3. RESULTS AND DISCUSSION

CHAPTER - I

3.1 PHYTOCHEMICAL STUDIES - GC-MS

Presently, there is increasing interest in identifying plant principles having hepatoprotective, nephroprotective and antioxidant properties against drug and chemical induced hepatorenal damages. The study of the bioactive constituents of higher plants represents a promising approach to the discovery of new drugs. The investigation of plants used as remedies in traditional folk medicine can be a useful tool to identify several biologically active molecules from the 250,000 higher plants species (Mbwamboo et al., 1996).

A variety of herbs and herbal extracts contain different phytochemicals with biological activities that can be a valuable therapeutic index, of their pharmacological activities. Most of the protective effects of fruits and vegetables have been attributed to phytochemicals, which are the non-nutrient plant compounds.

In recent years, Gas Chromatography-Mass Spectrometry (GC-MS) has been applied unambiguously to identify the structures of different phytoconstituents in plant extracts and biological samples with great success (Prasain et al., 2004). GC-MS is a reliable technique to identify the constituents of volatile matter, long-chain branched hydrocarbons, alcohols acids and esters (Anjali et al., 2009).
3.1.1 Phytochemical analysis of ethanolic extract of Caesalpinia sappan (CS)

The ethanol extract of CS was a complex mixture of many constituents and 19 compounds were identified in this plant by GC-MS. Phytoconstituents such as decanal, 1-octanol, 2-butyl-eugenol, benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-(synonyms: a-curcumene), 1,3-cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*,S*)]- (synonyms: zingiberene), a-Farnesene, cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)-(synonyms: a-bisabolene), cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*,S*)]- (synonyms: a-sesquiphellandrene), 1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl- (synonyms: nerolidol) undecanoic acid, a-cedren-9-a-ol, tetradecanoic acid, Spiro[4.5]dec-6-en-8-one, 1,7-dimethyl-4-(1-methyleneethyl)-, n-Hexadecanoic acid, L-serine, o-(phenylmethyl)-, 9,12-octadecadienoic acid (Z,Z)-, oleic acid, gingerol and heptacosane were identified in the ethanol extract of CS by relating to the corresponding peak area through coupled GC-MS (Table 2; Fig. 3). Most of the phytochemical compounds posses medicinal properties. For example, they exhibit antioxidant, antimicrobial, anti-tumor, hepatoprotective, hypocholesterolemic, and anti-inflammatory properties, as identified by Dr. Duke's Phytochemical and Ethnobotanical Databases.
3.1.2 Phytochemical analysis of ethanolic extract of *Clitoria ternatea* L. (*CT*)

The ethanol extract of *CT* was a complex mixture of many constituents and 16 compounds were identified in this plant by GC-MS. Phytoconstituents such as cyclohexane, 1,5,5-trimethyl-6 (2-propenyldiene; 8.62%), d-glycero-d-galacto-heptose (5.85%), 1H-azonine, octahydro-1-nitroso-4H-pyran-4-one (3.73%), 2,3-dihydro-3,5-dihydroxy-6-methyl (13.24%), 6-acetyl-a-d-mannose (3.79%), a-D-glucopyranoside, O-a-D-glucopyranosyl-(1.fwdarw.3)-a-D-fructofuranosyl (7.34%), desulphosinigrin (3.33%), decanedioic acid 3,8-dioxo-, dimethyl ester (2.43%), 9,12,15-octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[{(trimethylsilyl)oxy}methyl] ethyl ester, (Z,Z,Z) (3.59%), dodecanoic acid, 3-hydroxy (9.80%), n-hexadecanoic acid (38.29%), hexadecanoic acid, ethyl ester (7.05%), 9,10-secocholesta-5,7,10 (19%)-triene-3,24,25-triol, (3a,5Z,7E)-(3.44%), 9,12-octadecadienoic acid (Z,Z) (66.13%), and oleic acid (20.14%) 1-monolinoleoyl glycerol trimethylsilyl ether (27.89%) were identified in the ethanol extract of *CT* by relating to the corresponding peak area through coupled GC-MS (Table-3; Fig. 4).

In this plant also, most of phytochemical compounds have pharmacological properties such as antioxidant, hypcholesterolemic, anticancer, diuretic, hepatoprotective, and anti-inflammatory properties.
DISCUSSION

Table-2 and Table-3 and Fig. 3 and Fig. 4 show the GC-MS analysis of phytochemicals in the ethanol extract of CS and CT respectively. From GC-MS analysis it was found that both the extracts showed a positive response for the presence of flavonoids, glycosides, saponins, steroids and tannins.

This identification was done by comparison of their mass spectra on both columns with phytochemical and ethnobotanical databases libraries or with mass spectra from the literature (Adams, 2001; Jennings and Shibamoto, 1980) and home-made library. Most of the compounds (n-Hexadecanoic acid, Hexadecanoic acid, ethyl ester, Gingerol, Tetradecanoic acid, Benzene, and 1-(1, 5-dimethyl-4-hexenyl)-4-methyl- (Synonyms: a-Curcumene), Eugenol) belong to the group of antioxidant agents (Yoshida and Nihi, 2003). Based on data available in literature all these compounds could effectively contribute to the biological activities of Caesalpinia sappan and Clitoria leschenaultia takeda.

Since medicinal plants are endowed with complex chemical substances such as alkaloids, glycosides, flavonoids, saponins tannins etc., efforts are being made continuously to isolate and discover more potent plant principles for the treatment of various human ailments (Prajapathi et al., 2003). Different phytochemicals have been found to possess a wide range of activities; which may help in protection against chronic diseases. Most of the phytochemicals have the ability to inhibit lipid peroxidation and also possess hepato and nephroprotective properties.
It is hypothesized that the presence of phenolic compounds (Gonzalez de Mejia et al., 2004), saponins, glycosides and flavonoids (Yoshikawa et al., 2001) present in plant extracts are capable of inhibiting hepatocellular damage induced by hepatotoxins in both in vivo and in vitro studies.

Generally, the highest concentration of octadecadienoic acid, hexadecanoic acid, and oleic acid was detected with GC-MS. These compounds have been already reported as exerting hypoglycemic and hypolipidemic effects (Hussain et al., 2004; Dadu Khan Burdi et al., 2007), and also as having antioxidant and hepatoprotective activity (Muley et al., 2009).

Other nephroprotective medicinal plants were reported to inhibit xenobiotic induced nephrotoxicity in experimental animal models due to their potent antioxidant or free radical scavenging effects (Devipriya and Shyamaladevim 1999; Annie et al., 2005). In addition, alkaloids were found to strongly inhibit lipid peroxidation induced in isolated tissues via antioxidant activity (Kumaran and Karunnakaran 2007). The protection offered by the extract might be due to the presence of flavonoids and alkaloids (Donsky et al., 2007; Lucia et al., 2007).

It is believed that the active principles extracted from medicinal plants produce better, specific and safe drugs (Hasrat et al., 2004) and may reduce the risk of toxicity and maintain their therapeutic efficacy when put for use in clinical application (Nan et al., 2003).
The results of the preliminary phytochemical screening of CS and CT leaves extract reveal that both plants have quite a number of phytochemical constituents which may be responsible for many pharmacological actions.

CHAPTER II - IN VITRO STUDIES

3.2 HEPATOPROTECTIVE ACTIVITY

Cell culture methods have been widely used in toxicological studies, primarily in basal cytotoxicity assays, the results of which can be used to design more specific studies. Acetaminophen induced toxicity has been assessed in vitro using renal, hepatic, nervous and other cell cultures, including tumour cells. HepG2 cell lines have been proposed as an alternative model to hepatocytes and are used in various metabolic and drug toxicity studies (Sassa et al., 1987). They have the added advantages of being available in plenty, easy maintenance, rapid cryopreservation and the ability to retain drug metabolic and enzyme activities (Duthie et al., 1994). HepG2 cells possess many of the morphological and biochemical features of normal hepatocytes (Bouma et al., 1989). 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 (Moya et al., 2008).

3.2.1 Determination of effect of CT and CS extracts on the cell viability by Trypan blue staining assay

Fig. 5 depicts the cell viability by trypan blue assay in terms of percentage of cell death. The percentage of cell death significantly increased (P<0.001) in APAP induced group (Group 3) when compared to control (Group...
1). All the solvent extracts of CS showed decrease in the cell death after inducement with APAP. The ethanol extract treatment showed a better effect than choloform and ethyl acetate extracts (P<0.01). Of the three concentrations of the ethanol extract, 600 µg/ml was more effective (P<0.001) than other concentrations 200 µg/ml (Group 4) and 400 µg/ml (Group 5). Treatment with extract alone (Group 2) did not change cell viability when compared to control (Group 1).

The same trend was observed with CT extract. The ethanol extract of CT showed better effect on reducing the cell death by APAP than chloroform and ethyl acetate extracts as depicted in Fig. 5(a). Here also 600 µg/ml of ethanolic extract of CT showed better protective effect than other concentrations.

