Chapter VI

DISCUSSION
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The traditional medicine all over the world is now adays revalued by extensive activity of research on different plant species and their therapeutic principles. Experimental evidences suggest that free radicals (FR) and reactive oxygen species (ROS) can be involved in a number of diseases. As plants produce a lot of antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Hepatoprotective studies have attracted the attention of many research scholars in recent years. Animal experiments and clinical studies in this field are mostly centered on identification of the drugs as well as evaluation of various factors involved in hepatic dysfunction or degeneration. The present study is an attempt in identifying such a drug, its phytochemical screening, and isolation of phytoconstituents from active fraction, their structure elucidation and the hepatoprotective / curative studies of the active fraction.

In Ayurveda and Homeopathy many herbal drugs are used as preventive / curative in hepatitis. The present study was focussed on the screening of the plant Acalypha indica Linn (Euphorbiaceae) phytochemically and pharmacologically. The phytochemical screening of A. indica revealed
the presence of phytosterols, alkaloids, carbohydrates, saponins, tannins, diterpenes, flavonoids and gums (Table 4.2). The presence of some of the chemical constituents were reported earlier by various authors.247-251

In indigenous system of medicine the doses of crude extracts or any other drug are not determined after a systematic toxicity study. Hence an acute toxicity study was carried out in Albino mice and rats to determine LD$_{50}$ value. The maximum dose administered was 2gm/kg body weight. Based on this a pilot study of hepatoprotective activity of different extracts was conducted to find out the most effective one. Then a dose response assay was carried out to find out the minimum dose that could exert maximum hepatoprotective activity; this was selected for further programmed studies (100mg/kg). (Table 5.14& 5.15)

The gross behavioural studies of all the extracts showed that there were no stimulant effects in any of the doses given, but a depressant effect (ethanol extract) from 250mg/kg onwards; but practically no death was observed in any of the extracts. (Table 5.9 to 5.13)

In the present study a significant elevation of transaminases (Aspartate amino transferase – AST and Alanine amino transferase – ALT), alkaline phosphatase (ALKP) and total bilirubin in the serum of rats treated with CCl$_4$ was observed. This is due to necrosis or break down of hepatocytes$^{126,140}$. 
Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver.\textsuperscript{105}

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver diseases\textsuperscript{292}. The hepatotoxic effects of CCl\textsubscript{4} are largely due to the active metabolite, trichloromethyl radical.\textsuperscript{292-293} This activated radical binds covalently to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids (PUFA). This leads to the formation of lipid peroxides which in turn give products like malondialdehyde (MDA) that causes damage to the membranes. This lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl\textsubscript{4}.\textsuperscript{294-295} This is evidenced by an elevation in the serum marker enzymes mainly AST, ALT, and ALKP.

Carbon tetrachloride is a pharmacological tool used to produce liver damage in animal models. Its hepatotoxic action begins with changes in endoplasmic reticulum which results in loss of metabolic enzymes located in the intracellular structure.\textsuperscript{296-297}

Another explanation of hepatotoxicity of CCl\textsubscript{4} has been found to depend on its metabolism by Cytochrome P\textsubscript{450} to free radicals that initiate formation of lipid peroxides from membrane unsaturated lipids\textsuperscript{298} or conjugate with glutathione leading to its depletion.\textsuperscript{299} The datas available here
support this hypothesis as CCl₄ intoxication caused elevation of lipid peroxidation, leakage of AST, ALT and ALKP, depletion of GSH and other antioxidants, culminating in liver damage. There are reports which state that a single intraperitoneal injection of CCl₄ can produce an elevation in the activity of serum transaminases and alkaline phosphatase. Marked increase in the levels of transaminases and alkaline phosphatase in the CCl₄ poisoned rabbits as compared to normals was observed by Pandey and Chaturvedi. Recently Adamska and Mlynarezyk reported about increase in transaminases along with other hepatic enzymes like sorbitol dehydrogenase and glutathione reductase after CCl₄ administration. There are many other reports in support to the above findings. Srinivasan and Recknagel have described, a different aspect, about the increase in AST and ALT levels preferably in female rats, after CCl₄ intoxication.

