglobulin, plasminogen and produces most of the plasma proteins; and show
biosynthetic capabilities similar to those of normal hepatocytes (Dehn et al. 2004; Babich
et al. 2011). Further, they retain cell surface receptors (Peppard & Knap 1999); can
induce metallothionein in response to metals and organic compounds (Jahroudi et al.
1990; Miura et al. 1999). HepG2 cell line can synthesize, assemble and secrete
lipoproteins, and liver-specific serum proteins in serum-free culture medium (Dufresne et
HepG2 cell line has demonstrated the retention of cytochrome P450-dependent monooxygenase enzymes (Labruzzo et al. 1989; Roe et al. 1993; Schuetz et al. 1993; Duthie et al. 1994; Caro et al. 2001; Marf et al. 2001; Rodriguez-Anton et al. 2002) and glucuronic- and sulfate- conjugation abilities (Doostdar et al. 1988). Thus, they have the ability to carry out normal biotransformation reactions essential to the detoxification process. CYP450 activity can be enhanced by the culture medium used, and metabolic responses to dioxin (tetrachlorodibenzo-p-dioxin) and dibenzofuran (2,3,7,8-tetrachlorodibenzofuran) are similar to those found in primary human liver cell cultures, thus making this hepatic carcinoma cell line a potentially useful model for toxicological testing (Dehn et al. 2004).

HepG2 cell line also possess many of the morphological and biochemical features of normal hepatocytes (Krithika et al. 2009). Since it retains many of the phenotypic and genotypic characteristics of liver cells, this cell line has been used in various studies related to medicinal plants for their liver protecting property and it is suggested that the cellular damage in HepG2 cells by a variety of toxicants, involve lipid peroxidation as a major mechanism (Patlolla et al. 2009; Krithika et al. 2009; Noh et al. 2010).

In essence, HepG2 cell line display the characteristics of anchorage-sensitive hepatocytes, including secretion of differentiated gene products (Rollier et al. 1993), plasma membrane polarity (Maurice et al. 1988), and growth factor regulation of cell mobility (Tajima et al. 1992). HepG2 cell line has been well characterized for toxicity testing (Wilkening et al. 2003; Gerets et al. 2012; Dehn et al. 2004; Mersch-Sundermann et al. 2004) and are used in various metabolic and drug toxicity studies (Therapeutics et al. 2007). The model is also widely used to investigate the hepatoprotective property of crude plant extracts and their isolated compounds (Krithika et al. 2009).

Further, immortal cell lines are available in large quantities and are easily maintained in culture system as compared to primary hepatocytes or liver slices, making them an obvious choice for consideration as in vitro models. HepG2 cell line has an added advantage of rapid cryopreservation and ability to retain drug metabolic and enzyme activities on revival (Krithika et al. 2009). Besides, they yield quick results with reproducibility and reliability (Davila et al. 1998).
2.3 Assessment of drug-induced toxicity

Generally, hepatotoxicity involve different mechanisms of cytotoxicity (Kedderis 1996; Kaplowitz 2004). These includes direct effect on organelles like plasma membrane, mitochondria, endoplasmic reticulum, cytoskeleton and nucleus or indirect effect on cellular organelles through the activation and inhibition of signalling kinases, transcription factors and gene-expression profiles. The resultant intracellular stress leads to cell death caused by either apoptosis or necrosis.

2.3.1 Measurement of biochemical indices

The hepatic injury leads to appearance of variety of clinical and histopathological features which can be diagnosed biochemically (Singh et al. 2011). Biochemical indicators offer sensitive methods for determining these cellular disturbances. Most of these indicators fall into the categories of measuring viability, membrane integrity, metabolic function, or various nonspecific indicators that are associated with toxicity.

With regards to varied functions, liver contains a large pool of enzymes of which, a few are present in serum in very low concentrations with no known function in serum and behave like other serum proteins. These enzymes have characteristic rate of clearance. The elevation of a given enzyme activity in serum is thought to primarily reflect its increased rate of entrance into serum from damaged liver cells reflecting generalized damage to hepatocytes (Cordero-Pérez et al. 2013). Elevations in serum enzyme levels like alanine aminotransferase (ALT), aspartate aminotransferase (AST) alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) are considered relevant toxicity indicators whereas increases in both total and conjugated bilirubin levels are measures of overall liver function. Macroscopic and in particular histopathological observations and investigation of additional clinical biochemistry parameters allows confirmation of hepatotoxicity.