3.2.2 Determination of effect of CS and CT extracts on the cell viability by MTT assay

Fig. 6 and 6(a) depict the effect of CS & CT extract on the cytotoxicity of HepG2 cell lines as determined by MTT assay.

Treatment with acetaminophen (10mM) caused significant loss of viability of cells as measured by this assay. Pretreatment with MV, CT extracts (200 µg - 600 µg) before adding acetaminophen caused significant increment in viability of cells in a dose dependent manner. Both CT and CS extract treatments along with acetaminophen significantly increased cell viability by MTT assay. The highest concentration of CT and CS (600 µg) (Group 6) was most effective as (P<0.01) compared to other concentrations of extract.
Efficacies of different solvent extracts of CS and CT were tested. Of these, ethanol extract of CT and CS showed the most efficiency (P<0.001) in protecting the cells against APAP toxicity.

There was no significant difference in cell viability between cells incubated with plant extract (600 μg/ml) for 12 h and control cells, which indicates that both extracts (CT & MV) have no toxic effect up to 600 μg/ml.

The result indicates that moderate to good protection is offered by the extracts of CT and MV. The highest protection is observed in the ethanolic extracts of CT and MV. Hence ethanolic extract of CS and CT were used for the in vivo hepatoprotective studies.

Cells were treated with 10 mM APAP, a concentration previously associated with cytotoxicity and extensively used in in vitro cell culture system. (Hongslo et al., 1988: Shen et al., 1991, Boulares et al., 1996b Ryu et al., 2000). Nicod et al., 1997) also reported that APAP (5-30 mM) caused a significant concentration dependent decrease of cell viability (MTT assay) and depletion of intracellular GSH after 24 hr.

N-acetyl benzoquinine-imine, a metabolite of acetaminophen induces apoptosis, observed cell death while treating with acetaminophen in HepG2 cells (Manuela and neuman et al., 2002). Boulares et al. (1999b) have shown that acetaminophen alters cell proliferation in numerous cell types. Also APAP has been shown to induce DNA fragmentation in cultured hepatocytes (Shen et al., 1991).
In recent years, considerable attention has been directed on APAP-induced apoptosis. Hepatotoxic doses of APAP may alter the integrity of genomic DNA and may lead to cell death by apoptosis (Ray et al., 1996) compared the effect of APAP on two PC12 cell lines (expressed and non-expressed P4502E1). The major mode of death caused by APAP treatment was apoptotic (Hamid et al., 2002). Treatment with the extracts reduced toxicity induced by APAP.

3.3 NEPHROPROTECTIVE STUDIES

The Vero cell line established from kidney cells of the African Green Monkey (Cercopithecus aethiops), has a characteristic growth pattern in culture (Bianchi and Ayres, 1971), and has been used to study cell growth, differentiation (Genari et al., 1996; Haas et al., 2001; Lombello et al., 2000; Santos et al., 2001), cytotoxicity (Estacia et al., 2002) and cell transformation induced by different agents or conditions (Genari et al., 1996).

These cells are recommended for such investigations in standard protocols (ISO 10993-5, 1992-E) and provide an excellent model for studying nephrotoxicity in vitro because of their characteristic growth pattern and behaviour in culture. Since Vero cells have very well-defined properties, subtle alterations in their growth and morphology induced by acetaminophen are easily observed.

3.3.1 Determination of effect of CS and CT extracts on the cell viability by Trypan blue staining assay
Fig. 7 and 7(a) depict the cell viability by trypan blue assay in terms of percentage of cell death. The percentage of cell death significantly increased (P<0.001) in APAP induced group (Group 3) when compared to control (Group 1). All the solvent extracts of CS showed decrease in the cell death after inducement with APAP. However, the ethanol extract treatment showed better effect than choloform and ethyl acetate extracts (P<0.01). Of the three concentrations of the ethanol extract, 600 µg/ml was found to be more effective (P<0.01) than other concentrations (200 µg/ml (Group 4) and 400 µg/ml (Group 5). Treatment with extract alone (Group 2) did not change cell viability when compared with control (Group 1).

The same kind of trend was seen with CT extract. The ethanol extract of CT showed better effect in reducing the cell death by APAP than choloroform and ethyl acetate extracts as depicted in Fig. 7(a). Here also 600 µg/ml of ethanolic extract of CT showed better protective effect than other concentrations.

3.3.2 Determination of effect of CS and CT extracts on the cell viability by MTT assay

Cytotoxicity was measured based on the alteration of plasma membrane permeability. MTT is cleaved in the tetrazolium ring by the succinate tetrazolium reductase in active mitochondria. This yellow MTT is cleaved by all living, metabolically active cells and not by the dead cells thereby forming formazan, which form purple crystals and is directly proportional to cell number (Mosmann, 1983).
Figs. 8 and 8(a) depict the effect of CS and CT extract on the cytotoxicity of vero cell lines as determined by MTT assay.

Treatment with acetaminophen (10 mM) caused significant loss of viability of cells as measured by this assay. Pretreated with CT, CS extracts (200 μg - 600 μg) and acetaminophen caused significant increment in viability of cells in a dose dependent manner. Both CT and CS extract treatments along with acetaminophen significantly increased cell viability by MTT assay. The highest concentration of CT and CS (600 μg) (Group 6) was most effective when compared to other concentrations of the extract. The efficacies of different solvent extracts of CT and CS were also tested. Of these the ethanol extract of CT and CS showed most efficiency (P<0.001) in protecting the cells against APAP toxicity.

There was no significant difference in cell viability between cells incubated with plant extracts (600 μg/ml) for 72 h and control cells, which indicates that both the extracts (CT & MV) had no toxic effect up to 600 μg/ml. Thus the result indicates that moderate to good protection is offered by the extracts of CT and MV. The highest protection is observed in 600 μg/ml concentration of ethanolic extract of CT and MV.

From these results, it is proved that 600 μg/ml concentration of ethanolic extract of CT and CS had the most efficient effect against APAP induced cytotoxicity. Hence the ethanolic extract of CS and CT was used for the \textit{in vivo} studies.

\textbf{CHAPTER III - IN VIVO STUDIES}
3.4 SAFETY EVALUATION STUDY

Rats when fed with ethanol extract of CS & CT up to 2000 mg/ kg body weight p.o. exhibited no mortality or any sign of gross behavioral changes when observed initially for 72 h, and finally up to 15 days.

DISCUSSION

The safe evaluation study of ethanol extract of CS & CT showed that no mortality of rats occurred up to a limit dose of 2000 mg /kg body weight given p.o. This is an indication that the extract has low acute toxicity when administered p.o.

According to Clarke and Clarke (1977), substances with LD$_{50}$ of 1000 mg/kg body weight/oral route are regarded as being safe or of low toxicity. The high LD$_{50}$ obtained is an indication that the extract could be administered with a high degree of safety where the absorption might be incomplete due to inherent factors impeding absorption along the gastrointestinal tract (Dennis 1984).

3.5 ACETAMINOPHEN (APAP) INDUCED HEPATOTOXIC STUDIES IN ALBINO RATS

The serum activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and bilirubin were used as biochemical markers to assess early acute hepatic damage.

Many people all over the world are suffering from hepatic damage induced by alcohol and drug abuse. The liver plays a major role in the digestion,
metabolism and storage of nutrients. Today the increasing impact of liver disease, especially liver injury due to pharmacological treatment is being recognized. In recent years new insights have been gained into the pathological mechanisms of liver injury. In certain cases this forms the basis for novel therapeutic strategies (Gerbes et al., 2006).

The liver is the first organ to metabolize all foreign compounds and hence it is susceptible to different diseases. Some are rare but there are a few, including hepatitis, cirrhosis, alcohol related disorders and liver cancer which are fatal. A major cause of these disorders is the exposure to different environmental pollutants and xenobiotics e.g., Paracetamol (APAP), carbon tetrachloride (CCl₄), thioacetamide, alcohol etc. These toxicants mainly damage the liver by producing reactive oxygen species (ROS).

Acetaminophen (N-acetyl-para-aminophenol; Paracetamol, APAP) is one of the most widely and commonly used over-the-counter drugs for the relief of fever and headaches due to its antipyretic and analgesic properties, and is a major ingredient in cold and flu remedies. Though APAP is generally considered safe for human use at recommended doses, potentially fatal liver damages occurred in rare cases when an acute over-dose or even a normal dose was taken. Accordingly, APAP overdose is one the most common causes of drug poisoning world-wide. Excessive use of APAP can cause multiple organ damages, especially of the liver and kidney (Bertolini 2006; Yapar, et al., 2007). Other tissues have been shown to be affected by acetaminophen, for instance eye (Zhao et al., 1997), lung (Hart et al., 1998), testes (Boyd 1970), heart (Prescott, 1980) and lymphoid tissues (Cohen et al., 1997).
The excessive production of reactive oxygen free radicals have been known to produce tissue injury through covalent binding and lipid peroxidation and have been shown to augment fibrosis as seen from increased collagen synthesis (Geesin \textit{et al.}, 1990). Scavenging of free radicals by antioxidants could reduce the fibrosis process in the tissues (Thresiamma and Kuttan, 1996). Free radical may also be a contributory factor in a progressive decline in the function of the immune system (Pike and Kuttan, 1995). Cooperative defense systems that protect the body from free radical damage include the antioxidant nutrients and enzymes.