In liver, aspartate and alanine transferases are present in high concentration. Due to necrosis of hepatocytes or abnormal membrane permeability, these enzymes are released from the cell and their levels in the blood increase. Alanine amino transferase (ALT) is a sensitive indicator of acute liver damage and elevation of this in nonhepatic disease is unusual. ALT is more selectively a liver parenchymal cell enzyme than is aspartate amino transferase (AST) The transferases being more stable than other enzymes, are better indices for evaluating the extend of hepatic
Alkaline phosphatase is a membrane bound enzyme and its elevation in plasma indicates membrane disruption in the organ.\textsuperscript{9,10,11}

Liver tissue rich in both transaminases (AST and ALT) contain more ALT than AST. While both transaminases are elevated in sera of patients with acute hepatic diseases, ALT that is slightly increased by cardiac necrosis is a more specific indicator of liver damage.\textsuperscript{193, 307} According to Harper et al\textsuperscript{98} AST, ALT and serum bilirubin are the most sensitive tests employed in the diagnosis of hepatic diseases.

Rats treated with CCl\textsubscript{4} showed significant decrease in the levels of total protein and total albumin. Bahar Ahmed et al\textsuperscript{102} and Padma GM Rao et al\textsuperscript{268} and Ashok Shenoy et al\textsuperscript{107} have already given similar reports. Administration of \textit{Acalypha indica} increased the levels of total protein and albumin in the serum indicating hepatoprotective activity. Stimulation of protein synthesis has been advanced as a contributory mechanism accelerating the regeneration process and production of liver cells.\textsuperscript{308}

Histopathological reports revealed the presence of perivenular necrosis, congested sinusoids and inflammation of hepatocytes in CCl\textsubscript{4} -treated animals (after acute hepatoprotective studies) where as it was reversed by the administration of the test drug extract. In the test group normal architecture was retained and mild inflammation was observed. There are
many similar reports where plant drugs have produced such effects on CCl₄-treated animals.¹⁵¹,¹⁵₈,¹⁶²,²₆₈

Studies employing drugs and antioxidants in CCl₄ induced damage have proved that they act differently when given prior to or after CCl₄ poisoning. Hence two types of experiments had been conducted. In one, drug was given after CCl₄ poisoning and in the other CCl₄ was given after drug and the same biochemical pattern studied. This was the basis for curative study.

In the curative study also, there was significant reduction in the elevated levels of biochemical parameters and this is in agreement with that of other published reports¹⁰⁷,¹²⁷,²₆₈. Even though there was significant reduction in the elevated levels of parameters like AST, ALT, ALKP and serum bilirubin of pretreated animals, the results were more significant in the post-treated group. Compared to the toxin induced group the activities of the above said parameters were decreased to more than 50% in the post treated group where as the decrease was 32.28% to 50.43% in the pre-treated group. There are many reports of pre-treatment or post-treatment that resulted in significant hepatoprotective / curative effect.¹³⁰,³⁰⁹-³¹¹

Increased lipid peroxidation in liver was another phenomenon noted in experimental animals. There was significant increase in the activity of malondialdehyde in the liver of CCl₄-treated animals. Many reports of increased lipid peroxidation in animals treated with CCl₄ are available.⁸⁸,¹⁹⁸,¹⁹⁹
Dianzani et al.\textsuperscript{97} have demonstrated increased lipid peroxidation as a frequent feature in animal liver after administration of CC\textsubscript{4}.

The glutathione system is an important endogenous antioxidant found particularly in high concentration in the liver and is known to have key functions in protective process. The reduced form of GSH becomes readily oxidized to GSSG on interacting with free radicals. Excessive production of free radicals result in oxidative stress. It leads to damage of macromolecules. eg. free radicals can induce lipid peroxidation in vivo\textsuperscript{312}. In our study, elevated level of lipid peroxides and depletion in GSH concentration in the livers of rats induced with CC\textsubscript{4} and paracetamol were observed. In the curative studies pre as well as post treatment with \textit{Acalypha indica} extract reduced the increased levels of MDA. Also it enhanced the concentration of Glutathione (GSH), as well as free radical scavenging enzymes like Glutathione peroxidase (GPx) Glutathione reductase (GR) and Superoxide dismutase (SOD) in the drug treated groups establishing the anti-oxidant defence mechanism. Since \textit{A.indica} reduced the lipid peroxidation and increased the activity of GSH,GPx, GR and SOD of rats, it is possible that mechanism of hepatoprotective / curative action might be due to the presence of antioxidants like flavonols, polyphenols, kauranes etc. present in the extract.
It is well established that generation of reactive oxygen species (O$_2$, H$_2$O$_2$ and OH$^-$) from the incomplete reduction of molecular oxygen is closely related to cellular damage. But these species may not always lead to injury that depends on the status of antioxidant defense mechanisms\textsuperscript{313} such as SOD, GPx, Catalase, GR and GST. Superoxide dismutase, a principal protective enzyme dismutates to H$_2$O$_2$ and oxygen; H$_2$O$_2$ produced can then be decomposed enzymatically by catalase or by GPx. Glutathione peroxidase not only decomposes H$_2$O$_2$ but also interacts with lipid peroxides. Endogenous thiols and GSH in particular has been working as the most important defense against cytotoxicity\textsuperscript{314}. Since GR affects reduction of GSH, the level of this enzyme also is of importance in the detoxification of peroxides.