ALT plays role in amino acid metabolism and catalyzes the transfer of amino group from alanine to α-ketoglutarate generating glutamate and pyruvate, and
gluconeogenesis. ALT activity is the most commonly used biomarker of hepatotoxicity. In human sera it is normally present in the range of 5-50 U/L, however during the liver damage, the concentration increases. ALT is primarily found in the liver and is found with lower activities in skeletal muscles and cardiac tissue, making it a more specific test for detecting liver anomalies (Dufour et al. 2000a; Dufour et al. 2000b; Amacher 2002) as an indicator of hepatocellular necrosis.

AST is a liver enzyme that catalyzes the transfer of an amino group from aspartate to α-ketoglutarate generating oxaloacetate and glutamate. It is also reported to show activity in other organs like heart, muscle, brain and kidney (Nathwani et al. 2005). Normal levels of AST found in human sera are in the range of 7-40 U/L, the excess quantities are an indicator of hepatocellular necrosis, however non-specifically owing to the presence in other tissues as well (Ozer et al. 2008; Dufour et al. 2000a; Dufour et al. 2000b).

ALP present in biliary ducts, hydrolyzes monophosphates at an alkaline pH, and is usually eliminated in bile. Besides biliary duct, it is also found in other organs such as bone, placenta, kidney and intestine. Normal levels reported in human are in the range of 20-120 U/L, the excess presence is an indicator the inhibition of liver bile excretion process. This is due to the inability to excrete ALP through bile as a result of congestion or obstruction of the biliary tract as a consequence of hepatotoxicity. Elevation in ALP and/or bilirubin with little or no increase in ALT is reported to be a biomarker of hepatobiliary defects and cholestasis (Ramaiah 2007; Ozer et al. 2008).

GGT is an enzyme which is found in liver, kidney and pancreatic tissues, the enzyme concentration being low in liver as compared to kidney. It catalyzes transfer of γ-glutamyl groups to amino acids and short peptides. It is more useful clinically when compared to ALP. ALP is more sensitive but much less specific than GGT. The comparison of the levels of GGT and ALP determine the site of injury as that of bone or liver. Normal GGT level with an elevated ALP level is suggestive of bone disease as GGT is not found in bone while an elevated level of both the enzymes is suggestive of liver or bile duct disease especially cholestasis and biliary effects (Sheehan & Haythorn 1979). Normal levels of GGT in human are in the range of 0-51 U/L.
The other biochemical markers of hepatic injury includes monitoring the levels of total bilirubin and urobilinogen, as indicators of bilirubin metabolism impairment; and bile acids to monitor cholesterol metabolism (Thapa & Walia 2007; Geuken et al. 2004; Zollner et al. 2006). Further, prothrombin time is also measured as liver to differentiates between normal and damaged liver cells as it evaluates the functioning of proteins responsible for formation of blood clotting factors. Lactate dehydrogenase is reported to assists in energy production by catalysing the interconversion of pyruvate and lactate with concomitant inter-conversion of reduced nicotinamide adenine dinucleotide (NADH) and its oxidized form NAD+, and helps in detecting hepatocellular necrosis (Thapa & Walia 2007). Glutamate dehydrogenase is an enzyme that is involved in oxidative deamination of glutamate (Ozer et al. 2008; O’Brien et al. 2002). Albumin is the main protein in blood used as a supplementary test for hepatic biosynthetic functions (Thapa & Walia 2007). Additionally, the estimation of total proteins in the body is helpful in differentiating between a normal and damaged liver function as the majority of plasma proteins like albumins and globulins are produced in the liver (Thapa & Walia 2007). Serum F protein is a key enzyme involved in tyrosine catabolism (Neve 2003) produced in large amounts in liver. There are reports showing elevations in the serum F protein of patients with hepatocellular damage (Foster et al. 1989; Callaghan et al. 1994; Oliveira & Vindlacheruvu 1987; Beckett et al. 1989). Moreover, enzymes like arginase I (Ikemoto et al. 2001; Ashamiss et al. 2004), malate dehydrogenase (Zieve et al. 1985; Korsrud et al. 1972; Kawai & Hosaki 1990), purine nucleoside phosphorylase (Ozer et al. 2008; Ohuchi et al. 1995) and paraoxonase 1 (Ozer et al. 2008; Feingold et al. 1998) are also monitored as indicators of damage to specific functions of hepatocytes among others.