\textbf{RESULTS}

The serum activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and bilirubin were used as biochemical markers to assess hepatic damage.

The activity levels of serum ALT, AST, ALP and bilirubin were observed in control, APAP induced and Silymarin treated groups. In group 2 animals which were treated with APAP the levels of biochemical marker enzymes like ALT, AST and bilirubin were significantly elevated (P<0.001).

The activity level of ALP however, was significantly (P<0.001) elevated when compared to level in normal rats (Table: 4, Fig. 9).

Bilirubin, an endogenous organic anion, binds reversibly to albumin and is transported to the liver, where it is conjugated to glucuronic acid and excreted in the bile. It is derived primarily from catabolism of red blood cells,
heme and to a lesser extent from degradation of myoglobin, cytochrome, catalase and peroxidase.

Table 4; Fig. 9(a) depicts serum level of total birlubulin and total protein in control and experimental groups of rats. Rats intoxicated with acetaminophen (Group 2) showed a significant elevation (P<0.001) in total bilirubin and total protein. Pretreatment with CS extract (Group 3 & 4) resulted in reversal of the above changes to near normal.

The oxidative stress in the liver tissue was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS) and antioxidant defense enzymes.

The TBARS was assessed in normal, and APAP induced animals. Significant (P<0.001) elevation in the levels of TBARS was seen in APAP treated groups when compared to levels in normal animals (Table 5; Fig. 10).

The levels of antioxidant enzymes viz., Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione-S- transferase (GST) were estimated in normal, APAP induced and silymarin treated animals. In group 2 induced liver toxic animals the reduction of the antioxidant enzymes like SOD and CAT was significant although the levels of GPx and GST were found to have significantly decreased when compared to levels in normal animals (Table 5; Fig. 10 (a &b) ).

DISCUSSION
The liver is the largest organ in the vertebrate body and is the major site of xenobiotic metabolism and excretion. Liver injury can be caused by toxic chemicals, drugs and virus infiltration from ingestion or infection. The toxins absorbed from the intestinal tract gain access first to the liver resulting in a variety of liver ailments. Thus liver diseases remain one of the serious health problems (Karan et al., 1999).

Acetaminophen is known to cause hepatotoxicity in experimental animals and humans at high doses (Mitchell, 1988; Eriksson et al., 1992; Thompsen et al., 1995). The laboratory features of hepatotoxicity induced by APAP resemble other kinds of acute inflammatory liver diseases with prominent increase of ALT, AST and ALP levels (Davidson and Eastham, 1966).

The serum level of hepatic enzymes ALT, AST, ALP and total bilirubin levels were increased and reflected the hepatocellular damage in the APAP-induced hepatotoxicity animal model. This is indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman et al., 1978).

Oxidative stress is considered to play a prominent causative role in many diseases including liver damage (Kiso et al., 1984). Oxidative stress is the state of imbalance between the level of antioxidant defense system and production of oxygen derived species. Increased O$_2$ concentration and production of oxygen derived species such as superoxide radical ($^\bullet$O$_2^-$), Hydroxyl radical (OH$^*$) and hydrogen peroxide cause oxidative stress (Zhu et al., 2004).
Previous studies have demonstrated that oxidative stress is a major mechanism in the development of APAP-induced hepatotoxicity (Lin et al., 1998; Ahmad and Khater, 2001; Shanmugasundaram and Venkataraman, 2006).

Oxidative stress is now recognized to be associated with more than 100 diseases, as well as with the normal ageing process (Ghasanfari et al., 2006). There is a strong correlation between thiobarbituric acid reactive substances (TBARS) as a marker of lipid peroxidation and products that reflect oxidative damage to DNA (Chen et al., 2005). Increases in the formation of TBARS in iron (II) sulphate (10 µM)-induced oxidative stress, when compared to the normal, suggest possible damage of tissues with an overload of iron. Free iron in the cytosol and mitochondria can cause considerable oxidative damage by increasing superoxide production, which can react with Fe (III) to regenerate Fe (II) that participates in the fenton reaction (Fraga and Oteiza, 2002). Iron overload results in the formation of lipid peroxidation products, which have been demonstrated in a number of tissues, including the liver and kidneys (Houglum et al., 1990). Storage of iron in the liver leads to liver cirrhosis.

The body has an effective defense mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, CAT, GPx and GST. These enzymes constitute a mutually supportive defense team against ROS (Venukumar and Latha, 2002). APAP induced elevation of plasma AST and ALT activities could be regarded as an index of damage of the liver parenchymal cells. The balance between ROS production and these antioxidant defenses may be lost, and ‘Oxidative stress’ results, which through a series of events deregulates the cellular functions leading to hepatic necrosis. Under oxidative
stress, some endogenous protective factors such as GPx and catalase are activated in the defence against oxidative injury. This increase in enzyme activities was probably a response to increased ROS generation (Kyle et al., 1987). The increased levels of SOD, catalase and GPx observed point out the hepatic damage in the rats administered with APAP.

3.5.1 Effect of Caesalpinia sappanethanolic extract on APAP induced liver toxicity in albino rats

The liver is the most important organ in terms of biochemical activity in the human body. The liver has great capacity to detoxify and synthesize useful substances, and therefore, damage to the liver inflicted by hepatotoxic agents has grave consequences (Achliya et al., 2004). Many risk factors, including hepatic viruses, alcohol consumption and chemical agents, have a significant impact on the etiologies of liver diseases. Environmental pollution, bad dietary habits, and hepatic viruses have been considered to be the main factors that cause liver diseases (Day and Yeaman, 1994; Szabo, 2003).

There are several characteristic pathologies in the livers of patients with liver disease, including fatty liver, hepatitis, liver fibrosis, hepatocirrhosis and liver cancer. Liver fibrosis is the common end stage of most chronic liver diseases regardless of the etiology (Bataller and Brenner, 2005), and its progression leads to liver cirrhosis and liver cancer. Currently, it is believed that the early stage of liver fibrosis can be reversed, while liver cirrhosis cannot be. Therefore, preventing and eliminating the bad factors, and ameliorating fatty liver and liver fibrosis, are the most effective methods to prevent the liver from ultimately deteriorating (Freidman, 1993; Brenner et al., 2000).
Much progress has been made in the understanding of the pathogenesis of liver diseases, resulting in improved prevention and therapy with promising prospects for even more effective treatments. In view of the severe undesirable side effects of synthetic agents, there is a growing focus on following systematic research methodology and evaluating the scientific basis of traditional herbal medicines that claim to possess hepatoprotective activity (Shahani, 1999; Achliya et al., 2004).

Acetaminophen (APAP) is generally accepted as a safe analgesic and an antipyretic when administered within the therapeutic range; however, after an overdose absorption or in specific conditions (alcohol absorption, etc.), APAP is known to be hepatotoxic and nephrotoxic in humans and experimental animals (Curry et al., 1982; Keaton 1988; Vermeulen et al., 1992; Bonkovsky et al., 1994). It was suggested that APAP-induced hepatic injury is produced by the cytochrome P-450-generated reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), which reacts with sulphydryl groups and protein thiols. At high doses of APAP, NAPQI first depletes hepatocytes of intracellular glutathione (GSH) and then alkylates cellular macromolecules (Nelson and Bruschi 2003).

At present herbal drugs have an important role in health programmes worldwide; there is also a resurgence of interest in herbal medicines for treatment of various ailments including hepatotoxicity. India, the abode of ayurvedic system of medicine, assigns much importance to the pharmacological aspects of plants.
In the present study the folklore medicinal plant Caesalpinia sappan was selected to evaluate its hepatoprotective effects on APAP induced liver toxicity in *albino* rats.

**RESULTS**

The serum activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and bilirubin were used as biochemical markers for the assessment of early acute hepatic damage.

The activity levels of serum ALT, AST, ALP and bilirubin were observed in normal, APAP-induced and silymarin treated groups. In group 2 animals which were treated with APAP, the levels of biochemical marker enzymes like ALT, AST and bilirubin were significantly (*P*<0.001) elevated when compared to level in normal rats (Table 4; Fig. 9 & 9(a)).

The effect of *Caesalpinia sappan* on serum marker enzymes is presented in Table 4, fig 9 & 9(a). The serum levels of ALT, AST, ALP and total bilirubin were markedly significantly (*P*<0.001) elevated and that of protein levels were significantly (*P*< 0.001) decreased in acetaminophen treated animals, indicating liver damage. Administration of ethanolic extract of *Caesalpinia sappan* at the doses of 250 (Group 3) and 500 mg/kg (Group 4) remarkably significantly (*P*<0.01; *P*<0.001) reversed hepatotoxicity induced by acetaminophen.