**2.3.2 Measurement of oxidative stress**

As discussed earlier (section 2.2.2), APAP overdose depletes mitochondrial GSH levels (Pierro & Rossoni 2013) leading to generation of oxidative stress due to reactive oxygen and nitrogen species and elevated Ca2+ levels. These are well-known inducers of the mitochondrial MPT in hepatocytes (Hinson et al. 2010). The MPT is characterized by mitochondrial swelling, uncoupling of the oxidative phosphorylation, and formation of
pores in the inner mitochondrial membrane (Hinson et al. 2010) that eventually results in breakdown of the mitochondrial membrane potential, the inability to synthesize ATP, and ultimately, necrosis (Hinson et al. 2010).

Cells cope with oxidants such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH) nitric oxide (NO) and nitroxyl radicals (ONOO$^-$), through several detoxification mechanisms (Figure 2.2). A variety of endogenous and exogenous components act against free radicals to neutralize them. Broadly these include, endogenous enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) whereas non enzymatic and metabolic antioxidants such as GSH, L-arigine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc (Sen et al. 2010; Dröge 2002). Nutrient antioxidants belonging to exogenous antioxidants, which cannot be produced in the body but provided through diet or supplements viz. trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids etc (Sen et al. 2010). Vitamin E and C are the non-enzymatic antioxidants exist within normal cells as well as they can be supplied through diet. Further, metal binding proteins like ferritin, lactoferrin, albumin and ceruloplasmin as well as phytoconstituents also act as antioxidants within the cells (Sen et al. 2010).

Conventionally, the assessment of cellular antioxidant capacity involves the measurement of the activities of antioxidant enzymes SOD, CAT, GSH-Px, and GR as well as monitoring the level of the metabolite antioxidant GSH as the measures of cellular state of ROS generation (Dröge 2002). However, the generation of ROS can also be detected directly by employing a cost-effective, sensitive and automated flow cytometric technique involving the use of an intracellular dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Raza & John 2012; Khan et al. 2013; Birringer et al. 2010; Kim et al. 2012). DCFDA is a chemically reduced form of fluorescein used as an indicator for ROS in cells, to detect the generation of reactive oxygen intermediates. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent DCFDA is converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) which is then estimated using flow cytometer.
Figure 2.2 Generation of various types of ROS

APAP induced toxicity results in generation of ROS. \( \text{O}_2^- \) radical dismutate to \( \text{H}_2\text{O}_2 \) under the action of SOD, which forms \( \text{OH} \) by Fenton’s reaction. NO when combines with \( \text{O}_2^- \), forms deleterious ONOO. \( \text{H}_2\text{O}_2 \) when acted upon by catalase release water, but when acted upon by GSH-Px, oxidizes GSSG reduced GSH. GR restore reduced glutathione reducing nicotinamide adenine diphosphate NADP\(^+\).
2.3.3 DNA fragmentation, necrosis and apoptosis

Three major morphologies of cell death have been described: apoptosis (type I), cell death associated with autophagy (type II) and necrosis (type III). Apoptosis and cell death associated with autophagy can be distinguished by certain biochemical events (Krysko et al. 2008). However, necrosis is characterized mostly the absence of caspase activation, cytochrome c release and DNA oligonucleosomal fragmentations. However, the necrosis caused due to APAP-induced hepatocyte injury can be visualized by fluorescence imaging using acridine orange/ethidium bromide (AO/EB) staining (Kasibhatla et al. 2006). AO is a vital dye that stains both live and dead cells, whereas EB stains only those cells that have lost their membrane integrity. Using this technique, the necrotic cell death, extensive leakage of cellular content as well as formation of apoptotic bodies can also be visualized (Zainal Ariffin et al. 2009; Ma et al. 2013). Further, to monitor the cellular changes associated with APAP-toxicity, cell cycle analysis is carried out which depicts the effect of toxicity in terms of shift in the distribution of cells with growth phases (Vermeulen et al. 2003). The normal cell cycle includes G1 phase, S phase, G2 phase and M phase. Under the microscope, interphase cells simply grow in size, but different techniques reveals that the interphase includes G1, S and G2 phases (Norbury & Nurse 1992). Replication of DNA occurs in S phase which is preceded by a gap called G1 during which the cell is preparing for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis. G1, S, G2 and M phases are the traditional subdivisions of the standard cell cycle. PI staining technique used in cell cycle analysis includes intercalation of PI dye in the DNA of cells quantitatively (Štraser et al. 2013; Hsu et al. 2012). The cell cycle analysis is carried out using flow cytometer which separates the cells based on size (forward scatter) and complexity (side scatter). Typically, when cells undergo apoptotic event, the cells shrink in size, resulting in reduction of its diameter. This phenomenon is captured using this instrument which gives rise to an extra peak of smallest cells to the extreme left of other peaks which is labelled G0 phase, which signifies apoptosis.
2.4 Phytomedicine for hepatoprotection

As it is known, that liver dysfunction results in impairment of toxin detoxification, leading ultimately to toxin accumulation in vital organs and consequently causing disseminated damage to them; it has thus become particularly imperative that a conscious effort be made to prevent exposure to these toxic substances, or where unavoidable, mitigate the associated liver injury by the use of plants or allopathic drugs known to possess universally accepted hepatoprotective potency. However, there is currently no allopathic drug that is effective for the treatment of liver diseases (Okwuosa et al. 2014). On the other hand, Medicinal plants play significant role in the treatment/management of liver disorders and many of them have shown significant hepatoprotective potency. Modern science has examined numerous plant extracts for this purpose and has confirmed the traditional experience by discovering the mechanisms and modes of action of these plants (Davila et al. 1998). In spite of the tremendous advances seen, very few significant and safe hepatoprotective agents are available in modern therapeutics.

Use of herbal medicine for the treatment of various ailments dated back to thousands of years. Out of the 250,000 higher plant species, about 70,000 species have been used in different traditional medicinal formulations (Dey et al. 2013). Plants have emerged as a great source of pharmaceutical products. In China, the plant-based pharmaceutical industry accounts for one-third of the entire pharmaceutical business and in Malaysia, the annual market of herbal medicine is around 1 billion Malaysian ringgit. In United States alone, the estimated plant-derived pharmaceutical market is of US$9 billion per year (Dey et al. 2013). There has been increasing scientific and industrial interest in ethnobotanical medicine during the past few decades and thus, the global market of herbal medicine is expected to increase from $19.5 billion in 2008 to $32.9 billion in 2013 with an annual growth rate of 11% (Dey et al. 2013).

The Indian Ayurvedic medicinal system dates back to the mid-second millennium BCE describing the usage of various plants based products for the ailment of numerous diseases. About 77 herbal formulations are found in Ayurveda having hepatoprotective properties (Dey et al. 2013), among which many of them have been tested for their
hepatoprotective capacity. Many Indian ancient medicinal texts emphasise on the hepatoprotective capacity of certain plants such as Kalmegh, *(Andrographis paniculata)*, Bhuia Amla *(Phyllanthus niruri)*, Indian bearberry *(Berberis aristata)*, Turmeric *(Curcuma longa)*, Kutki *(Picrorhiza kurroa)*, Mulethi *(Glycyrrhiza glabra)*, Punarnava *(Boerhavia diffusa)*, Tulsi *(Ocimum sanctum)*, Chicory *(Cichorium intybus)*, Bhringa Raja *(Eclipta alba)*, Kanak champa *(Pterospermum acerifolium)*, Guduchi *(Tinispota cordifolia)*, Chirayata *(Swertia chirata)*, etc (Dey et al. 2013).

Various bioactive compounds from plant sources possessing antioxidant, anti-cancer, immunostimulatory effect are also being tested for their possible hepatoprotective potential. A wide variety of flavonoids such as quercetin *(Helichrysum arenarium)*, myricetoside C *(Cercis siliquastrum)*, stachyrin *(Stachys recta)*, eupatolin *(Artemisia capillaris)*; alkaloids such as atropine *(Datura metel)*, pilocarpine *(Aristoclochia elementis)*, berberine *(Beriberis vulgaris)*; organic acids and lipids such as glycolic acid *(Cynara scolymus)*, dihydrocholic acid *(Curcuma longa)* have shown potent anti-hepatotoxic activity (Valan et al. 2010).