The oxidative stress in the liver tissue was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS), and antioxidant defense enzymes viz., Superoxide dismutase (SOD), catalase (CAT), glutathione
peroxidase (GPx) and glutathione-S- transferase (GST) in APAP administrated as well as plant extract treated groups.

Acetaminophen treatment (Group 2) caused a significant (P<0.001) decrease in the level of SOD, Catalase, GPx, GSH and GST in liver tissue when compared with control group (Group 1). The treatment of CS at the doses of 250 (Group 3) and 500 mg/kg (Group 4) resulted in a significant (P<0.01; P<0.001) increase of SOD, catalase, GPx, GSH and GST when compared to Group 2 (Table 5; Fig. 10, 1 (a, b)). The standard drug, silymarin treated animals (Group 5) also showed a significant (P<0.001) increase in antioxidant enzymes levels compared to Group 2.

Analysis of TBARS level showed a significant (P<0.001) increase in the acetaminophen treated rats. After administration of the plant extract (250 mg/kg (Group 3) & 500 mg/kg (Group 4)), the levels of TBARS were found to have decreased significantly (P<0.01, P<0.001; Table 5; Fig. 10).

**DISCUSSION**

In the assessment of liver damage by APAP hepatotoxin, the levels of enzymes such as ALT and AST are taken as indicators. Necrosis or membrane damage releases the enzymes into circulation; therefore, its quantity can be measured in serum. ALT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore ALT is more specific to the liver, and thus a better parameter for detecting liver injury.

Leakage of cellular enzymes into plasma is a hallmark sign of hepatic injury or damage. In addition, the extent and type of liver injury or damage can
be assessed based on the presence or absence of specific marker enzymes in the blood streams. Generally, measurement of ALT, AST and ALP are commonly used as marker enzymes of hepatotoxicity (Asha et al., 2004; Yen et al., 2007). The significant decrease in the levels of biochemical marker enzymes like ALT, AST, ALP and bilirubin in plant extract administered animals might be due to decreased leakage of the enzymes in liver cells. This suggests that the Caesalpinia sappan plant extract could repair the hepatic injury and or restore the cellular permeability, thus reducing the toxic effect of APAP induced liver toxicity and preventing enzymes leakage into the blood circulation.

Other investigators have reported similar observations (Lin et al., 1998; Maiti et al., 2007; Ahmed and Khater, 2001; Shanmugasundaram and Venkataraman, 2006).

The significant depletion of levels of TBARS and lipid peroxides in the liver tissue of the plant extract administered animal group might be due to reduced lipid peroxidation and or elevation of tissue antioxidant defense enzymes activity levels, indicating that the plant extract could reduce the generation of free radicals and increase free radicals scavenging mechanism.

Biological systems try to protect themselves against several toxicants. These include free radical scavengers and chain reaction terminators; enzymes such as SOD, CAT, and GPx system. These enzymes constitute a mutually supportive team of defense against ROS (Venukumar and Latha, 2002). The balance between ROS production and these antioxidant defenses may be lost, and ‘oxidative stress’ results, which through a series of events deregulates the cellular functions leading to hepatic necrosis. Under the oxidative stress, some
endogenous protective factors such as GPx and catalase are activated in the defence against oxidative injury. This increase in enzyme activity was probably a response towards increased ROS generation (Kyle et al., 1987).

CAT is a key component of the antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radicals induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Therefore, a reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury (Thabrew et al., 1897). SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide thus diminishing the toxic effect caused by this radical.

In this study, significant increase in the levels of antioxidant enzymes was seen which indicates the free radical scavenging properties of Monochoria vaginalis. These findings also indicate that CS may be associated with decreased oxidative stress and free radical mediated tissue damage.

It has been recognized that the use of natural products is an important preventive approach to minimize the pathological consequences of oxidative stress. One of the many key considerations that need to be addressed in evaluating the hepatoprotective potential of a natural product is its inherent
toxicity as well as its efficacy in protecting at low concentration, preferably capable of inhibiting LPO process. In the present study, the plant extract did not show any damage to the system but elicited protective effects.

In conclusion, the hepatoprotective effect of Caesalpinia sappanleaf extract can be correlated directly with its ability to reduce the rate of lipid peroxidation and enhance antioxidant defense status. The findings of this study suggest that CS can be used as a safe, cheap, and effective alternative chemopreventive, protective agent in the management of liver diseases.

3.5.2 Hepatoprotective effect of Clitoria ternatea L. (CT) ethanolic extract on APAP induced liver toxicity in albino rats

The effect of Clitoria ternatea L. (CT) on serum marker enzymes is presented in Table 4, Fig 9. The serum levels of ALT, AST, ALP and total bilirubin were markedly significantly (P<0.01) elevated and that of protein levels significantly (P<0.001) decreased in acetaminophen treated animals, indicating liver damage. Administration of ethanolic extract of Clitoria ternatea L. (CT) at the doses of 250 (Group 6) and 500 mg/kg (Group 7) remarkably significantly (P<0.01; P<0.001) prevented hepatotoxicity induced by acetaminophen.

Analysis of TBARS reaction showed a significant (P<0.001) increase in the acetaminophen treated rats. Treatment with CT (250 mg/kg; Group 6 & 500 mg/kg Group 7) significantly (P<0.01; P<0.001) prevented the increase in TBARS level which was brought back to near normal (Table 5, Fig. 10).
Acetaminophen treatment caused a significant (P<0.001) decrease in the level of SOD, catalase, GPx and GST in liver tissue when compared to the control group. The treatment with CT at the doses of 250 and 500 mg/kg resulted in a significant (P<0.05; P<0.01) increase of SOD, catalase, GPx and GST when compared to Group 1 (Table 5; Fig. 10 (a, b)).

The standard drug, silymarin treated animals also showed a significant (P<0.001) increase in antioxidant enzymes levels compared to Group 1. Morphological observations showed an increased size and enlargement of the liver in acetaminophen treated groups. These changes were reversed by treatment with silymarin and also with CT at the two different doses tested groups.

**DISCUSSION**

Acetaminophen a widely used antipyretic analgesic drug produces acute hepatic damage on accidental over dosage. In overdose situations, however, glutathione levels are exhausted and NAPQI can directly modify susceptible protein residues in what is widely believed to be the first step in a cascade of biochemical events leading to hepatocyte death (Bessems et al., 2001; Adams et al., 2001).

In the present study APAP treated animals developed a significant hepatic damage and oxidative stress, which resulted in a marked increase in serum AST, ALT, SALP and total bilirubin levels. This is indicative of cellular
leakage and loss of functional integrity of cell membrane in liver (Drotman et al., 1978). However the total protein level was decreased.

There was a significant (P<0.001) restoration of these enzyme levels on administration of the ethanol extract in a dose dependent manner and also by silymarin at a dose of 25 mg/kg. The reversal of increased serum enzymes in acetaminophen induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew et al., 1987; Maiti et al., 2007). Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretory mechanism of the hepatic cells, as well as repair of hepatic tissue damage caused by APAP. This indicates that the anti-lipid peroxidation and/or adaptive nature of the systems is brought about by plant extract against the damaging effects of free radical produced by APAP.

The increase in TBARS level in liver induced by acetaminophen suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with CT significantly reverses these changes. Hence it is likely that the mechanism of hepatoprotection of CT is due to its antioxidant effect.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury (Curtis and Mortiz 1972). SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It
scavenges the superoxide anion to form hydrogen peroxide thus diminishing the toxic effect caused by this radical. The ethanolic extract of CT causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and its highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance and Greenstein, 1992). Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. A higher dose (500 mg/kg) of CT and silymarin increases the level of CAT.

Reductions of both GPx & GSH activity in APAP-treated rats as observed in this study indicate the damage to the hepatic cells. Administration of CT extract promoted the reactivation of hepatic glutathione reductase enzyme in APAP-treated rats. The increase in the level of GSH level after the administration of plant extract to such APAP treated rats was due to the protective effect of the ethanol extract of MV.

Severe centrilobular necrosis and fatty infiltration in hepatocytes was produced by acetaminophen. Treatment with different doses of ethanolic extract of CT produced only mild degenerative changes and absence of centrilobular necrosis, indicating CT treatment significantly reversed these signs of inflammation and necrosis, suggesting that CT treatment conferred hepatoprotectivity.
3.6 LIVER MITOCHONDRIAL STUDIES

3.6.1 Effect of CS and CT extract on liver mitochondrial antioxidants status in control and experimental animals

Oxidative stress affects cellular integrity only when antioxidant mechanisms are no longer capable of coping with the generation of free radicals (Adachi and Ishii, 2002). Acetaminophen administration induces oxidative stress by either enhancing the production of reactive oxygen species and/or decreasing the level of endogenous antioxidants. The deleterious effects of O2 and OH radicals in oxidative stress can be counteracted by antioxidant enzymes such as SOD, CAT and GPx. In addition to the enzymes, GST provides GSH and helps to neutralize the toxic electrophiles (Ananthan et al., 2004).