The current treatment for hepatotoxicity includes drugs which influence the CYP450 enzyme system either by inhibiting or inducing the metabolic activity of enzymes (Parameswari et al. 2013). Methionine, cysteamine, and N-acetyl cysteine (NAC) can detoxify NAPQI and decreased the risk of liver damage in randomized trials. However, methionine and cysteamine resulted in more adverse effects on gastric and nervous system compared to NAC (Brok et al. 2006). NAC is used to treat APAP-induced hepatotoxicity with proven effect as an antidote. Numerous reports suggests that oral and intravenous administration of NAC prevents hepatotoxicity, and works by replenishing GSH stores, binding directly to APAP toxic metabolite and enhancing nontoxic sulfate conjugation in liver cells showing antioxidant as mechanism of action (Smilkstein et al. 1988; Algren 2008). NAC acts as a precursor molecule for synthesis of GSH, which is an intracellular antioxidant molecule (Pierro & Rossoni 2013). However, it has been reported that, the timing of NAC therapy post-toxicity should be considered vital, else, it increases the risk of developing fulminant hepatic failure and death. Herbal medicines have put forth few formulations for liver disorders which have gained popularity and much importance in recent years because of their safety, efficacy and cost effectiveness as well as better compatibility (Bent 2008). The World Health Organization
(WHO) estimates that up to 80% of the world’s population, mostly in developing countries, rely on traditional medicine practices for its health care needs (Musila et al. 2004). Hence, development of plants based drugs are given importance in global markets (Kushwah et al. 2013).

Plants present a large source of natural antioxidants that might serve as leads for the development of novel drugs. Naturally occurring antioxidants are of interest in medicinal preparations for use in hepatoprotection against APAP-induced toxicity since it is associated with extensive generation of ROS. Fruits and vegetables show the presence of natural antioxidants which are related to three major groups: vitamins, phenolics and carotenoids and the ability of these compounds to scavenge ROS is well documented (Thaipong et al. 2006). Due to the presence of the conjugated ring structures and hydroxyl groups; these compounds have the potential to function as antioxidants by scavenging or stabilizing free radicals through hydrogenation or complexing with oxidizing species and retard oxidative regeneration of membrane lipids (Khan et al. 2012).

Silymarin, which is a widely reported herbal extract used for treatment of liver diseases, is obtained from the seeds of *Silybum marianum* (Shaker et al. 2010). Silymarin is composed of: silibinin, isosilibinin, silydianin and silychristin. It is reported to be protective against CCl₄, APAP, phalloidin, galactosamine and thioacetamide including alcoholic liver diseases (Shaker et al. 2010). Thus, silymarin is used as a standard agent for comparison in the evaluation of hepatoprotective effects of herbal products (Pradeep et al. 2007). Quercetin, ubiquitous in plant kingdom, has been widely reported to show both antioxidant and hepatoprotective potential. It stops propagation of lipid peroxidation, increases GSH levels and antioxidant enzyme function and prevents Ca²⁺ dependent cell death (Bentz 2009; Ansari et al. 2010). Further, vitamin E reported to be present in variety of fruits and vegetables is a class of phytoconstituent, that competes for deleterious peroxyl radicals scavenging them much faster before they can scavenge polyunsaturated fatty acids (Burton & Traber 1990) protecting the membrane from radical damage. Essential oil extracted from different species of the genus Rosa (Rosaceae) increase the secretion of bile fluid and major organic components of bile acting as a hepatoprotectant (Achuthan et al. 2008). (+) Borneol, isolated from *Dryobalanops aromatica* (Dipterocarpaceae), is a bicyclic monoterpenoid, which show
cholertic property (Spiridonov 2012). Phyllanthin, rutin, kaempferol, gallic acid, gallic acid, ellagic acid, phenazine and phenazine derivatives, lupeol, resveratrol, etc are other phytoconstituents reported to be hepatoprotective and successfully isolated from the plant sources among others (Sarin et al. 2014; Gurocak et al. 2013; Kasdallah-Grissa et al. 2006). However, a large proportion of phytoconstituents responsible for the activity with their mechanism of action remains to be identified.

As, chronic hepatic diseases stand as one of the foremost health troubles worldwide, with liver cirrhosis and drug induced liver injury accounting ninth leading cause of death in western and developing countries and since therapies developed along the principles of western medicine are often limited in efficacy, carry the risk of adverse effects, and are often too costly, especially for the developing world; treating liver diseases with plant derived compounds which are accessible and do not require laborious pharmaceutical synthesis seems highly attractive.

As oxidative stress plays an important role in APAP-induced hepatotoxicity, the use of antioxidants are expected to offer better protection to respond to the liver damage. Extensive literature revealed that there are very few plants which demonstrate hepatoprotective activity with active phytoconstituents that are well characterized (Yahya et al. 2013; Nithianantham et al. 2011; Sumathi et al. 2011; Karami et al. 2013). The elucidation of mechanism of hepatoprotection has also been an area less explored.

2.5 *Brassica juncea* Czern. and Coss.: Possible role in hepatoprotection

*B. juncea* belongs to the Cruciferae family of Angiosperms (Kumar et al. 2011), which is a large family with over 3,000 species grouped in 350 genera, including a group of edible plants. The name ‘Cruciferae’ is derived from the fact that the petals of the flowers of these plants display a distinctive cross form arrangement (Cartea et al. 2011). The *Brassica* genus is economically important within the family, containing 37 different species. The taxonomy of this genus is complex (Soengas et al. 2011). The classification
of the genus *Brassica* and its allied genera has been reported indicating their subgenera, sections, species and subspecies (Gomez-Campo 2003). Six groups of interrelated species are included in genus *Brassica* and the cytology of these species has been studied and the relationships among their genomes has been established (Soengas et al. 2011). The three diploid *Brassica* species: *Brassica nigra* (L.) Koch (2n=16), *Brassica oleracea* L. (2n=18) and *Brassica rapa* L. (2n=20), form the classic Triangle of U. Three amphidiploid species rose following natural hybridization of these species in different combinations, namely *Brassica* carinata A. Braun (2n=4x=34), *Brassica juncea* (L.) Czern. (2n=4x=36) and *Brassica napus* L. (2n=4x=38) (Cartea et al. 2011). The economically important vegetable species are *B. oleracea* consisting of vegetable and forage forms, such as kale, cabbage, broccoli, Brussels sprouts and cauliflower; *B. rapa* includes turnip, Chinese cabbage, pak-choi, and oil-seeds; *B. napus* crops are mainly used as oilseed (rape-seed); and the mustard group formed by *B. carinata*, *B. nigra* and *B. juncea*, which are used as condiments by virtue of their seeds (Cartea et al. 2011).

In general, plant can be taxonomically defined as follows:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae - plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subkingdom</td>
<td>Tracheobionata – vascular plants</td>
</tr>
<tr>
<td>Superdivision</td>
<td>Spermatophyta – seed plant</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliophyta – flowering plants</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida – dicotyledons</td>
</tr>
<tr>
<td>Subclass</td>
<td>Dilleniidae</td>
</tr>
<tr>
<td>Oder</td>
<td>Capparales</td>
</tr>
<tr>
<td>Family</td>
<td>Brassicaceae – mustard family</td>
</tr>
<tr>
<td>Genus</td>
<td>Brassica L. – Mustard</td>
</tr>
<tr>
<td>Species</td>
<td><em>Brassica juncea</em> (L.) Czern. and Coss. – Indian mustard</td>
</tr>
</tbody>
</table>