RESULTS

Table 6 and, Figs. 11, 11(a) depict the mitochondrial antioxidant status in control and experimental groups of rats. The activities of enzymic antioxidants (SOD, GPx and GST) were significantly lowered (P<0.001) in acetaminophen intoxicated rats (Group 2) when compared to control (Group 1). Pretreatment with CS extract at 250 mg/kg (Group 3) and 500 mg/kg (Group 4) significantly elevated the antioxidant status (P<0.01 and P<0.001) respectively.

Figs. 11, 11(a) show the activities of liver mitochondrial antioxidant enzymes namely, SOD, GPx and GST in control and experimental groups of rats. The activities of antioxidant enzymes were found to be significantly lowered (P<0.001) in acetaminophen intoxicated rats (Group 2) when compared
to control (Group 1). Pretreatment with CT extract at two different doses 250 mg/kg and 500 mg/kg (Group 6 & 7) significantly increased the antioxidant status (P<0.01; P<0.001) respectively. However the standard drug, silymarin treated animals also showed a significant (P<0.001) increase in liver mitochondrial antioxidant enzymes (SOD, GPx and GST) levels compared to Group 1.

**DISCUSSION**

SOD is a major intracellular enzyme, which protects against oxygen radicals by catalyzing the removal of the superoxide radicals. Acetaminophen administration has been shown to increase superoxide generation in liver mitochondria. The enhanced superoxide generation increases lipid peroxidation and induces mitochondrial dysfunction in rats that have been subjected to acetaminophen intoxication. Superoxide radicals produced by the respiratory chain are readily dismutated by mitochondrial SOD leading to the production of H$_2$O$_2$ (Kowaltowski and Vercesi, 1999; Yen et al., 1999) have also reported that mitochondrial SOD present in the mitochondrial matrix is a primary antioxidant enzyme that scavenges superoxide anions. Declined activity of SOD was noticed in rats intoxicated with acetaminophen which could be due to increased production of superoxides.

GPx metabolises peroxides such as H$_2$O$_2$ and protects cell membrane from lipid peroxidation. GPx has been shown to be an important adaptive response to condition of increased peroxidative stress (Ananthan et al., 2004). Mitochondria rely heavily on GPx activity for protection against the toxic effect of H$_2$O$_2$ production (Arai et al., 1984). GST is a group of
isoenzymes capable of detoxifying various exogenous and endogenous substances by conjugation with GSH (Vos and Van Bladeren, 1990). The depletion in the activity of these enzymes may result in the involvement of deleterious oxidative changes due to accumulation of toxic products (Venkateswaran and Pari, 2003). The results of the present study indicate that the GSH-dependent defense system in the mitochondria is defective in acetaminophen administered rats. The reduced activities of GPx and GST observed may be partly due to the lack of the substrate GSH along with the high degree of peroxides being formed (Ross, 1988) and also because of the oxidative modification of their protein structures.

Pretreatment with CS and CT extract elevated the antioxidant status in liver mitochondria. This may be due to the ability of CS and CT extract to interact with hydroxyl, superoxide, alkoxy and peroxy radicals, subsequently scavenging them indicating that both the plants have a potent antioxidant property.

3.6.2 Effect of CS and CT extract on the activities of mitochondrial enzymes in control and experimental animals

Mitochondrial membrane lipid peroxidation results in irreversible loss of mitochondrial functions such as mitochondrial respiration, oxidative phosphorylation and ion transport (Kowlatowski and Vercesi, 1999). Previous studies demonstrated that acetaminophen - induced hepatotoxicity resulted in dysfunctions in oxidative energy metabolism of rat liver mitochondria (Katyare and Satav, 1989; Burcham and Harman, 1990). Both in vivo and in vitro studies on the effect of acetaminophen and its metabolite NAPQI
showed alterations in cellular energy metabolism with changes in the respiratory rates, depletion of mitochondrial ATP content and alteration of energy coupling (Vendemiale et al., 1996). Enhanced mitochondrial lipid peroxidation has been reported to inactivate succinate oxidase, succinate dehydrogenase, isocitrate dehydrogenase and the components of the respiratory chain (Tretter et al., 1987).

**RESULTS**

Table 7 and Fig. 12, 12a represent the activities of TCA cycle enzymes (ICDH, α-KDH, SDH and MDH) and respiratory chain marker enzymes (NADH dehydrogenase and cytochrome C oxidase) in liver mitochondria of control and experimental groups of rats. A significant decrease (P<0.001) in enzyme activities brought about by acetaminophen intoxication (Group 2) in comparison with control (Group 1) was found to be significantly increased in group 3 (250 mg/kg; P<0.01) and group 4 (500 mg/kg; P<0.001) when pretreated with Caesalpinia sappan extract.

The activity of TCA cycle enzyme (ICDH, α-KDH, SDH and MDH) and respiratory chain marker enzymes (NADH dehydrogenase and cytochrome C oxidase) in the APAP treated group was significantly (P<0.001) decreased when compared to the normal animals (Group 1). Treatment with the ethanol extract of CT significantly (P<0.01 & P<0.001) (250 and 500 mg/kg; Group 6 & 7) prevented decrease in the level of respiratory chain markers and TCA cycle enzyme (Fig. 12, 12a) compared to the APAP induced rat (Group 2).

However the standard drug, silymarin treated animals also showed a significant (P<0.001) increase in TCA cycle enzymes (ICDH, α-KDH, SDH and
MDH) and respiratory chain marker enzymes (NADH dehydrogenase and cytochrome C oxidase).

DISCUSSION

TCA cycle enzymes

The activities of the enzymes (ICDH, α-KDH, SDH and MDH) involved in the aerobic oxidation of pyruvate in mitochondria were significantly lowered in acetaminophen intoxicated rats as compared with those in control. ICDH catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, NAD dependent ICDH requires Mn$^{2+}$ or Mg$^{2+}$ for its activity (Katyare and Satav, 1989). The decrease in pyruvate dehydrogenase activity leads to decrease in the formation of acetyl CoA and subsequently isocitrate. The low availability of the substrate, isocitrate may be the reason for the decrease in the activity of ICDH. The impairment in ICDH activity may also probably be due to acute decline in Mg$^{2+}$ or xenobiotic mediated oxidative damage. α-KDH is a mitochondrial enzyme complex that oxidatively decarboxylates α-ketoglutarate to succinyl CoA in the TCA cycle. Diminished α-KDH activity may underlie the mitochondrial abnormality in numerous disorders (Huang et al., 2003). Impairment of α-KDH was expected to reduce production of NADH by the TCA cycle and thus diminish mitochondrial membrane potential and the subsequent ability of the cell to synthesize ATP. SDH is known to contain a number of cysteine rich sulphur clusters and can be inhibited by a number of agents that modify sulphhydryl groups. NAPQI directly interacts with sulphhydryl groups on SDH, causing the loss of its activity (Burcham and Harman, 1990). The observed decrease in the activity of SDH may be due to the decrease in the level
of succinyl CoA, which resulted in the inhibition of α-KDH activity (Arathi and Sachdanandam, 2003).

Impaired activities of TCA cycle enzymes in liver suggest an impaired channeling and hence decreased oxidation of glucose via TCA cycle. Decreased availability of acetyl CoA from pyruvate owing to impaired glycolysis during liver injury may be the reason for the impaired activities of citric acid cycle enzymes. The rats pretreated with CS and CT extract showed considerable protection against impaired TCA cycle enzyme activities probably by preventing the excessive generation of NAPQI with concomitant improvement in the mitochondrial antioxidant defense system thereby protecting critical nucleophilic sites on the enzymes against toxic electrophilic metabolites.

**Respiratory marker enzymes**

Mitochondria carry out a variety of functions of which oxidative phosphorylation is the most important. Detoxification of oxygen via its reduction into H₂O₂ by the cytochrome oxidase system takes place in mitochondria. NADH dehydrogenase, a flavin-linked dehydrogenase and constituent of complex of the electron transport chain passes electrons from NADH to cytochrome Q. Cytochrome c oxidase; the terminal enzyme of the respiratory chain requires phospholipids for its optimal activity. Cytochrome C oxidase donates electrons directly to molecular oxygen and constitutes complex IV. These two enzymes are located in the inner mitochondrial membrane and their role is ultimately linked to the production of energy rich compounds like ATP. A significant decline in the activities of these enzymes as observed in this study would result in the inhibition of electron flow
from NADH to oxygen. It has also been reported that reduction in functioning of mitochondrial enzymes may be related to a defect in the mitochondrial energy production that would impair protein synthesis and energy production (Schultz and Chan, 2001). NAPQI, the toxic metabolite of acetaminophen was reported to arylate and oxidize essential protein sulphhydryls in the mitochondrial respiratory chain thereby limiting the ability of the mitochondria to meet the energy demand of the cell and affecting cellular energy homeostasis (Streeter et al., 1984).