*B. juncea*, also known as Indian mustard, Chinese mustard, oriental mustard, leaf mustard, or mustard green, (Kumar et al. 2011) is an economically important plant that has been well known in India for centuries for its medicinal and nutritive values. Various parts of the plants are edible and used in a range of folk medicines and spices.
The mustard seeds have been used traditionally for the treatment of muscular rheumatism, inflammatory neuralgic affections, vomiting and dengue (John 2011). The seeds have also been used as folkloric medicine against jaundice in the Jalgaon district of Maharashtra and by the Sugali tribes of the Yerramalais forest of the eastern ghats of Andhra Pradesh, India (Basha et al. 2011; Pawar 2012). There are various polyherbal formulations of mustard discussed in Ayurveda medicine. A decoction of *Moringa oleifera* root (1 in 20) with the addition of bruised mustard seed is useful in doses of 1–2 ounces in ascites due to liver and spleen diseases (Singh & Panda 2005). A fresh root of *M. oleifera* mixed with mustard seeds and green ginger is used as a counter irritant and blistering agent (Singh & Panda 2005). A paste of equal parts mustard, horseradish seeds, hemp seeds and barley mixed with sore buttermilk is a useful application to the scrofulous glands of the neck. According to Ayurveda medicine, mustard preparations are mild laxatives, diuretics and liver-bile stimulators (Desai 2005) and have been also documented to purge the toxins out of the body (Mishra et al. 2012). The leaves and seeds of these plants are edible and diverse medicinal uses of seeds are also well known in other countries. In China, mustard seed is a folk remedy for arthritis, foot ache, lumbago and rheumatism. It is also used in the treatment of tumours; leaves are used in soups for bladder infections, inflammation or haemorrhage. Most such traditionally known uses of the plant have been centred on its seeds, however the leaves of the plant are known to be used in some part of the world. The leaves are used in folk medicines as stimulants, diuretics and expectorants (Kumar et al. 2011). In Korea, the seeds are used for abscesses, cold, lumbago, rheumatism and stomach disorders. The seeds are also reported to be hypoglycemic (Thirumalai et al. 2011), antioxidant (Dubie et al. 2013), anti-diabetic (Grover et al. 2003), hyperglycemic (Grover et al. 2002), anxiolytic (Yoon et al. 2007), goiterogenic (Tripathi et al. 2008) and hepatoprotective (John 2011). The seed paste is used to treat backache, arthritis, paralysis, styes, edema of the lungs and liver, aperient, stimulant and emmenagogue (Mishra et al. 2012; Joy et al. 1998). The oil is used in cosmetics for hair control (Yu et al. 2003). The leaves, seeds and stems have been shown to reduce the severity of asthma and high blood pressure, restore normal sleep attacks and prevent heart attack in patients suffering from atherosclerosis or patterns in women experiencing symptoms of menopause, and reduce the frequency of
migraine diabetic heart disease (Ufelle et al. 2011). The hepatoprotective activity of aqueous extract of mustard leaves have been evaluated against CCl₄-induced hepatic damage in albino rats (Walia et al. 2011).

Taken together, these reports strongly suggest the therapeutic potential of seeds against various disorders.

**Phytochemical profile**

The rich phytochemical profile of *B. juncea* and related species indicate the presence of various phytoconstituents reported to show varied pharmacological properties along with antioxidant potential (Soengas et al. 2011; Podsdek 2005; Cartea et al. 2011).

Glucose-derived functional group attached to a sulphonated oxime through a side chain which may be either aliphatic, aromatic or heterocyclic, are termed glucosinolates (GLSs). *Brassica* vegetables are the largest source of GLSs in plant kingdom (Verkerk et al. 2009). More than 200 individual GLSs have been identified in the Brassicaceae plants, and many of them are reported to be present in *B. juncea*. GLSs are water soluble aminos, which in presence of the myrosinase enzyme generate isothiocyanates, thiocyanates or nitriles (Borek et al. 1994). The major pungent chemical constituent of mustard oil is an anticancer volatile phytochemical allyl-isothiocyanate which is formed from its GLS precursors namely sinigrin and glucoraphanin (Yu et al. 2003; Manson et al. 1997; Fabre et al. 1997). Sinigrin on hydrolysis by myrosinase yields allyl isothiocayante, glucose and potassium bisulphate. Three native GLSs, isolated from mustard seeds include *p*-hydroxybenxyl glucosinolate, 9-(methoxysulfonyl)nonyl glucosinolate and 8-(methoxysulfonyl)octyl glucosinolate (Fabre et al. 1997). The other classes of bioactive phytoconstituents reported to be present include glycosides, flavonoids, phenolic compounds, sterols, triterpene alcohols, proteins and carbohydrates (Das et al. 2009; Jung et al. 2009; Li et al. 2000; Sang et al. 1984; Yokozawa et al. 2003). The most abundant phenylpropenoids reported in the species include flavonoids and hydrocinnamic acids (Cartea et al. 2011). Quercetin, Kaempherol and Isorhamnetin are the main flavonoids reported to be present as *O*-glycosides (Hollman 2000; Aron &
Kennedy 2008). Anthocyanins like pelargonidium, cyanidin, delphidin, peonidin, petunindin and malvidin are present in the plant species (Scalzo et al. 2008; Moreno et al. 2010; Tatsuzawa et al. 2006). It has also been reported that flavonol aglycone spectrum of *B. juncea* is unique, not present in any vegetables including the other plants of the same family (Yang et al. 2008). Further, among other phenolic compounds present in the mustard meal include sinapic acid and sinapine, which are also water soluble antioxidants (Das et al. 2009). Napin and crusiferin are the major proteins present in the seeds as storage proteins (Kumar et al. 2011). An antifungal protein jucin and *B. juncea* glyxalase I are the other proteins isolated from the seeds (Deswal & Sopory 1999; Ye & Ng 2009). The seeds also contain glogins and mucilage (Khan & Abourashed 2010). The mustard oil consists mainly of glycerides of various fatty acids including erucic, eicosanoic, arachidcmonadecanoic, behenic, oleic, palmitic, α-linolenic and arachidonic acids; with high content of erucic acid (Joardar & Das 2007). Isothiocyanates such as allyl isothiocyanates, 3-butenyl isothiocyanate and phenethyl isothiocyanate are the main constituents of essential oil (Yu et al. 2003). The sulphides present in essential oil includes diallyl trisulfide, diallyl disulphide and diallyl sulphide (Yu et al. 2003). Phytosterol content of mustard oil is very high consisting mainly of brassicasterol, free campesterol, β-sitosterol, Δ5-avenasterol and traces of Δ5-stigmasterol reported to be useful for combating hypercholesterolemia (Sabir et al. 2003). Further, *Brassica juncea* has been reported to possess variety of vitamins such as vitamin C, β-carotene, thiamine, riboflavin and niacin (Kumar et al. 2011). Thus, seeds act as good source of natural antioxidants due to the high levels of carotenoids, tocopherols, ascorbic acid, phytosterols and sulphur containing compounds. The presence of phenolic compounds flavonoids, anthocyanin, cinnamic acids, etc further strengthen the therapeutic potential of plant contributing to the antioxidant property owing to their chemical and structural characteristics (Korkina 2007).

### 2.6 Assessment of antioxidant activity of plants

The antioxidant capacity of phytoconstituents can be detected by various *in vitro* techniques. Antioxidant compounds from plant extract can act either by free radical scavenging, chelating of transitional metal, as reducing agents and as activators of
antioxidant defence enzyme systems to suppress radical damage in biological system (Halliwell & Gutteridge 2007). Thus, on the basis of the type of chemical reactions involved, major *in vitro* antioxidant capacity assays can be divided into hydrogen atom transfer (HAT) reaction based assays and single electron transfer (ET) reaction based assays (Huang et al. 2005). ET-based assays measure the reducing capacity of antioxidants which include 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, Ferric ion reducing antioxidant power (FRAP) Assay, 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay and the HAT based assays quantify hydrogen atom donating capacity viz. oxygen radical absorbance capacity (ORAC) assay (Huang et al. 2005).

DPPH is a stable organic nitrogen radical with a uv-vis absorption maximum at 515 nm. Upon reduction, the purple colour of the solution fades turning yellow; the reaction progress is conveniently monitored by a spectrophotometer (Clarke et al. 2013). FRAP assay based on electron-transfer reactions uses a ferric salt, Fe(III)(TPTZ)$_2$Cl$_3$ (TPTZ = 2,4,6-tripyridyl-s-triazine) as an oxidant (Thaipong et al. 2006). The redox potential of species which can lead to potential problems as many metal chelators in food extract could bind Fe(III) and form complexes that are also capable of reacting with antioxidants. ABTS$^-$ is an oxidant used in the assay, generated by persulfate oxidation of ABTS. The colourless ABTS molecule is converted into the blue-green colored radical, ABTS$^+$, by oxidation of one-electron, which has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm, as well as the more commonly used maximum at 415 nm. Further, the ORAC assay is based upon the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo- compounds such as [2,2’-azobis(2-amidino-propane) dihydrochloride (AAPH)] (Cheerens & Iller 2006; Thaipong et al. 2006). Using ORAC assay lipophilic, hydrophilic, and total antioxidant capacity of a substance are measured; thereby making it ideally suited to measure the antioxidant capacity of a substance (Thaipong et al. 2006; Klein et al. 2013; Hiraganahalli et al. 2012; Anthony & Saleh 2013).