The decrease in the activity of NADH dehydrogenase may be due to acetaminophen induced depletion of reducing equivalents NADH and NADPH, which are necessary for the formation of GSH from GSSG. Thus a decline in the levels of reducing equivalents decreases mitochondrial GSH content and thereby leads to loss of cytochrome C oxidase (Arathi and Sachdanandam, 2003; Schultz and Chan, 2001) reported that a decrease in mitochondrial content could result in the concomitant loss of oxidative phosphorylation capacity.

Acetaminophen induced the release of cytochrome C from mitochondria to the cytosol (Ferret et al., 2001; Adams et al., 2001; Knight and Jaeschke, 2002). It is known that cytochrome C translocation from mitochondria is a multistep process involving an increase in outer membrane permeability, the diffusion of a small pool of unbound cytochrome C and the dissociation of the bulk of the mitochondrial cytochrome c from cardiolipin binding site followed by its release from mitochondria (Ott et al., 2002). Any change in the lipid composition of the mitochondrial membrane may also decrease the activity of cytochrome C oxidase as in acetaminophen intoxicated rats (Arai et al., 1984).
Prior oral administration of CS and CT extract increased the activities of respiratory chain enzymes that highlight the protection rendered by the extract in combating the mitochondrial membrane damage induced by acetaminophen.

3.7 LIVER MICROSOMAL ENZYME STUDIES

3.7.1 Effect of CS and CT extract on microsomal enzymes in control and experimental animals

Biotransformation is the process of elimination of xenobiotics by the conversion of lipophilic molecules to hydrophilic molecules. Enzymes that catalyse the biotransformation reactions are called as drug metabolizing enzymes localized in microsomal fraction of the cell. The metabolism of xenobiotics often occurs by more than one sequential reaction. Any defects in the function of detoxification system may affect homeostasis of the cell. Acetaminophen is reported to affect the physical structure of the membranes of liver microsomes in vivo and in vitro (Minamide et al., 1992).

RESULTS

Table 8 and Fig. 13 represent the activities of heme oxygenase in liver microsomes of control and experimental groups of rats. A significant increase (P<0.001) in the activity of heme oxygenase was noticed in rats that ingested acetaminophen (Group 2) when compared to control (Group 1). On treatment with Caesalpinia sappanexract at two different doses (250 mg/kg and 500 mg/kg) before the toxic insult of acetaminophen was brought back to near normal as that of control.
Table 8 and Fig. 13 show the level of heme oxygenase in liver microsomes of control and experimental animals. The level of heme oxygenase was significantly increased (P<0.001) in liver microsomes of rats challenged with acetaminophen (Group 2) when compared with control rats (Group 1). Upon prior oral administration with CT extract at two different doses (250 mg/kg and 500 mg/kg; Group 6 and 7), the level of heme oxygenase was significantly decreased (P<0.01, P<0.001) respectively.

However the standard drug, silymarin treated animals also showed a significant (P<0.001) increase in heme oxygenase when compared with APAP induced group (Group 2).

**DISCUSSION**

Puntarulo and Cederbaum (1988) have found a close parallelism between formation of malondialdehyde and loss of microsomal enzyme activities. Peroxidation of membrane lipids has been shown to repress the membrane fluidity (Lee and Yu, 1998). Therefore the observed decrease in the activities of xenobiotic metabolizing enzymes in acetaminophen intoxicated rats could be due to the laceration in membrane fluidity.

Heme oxygenase, the rate limiting enzyme in heme catabolism catalyses the degradation of heme into biliverdin. The induction of heme oxygenase is a general response to oxidative stress. Increased activity of heme oxygenase leads to release of free iron, which is involved in Fenton’s reaction and thereby enhances oxidative damages. The reduction in the activity of heme oxygenase by both CS and CT extract may be related to the observation that the
heme oxygenase gene has a binding motif known as heme response element’, which includes the binding site for the well defined transcription factor, NF-kB (Sen and Packer, 1996). The activation of NF-kB is a free radical mediated process, which would have been inhibited by CS and CT extracts.

3.8 LIVER LYPOSOMAL ENZYME STUDIES

3.8.1 Effect of CS and CT extract on lysosomal enzymes in control and experimental animals

Lysosomes are a group of cytoplasmic organelles, present in numerous tissues characterized by their content of acid hydrolases, which are capable of digesting foreign substances (e.g. viruses and bacteria), damaged or non-functional cellular compartment and cancer cells. They play an increasingly prominent role in the field of pathology. Lysosomal membrane is reported to contain large amount of glycoproteins, which play an important role in its structure and function (Chen et al., 1985).

The intact lysosomal membrane also has oxidoreductase enzymes, thiol containing proteins and ubiquinones. The loss of integrity of lysosomal membrane and subsequent discharge of enzymes into the blood stream is a characteristic feature of hepatic diseases including acetaminophen intoxication. The measurement of the released lysosomal enzymes is a method of testing the integrity of lysosomes.

RESULTS

The activities of lysosomal enzymes (β-D-glucuronidase and β-D-galactosidase in liver of control and experimental groups of rats were shown
in Table 9: Fig. 14. On acetaminophen administration (Group 2), the activities of the lysosomal enzymes have been found to be significantly increased (P<0.001) when compared to control rats (Group 1). The activities of theses enzyme were found to be at near normal levels when pretreated with Caesalpinia sappan extracts at two different doses of 250 mg/kg and 500 mg/kg (Group 3 & 4).

The level of liver lysosomal enzymes (β-D-glucuronidase and β-D-galaactosidase) of control and experimental groups of rats is depicted in Figure 10. The level of lysosomal enzyme was considerably high (P<0.001) in acetaminophen-intoxicated rats (Group 2) when compared to control (Group 1). Pretreatment with CT extract at two different doses (250 & 500 mg/kg; Group 6 & Group 7) brought down the level of lysosomal enzyme to the same level as in the control.

However the standard drug, silymarin treated animals also showed a significant (P<0.001) increase in liver lysosomal enzymes (β-D-glucuronidase and β-D-galaactosidase when compared with APAP induced group (Group 2).

**DISCUSSION**

β-D-glucuronidase is a marker of lysosomal integrity and is released due to the presence of oxygen free radicals (Kalra et al., 1988). This enzyme seems to be an important index for the examination of the integrity of the lysosomal membrane and is responsible for the tissue damage and necrosis of hepatic tissue (Yasuda et al., 2000; Kanase et al., 1994).
Ricciutti (1972) suggested that the intracellular release of lysosomal enzymes precedes cellular death by initiating cellular injury process ultimately causing tissue necrosis. Increased activity of lysosomal enzymes in liver tissue may be due to the abnormal fragility of lysosome in hepatotoxic conditions. These enzymes trigger cell death in a variety of systems. Lysosomal hydrolases initiate apoptosis in diverse systems (Tata, 1994; Shibata et al., 1998). Lysosomal rupture and consequently the amount of hydrolytic enzymes released into the cytosol may induce reparable sub-lethal damage, apoptosis or necrosis (Brunk and Svensson, 1999; Kessel and Poretz, 2000).

The recouping of lysosomal enzymes upon pretreatment with CS and CT extract may be due to the stabilizing property of the extract on lysosomal membrane, which may protect the rapid leakage of enzymes and obstruct the rise in enzyme activity. The membrane stabilizing action of the extract may be due to the presence of flavonoids. The extract may modify the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and thereby prevent the discharge of acid hydrolases.

CHAPTER IV - NEPHROPROTECTIVE STUDIES

3.9 NEPHROPROTECTIVE EFFECT OF CS AND CT EXTRACT ON APAP INDUCED NEPHROTOXICITY IN ALBINO RATS

Acetaminophen remains one of the most effective, over-the-counter chemotherapeutic analgesic-antipyretic agents belonging to the para-aminophenol class of the non-steroidal anti-inflammatory drugs (NSAIDs) (Jackson-Robert II and Morrow, 2001).
Its acute or chronic high doses are reported to produce hepatotoxicity, but impairment of renal function by acetaminophen as the main untoward effect is being increasingly reported (McLaughlin et al., 1998; Fored et al., 2001). Acetaminophen nephropathy is characterized by alterations in urine volume, in glutathione status, creatinine clearance and increase in products of lipid peroxidation.

Acetaminophen nephropathy is closely associated with a significant decrease in the renal tissue concentration of glutathione and nitric oxide overproduction (Abdel-Zaher et al., 2007). Research into the etiopathological basis of acetaminophen nephrotoxicity has recently been encouraged (Henrich et al., 1996). However, despite recognition of acetaminophen nephrotoxicity and concerted scientific efforts directed into developing therapeutic or prophylactic agents to protect against acetaminophen nephrotoxicity, conventional chemotherapeutic options available to either treat or prevent its development, are still limited. In the absence of reliable and effective modern nephroprotective drugs and available traditional medicines employed for the disease treatment, concerted efforts are currently channelled toward exploring complementary or alternative chemotherapy in the disease treatment and/or prevention.

Herbal medicines have recently attracted much attention as alternative medicines useful for treating or preventing lifestyle related disorders of nephrotoxicity and relatively very little knowledge is available about their mode of action. There has been a growing interest in the analysis of plant products which has stimulated intense research on their potential health benefits.
Effect of CS and CT extract on serum urea, uric acid and creatinine concentrations

The serum levels of urea, uric acid and creatinine concentrations were used as biochemical markers to assess renal damage. Serum urea and creatinine concentrations were significantly increased (P<0.001) in the APAP treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity (Table 10; Fig. 15, 15(a)). Treatment with the ethanol extract of CS showed significant (P<0.01 & P<0.001) (Group 3 and 4) decrease in concentrations of serum urea and creatinine compared to the APAP treated group. However, the levels of uric acid (UA) significantly decreased (P<0.001) in the APAP treated groups (Group 2, Fig. 15(a)), when compared to the control group. Treatment with ethanol extract of CS significantly (P<0.01 & P<0.001) (Group 3 & 4 respectively) increased the uric acid levels, compared to the APAP treated group.

Rats treated with plant extract alone (group 5) did not show any significant effect on serum urea, uric acid and creatinine levels when compared to control. The activities of serum Urea and creatinine were found to be significantly increased (P<0.001) in APAP intoxicated rats (Group 2) and were brought back to near normal significantly (P<0.01 and P<0.001; Groups 6 and 7) upon CT pretreatment.

The levels of uric acid (UA) decreased significantly when compared with control, in APAP treated animals. However on treatment with CT extract at dose level of 250 mg and 500 mg, the level of uric acid increased significantly P<0.01 and P<0.001 in Group 6 and 7 respectively.
3.9.1 Effect of the CS and CT extract on kidney antioxidant status

The oxidative stress in the renal tissue was assessed by measuring the levels of thiobarbituric acid reactive substances (TBARS), lipid hydro peroxides and antioxidant defense enzymes viz., Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione-S- transferase (GST) in APAP administrated as well as plant extract treated groups.

The activity of CAT in the APAP treated group was significantly (P<0.001) decreased when compared to the normal animals (Group I). Treatment with the ethanol extract of CS at two different doses (250 mg/kg and 500 mg/kg; Group 3 and 4) significantly (P<0.01 & P<0.001) prevented the decrease in the level of catalase activity (Fig.15b) compared to the APAP induced rat (Group 2).

Likewise, the decreased GPx activity as a result of the treatment with APAP was also restored by the ethanol extract of CS (P<0.01 & P<0.001) (Fig. 15b) for Groups 3 & 4 as compared to the normal group. Renal SOD activity was decreased significantly (P<0.001) in the APAP treated (group 2) animals as compared to the normal group. Treatment with the extract (250 & 500 mg/kg) (Groups 3 & 4) significantly (P<0.01 & P<0.001 respectively) elevated the SOD levels as compared to the APAP induced (Group 2) animals (Table 10(a) and Fig. 15c).

The GSH and TBARS levels of APAP and extract treated animals are presented in (Table 10a and Fig. 15d). The GSH level reduced significantly (P<0.001) along with an increase in TBARS concentration in the APAP treated group as compared to the Group 1. However, on treatment with ethanol extract
of MV, the GSH level was found to be enhanced significantly (P<0.01 & P<0.001) and the TBARS contents were reduced in Groups 3 & 4 as compared to the induced group (Group 2) (Table 10a; Fig.15b and 15d).

The activities of CAT were found to be significantly increased (P<0.001) in APAP intoxicated rats (Group 2) and were brought back to near normal significantly (P<0.01 and P<0.001; Groups 6 and 7; 250 mg/kg and 500 mg/kg) upon CT pretreatment.

The decreased GPx activity on treatment with APAP (Group 2) reverted to normal level significantly upon pretreatment with two different doses of CT extract respectively (P<0.01 & P<0.001; Groups 6 & 7; 250 mg/kg & 500 mg/kg).

The levels of GSH and TBARS were decreased significantly in rats induced with APAP (Group 2). Pretreatment with CT extract at two different doses (250 mg/kg and 500 mg/kg) resulted in significant increase (P<0.01 & P<0.001) in the levels of both GSH and TBARS.

Rats treated with plant extract alone (group 5) did not show any significant effect on serum urea, uric acid and creatinine levels when compared to control (Group 1).

### 3.9.2 Effect of ethanol extract of CS and CT on hematological parameters

APAP caused a significant (P<0.001) decrease in the PCV levels (Fig. 15e) resulting in acetaminophen associated nephropathy. Administration of
ethanolic extract of CS significantly (P<0.001) increased the PCV values recorded for APAP hematotoxicity and also caused a significant (P<0.01) increase in MCV levels in APAP treated animals (Group 2, Table 10b; Fig. 15e).

However, the administration of ethanol extract of Caesalpinia sappan (Groups 3 & 4) reversed the significant (P<0.001) increase in MCV levels. Further, in the APAP treated group (Group 2), the levels of PLC&MCHC decreased significantly (P<0.001) when compared with control (Group 1) (Table 10 b; Fig. 15f, g). By the administration of ethanol extract of CS at two different doses (250 & 500 mg/kg) these levels returned to normal significantly (P<0.01 & P<0.001). Also the levels of PLC, APAP treated animals are decreased significantly (P<0.001) when compared with Group 1, even though by the administration of ethanol extract of CS (250 mg & 500 mg), the PLC level was increased significantly (P<0.05 & P<0.01 respectively) (Table 10b; Fig. 15f).

On pretreatment with CT extract at two different doses (250 mg & 500 mg/kg; Groups 6 & 7) decreased level of PCV were significantly increased (P<0.01 & P<0.001) in addition to a significant (P<0.001) increase in MCV levels in APAP treated animals.

Further, in the APAP treated group (Group 2), the levels of PLC & MCHC decreased significantly (P<0.001) when compared with control (Group 1) (Fig. 15F, g). By the administration of ethanol extract of CT at two different doses (250 & 500 mg/kg) these levels returned to normal significantly (P<0.01
& P<0.001). By the administration of ethanol extract of CT (250 mg & 500 mg), the PLC level increased significantly (P<0.05 & P<0.01 respectively) (Fig. 15f).

However plant extract alone treated rats (Group 5) did not show any significant changes in hematological parameters (PCV, PLC, MCV, DLC and MCV).

**DISCUSSION**

Acetaminophen overdose is often linked to many metabolic disorders including serum electrolyte, urea and creatinine dearrangements. Increased concentration of serum urea and creatinine are considered for investigating drug induced nephrotoxicity in animals and man (Rai *et al.*, 2006). The reason behind acetaminophen toxicology is the CYP-mediated conversion of acetaminophen to a highly reactive quinone imine, *N*-acetyl-*p*-benzoquinone imine. The fundamental role of NAPQI in the toxicity of acetaminophen has been supported by many subsequent studies (Dahlin *et al.*, 1984, Streeter *et al.*, 1984, Lowry *et al.*, 1951). Blood urea nitrogen is found in the liver protein that is derived from diet or tissue sources and is normally excreted in the urine. In renal disease, the serum urea accumulates because the rate of serum urea production exceeds the rate of clearance (Mayne *et al.*, 1994). Elevation of urea and creatinine levels in the serum was taken as the index of nephrotoxicity (Anwar *et al.*, 1999, Bennit *et al.*, 1982, Ali *et al.*, 2001). Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatinine breakdown (Mayne *et al.*, 1994). Thus serum urea concentration is often considered a more reliable renal function predictor than serum creatinine. In the present study, administration of APAP to rats resulted in nephrotoxic condition and development of oxidative stress
damage in renal tissues. In this study, APAP induced nephrotoxicity showed a significant (P<0.01) increase in the serum urea and creatinine concentrations in group 2 (APAP induced) rats when compared to the normal group (Group 1).

Oral administration of plant extract significantly (P<0.001) increased the uric acid level in groups 3 and 4 respectively, when compared to the APAP induced rats (Group 2).

Thus, oxidative stress and lipid peroxidation are early events related to radicals generated during the hepatic metabolism of APAP. Also the generation of reactive oxygen species has been proposed as a mechanism by which many chemicals can induce nephrotoxicity (Somani et al., 2000). Previous studies have clearly demonstrated that acute APAP overdose increases the lipid peroxidation and suppresses the antioxidant defense mechanisms in renal tissue (Abdel-Zaher et al., 2007). However, in the APAP treated animals, the TBARS levels are increased significantly, when compared to control rats. On administration of ethanol extract of CS and CT, the levels of TBARS decreased significantly when compared to APAP induced rats.

During kidney injury, superoxide radicals are generated at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages kidney. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism (Linares et al., 2006). The present study also demonstrated that acute APAP overdose resulted in a decrease in the SOD, CAT and GST activities, when compared to control rats. It is due to enhanced lipid peroxidation or inactivation of the antioxidative enzymes. When the rats were treated with the
ethanol extract of CS and CT the reduction of SOD, CAT and GST activity increased significantly when compared to the induced group (P<0.001) (Group 2).

Current evidence suggests that intracellular GSH plays an essential role in detoxification of APAP and prevention of APAP-induced toxicity in the liver and kidney (Newton et al., 1996, Richie et al., 1992). However, APAP was found to increase the microsomal superoxide and hydrogen peroxide production in mice. The generation of the reactive oxygen species appears as an early event which precedes intracellular GSH depletion and cell damage in APAP hepatotoxicity (Manov et al., 2003). APAP administration also caused a significant decrease in GSH content. Administration of ethanol extract of CS and CT helped to increase the GSH depletion induced by APAP.

Other nephroprotective medicinal plants have been reported to inhibit xenobiotic-induced nephrotoxicity in experimental animal models due to their potent anti-oxidant or free radicals scavenging effects (Devipriya et al., 1999, Annie et al., 2005). In addition, alkaloids have also been reported to strongly inhibit lipid peroxidation induced in isolated tissues via its antioxidant activity (Kumaran et al., 2007). The protection offered by the extract could have been due to the presence of flavonoids and alkaloids (Lucia et al., 2007).

The activity elicited by the extract might be due to its ability to activate antioxidant enzymes. The findings suggest the potential use of the ethanol extract of Caesalpinia sappanand Clitoria ternatea L. (CT) as a novel therapeutically useful nephroprotective agent. Therefore, further studies to
elucidate their mechanisms of action should be conducted to aid the discovery of new therapeutic agents for the treatment of renal diseases.

The various blood cells (erythrocytes, leucocytes, and platelets) are produced at a turnover rate of about 1 to 3 million per second in a healthy human adult and this value could be altered in certain physiological or pathological states including hemolytic anemia or suppressive inflammation (Guyton, 1991). Certain drugs including alkylating cytotoxic agents could also affect blood formation rate and the normal range of hematological parameters (Adeneye et al., 2008). Treatment with APAP oral dose significantly increased the PCV, DLC & MCV levels. After administration of CS and CT extract these levels decreased significantly compared to the APAP induced group, whereas the levels of granulocyte, MCH, MCHC and PLC were decreased significantly in the APAP treated group, compared to the control group. However after administration of CS and CT extract these levels are significantly increased compared to the APAP treated group. However this study shows that the CT extract could contain candidate molecules reversing the hematotoxic effect of acetaminophen, with ensuing improvement of hematopoiesis.

The recorded hematotoxicity could be secondary to the deleterious effect of acetaminophen on organs of hematopoiesis in the body which include liver and kidneys. Literature has shown acute or chronic large dose acetaminophen to be associated with overproduction of a highly reactive intermediate, N-acetyl-\(p\)-benzoquinone-imine (NAPQI), which covalently bound to macromolecules of renal tissues (Prescott, 1980; Fored et al., 2001) resulting in acetaminophen-associated nephropathy (Emeigh Hart et al., 1996).
However, oral treatment with CS and CT extract reversed the significant decrease in the PCV value recorded for acetaminophen hematotoxicity and also caused a significant ($P<0.001$, $P<0.001$) dose related increase in the TLC, and PCV, respectively. This study showed that the extract could contain active biological principle(s) reversing the hematotoxic effect of acetaminophen, with subsequent enhancement of hematopoiesis. The biological principle(s) could also be mediating hematopoietin-like effect or enhancing the release of hematopoietin from hematopoetic organs such as the kidneys or liver.

CHAPTER V - HISTOPATHOLOGICAL STUDIES

3.10 HISTOPATHOLOGICAL STUDIES OF APAP INDUCED TOXIC LIVER OF RATS AFTER TREATMENT WITH ETHANOLIC EXTRACTS OF CAESALPINIA SAPPAN (CS) AND Clitoria ternatea L. (CT)(CT)

Histopathological studies also provide supportive evidence for biochemical analysis. Histopathological examination of liver sections of normal groups showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (Plate -1(A). Disarrangement of normal hepatic cells with necrosis, massive fatty changes, vacuolization, ballooning degeneration, broad infiltration of the lymphocytes and kuffer cells around the central vein and the loss of cellular boundaries are observed in APAP intoxicated liver (Plate -1(B).

Histology of liver from rats which received Caesalpinia sappanethanolic extract at 250 mg/kg (Group 3) showed mild degenerative changes and absence of centrilobular necrosis (Plate - 1(C). Histology of liver
from rat which received Caesalpinia sappan ethanolic extract at 500 mg/kg (Group 4 showed normal hepatocytes with mild inflammation [Plate - 1(D)]. Liver section of rat treated with silymarin at 25 mg/kg showed less vacuole formation reduced sinusoidal dilation, less disarrangements and degeneration of hepatocytes [Plate - 1(E)]. All these results indicate the hepatoprotective potential of MV.

In the Clitoria ternatea L. (CT) extract treated rats, the liver cells showed complete reversal of toxic effects. No necrosis, vacuolization, ballooning degeneration or broad infiltrations of the lymphocytes were observed in this group. The central vein and portal triads appear normal. Some of the hepatocytes showed binucleation suggesting regenerative activity with feathery degeneration of hepatocytes. Micro and macro vesicular type of fatty droplets were also not seen (Plates: 1(F&G)).

This study was undertaken to demonstrate the restoration ability of ethanolic extract of CS & CT on the liver damage induced by APAP.

Histological changes such as steatosis (Fatty changes in hepatocytes), perivenular fibrosis and significant pathomorphological alteration were observed in APAP induced liver damaged groups. These changes can alter the properties of a cell. Morphologic features that include vacuolization, ballooning degeneration, inflammatory cell infiltrate and mixed macro and micro vesicular steatosis were observed in the liver of APAP induced liver damaged groups. Fat accumulation in the liver induced by APAP administration also seems to be associated with depletion of hepatic S-adenosylmethionine (SAM) leading to serious biochemical disturbances
including inhibition of essential methylation reaction. Inhibition of these methylation reactions lead to steatosis, apoptosis and accumulation of damaged proteins with isoasparty residues. Hepatic damage observed in the present study may be partially attributed to cytochrome P450 generated metabolic cytochrome-P450 dependant enzyme activities in the liver that tend to be present at their greatest concentration near the central vein and at their lowest near the peripheral site (Pieffer et al., 1979). This could be due to the formation of highly reactive free radicals because of oxidative threat caused by APAP. The free radical generation would lead to auto-oxidation of the fatty acid present in the cytoplasmic membrane phospholipids and cause functional and morphological changes in the cell membrane (Pandit et al., 2004). The accumulated hydroperoxides can cause cytotoxicity, which is associated with the peroxidation of membrane phospholipids by lipid peroxides (Kaneko et al., 2003).

All the changes observed were very much reduced in the animals after the oral administration of ethanolic extracts of MV. Histopathological observations suggested the ability of these extracts to condition the hepatic cells to a state of accelerated regeneration thus decreasing the leakage of ALT, AST and ALP into the circulation and increasing the antioxidant defense enzymes. In CT plant extract administered animals the hepatic cell architecture appeared to be almost normal. Based on the above results, it could be concluded that CT ethanolic extract is a more potent hepato-stimulant exerting a significant hepatoprotective action against APAP induced liver toxicity.

3.11 HISTOPATHOLOGICAL STUDIES OF APAP INDUCED NEPHROTOXICITY IN ALBINO RATS AFTER TREATMENT
WITH ETHANOLIC EXTRACTS OF _CAESALPINIA SAPPAN_ (CS) AND _Clitoria ternatea_ L. (CT)

The biochemical results were also confirmed by the histological pattern of normal kidney showing normal tubular brush borders and intact glomeruli and Bowman’s capsule (Plate 2(A)). Severe tubular necrosis and degeneration is shown in the renal tissue on treatment with acetaminophen (Plate 2(B)). The rats treated with ethanol extract of _Caesalpinia sappan_ (250 mg/kg body weight) showed normal tubular pattern with a mild degree of swelling, necrosis and degranulation (Plate 2(C)). Treatment with the ethanol extract of _Caesalpinia sappan_ (500 mg/kg body weight) ameliorated the toxic manifestations in the kidney (Plate 2(D)).

The rats treated with ethanol _Clitoria ternatea_ L. (CT) extract (250 mg/kg body weight) showed normal tubular pattern with a mild degree of swelling, necrosis and degranulation [Plate 2(E)]. Treatment with the ethanol extract of CT (500 mg/kg body weight) ameliorated the toxic manifestations in the kidney [Plate 2(F)], whereas administration of CT extract (500 mg/kg) alone did not show any significant changes in the renal tissues [Plate 2(G)].

APAP-induced nephrotoxicity was evidenced by biochemical measurements and histopathological changes that coincide with the observations of other investigators (Gardner _et al._, 2002; Newton _et al._, 1983; Trumper _et al._, 1998). The biochemical results were also confirmed by the histological findings which showed preservation of the glomeruli and the surrounding Bowman’s capsule and mildly swollen tubules. Other nephroprotective medicinal plants have been reported to inhibit xenobiotic-induced nephrotoxicity in experimental
animal models due to their potent anti-oxidant or free radicals scavenging effects (Devipriya et al., 1999, Annie et al., 2005).