An effective defense against oxidative damage is the GSH cycle. As reported earlier GSH is oxidized to GSSG during detoxification of peroxidies by GPx and GST and then reduction of GSSG to GSH by GR. Increased amount of GSSG is transported out of cells to maintain the normal ratio. But when accumulated inside the cell, GSSG creates oxidative stress and thereby various cellular components become vulnerable to damage by ROS, mainly membrane lipids, proteins and DNA. Decreased activities of GST, GPx, GR and SOD in CCl$_4$ treated rats may increase their susceptibility to oxidative injury. The GSH / GSSG ratio is maintained by enzymatic activities of GR and GPx. Glutathione Reductase (GR) converts GSSG to GSH in presence of
NADPH while GPx acts as an antioxidant. GSH also participates in the transport of amino acids via the $\gamma$-glutamyl cycle and protein synthesis, where in the first enzyme involved is $\gamma$-glutamyl transpeptidase. The overexpression of antioxidant drugs is indicative of their ability to reactivate hepatocellular antioxidant defence in the liver. The protection offered by the extract may be due to the presence of molecules having glycosidic linkage or $\text{-OH}$ moieties which can react directly with chain carrying radicals terminating their propagation accounting for their antihepatotoxicity.

It is to be mentioned that histochemical lesions of CCl$_4$ poisoning could very well be reduced by the administration of $A.indica$ extract. If perivenular necrosis and ballooning degeneration were produced in the toxin treated group, only mild inflammation with slight degenerative changes was observed in the preventive group and normal architecture was visible in the protective and curative groups. It is likely that the protective effect of this drug could prevent fatty infiltration, but not enzyme leak.

Liver injury by CCl$_4$ is known to involve both fatty infiltration and centrilobular necrosis and these are two distinct and independent phenomena. Substances, which prevent fatty changes, need not necessarily prevent liver necrosis and vice versa. A wide array of compounds has been tested against CCl$_4$ liver injury and it has been found that some may prevent necrosis, others
change fatty contents and yet a third group may prevent both. In the present study *A. indica* could prevent necrosis and fatty changes.

An obvious sign of hepatic injury is the leakage of cellular enzymes into the plasma. It is established that serum enzymes such as ALT and AST levels are elevated in paracetamol- induced hepatotoxicity. In the present study elevation in the levels of AST, ALT and ALKP were found in paracetamol treated animals. But after drug treatment this condition was reversed. The observed reversal produced by the drug in serum biochemical parameters may be a manifestation of the reduction in the cell membrane disturbances.

Paracetamol produce hepatic necrosis when ingested in very large doses. It is metabolised in the liver primarily to glucuronide and sulphate conjugates. Paracetamol toxicity is due to the formation of an active metabolite N-acetyl – p-benzoquinone imine, by the cytochrome P_450 microsomal enzyme system which results in an oxidative stress producing liver glutathione depletion and hepatic necrosis. So under oxidative stress, the hepatocytic membrane appears to be the critical locus of damage and oxidative alterations are responsible for membrane damage in paracetamol- induced hepatotoxicity in rats. In the present study paracetamol intoxication resulted in depletion of glutathione and hepatic necrosis which is in concurrence with published reports. Hence it
can be postulated that co-valent bonding of paracetamol and its metabolites to cellular proteins may induce a series of events, which produce hepatocellular necrosis. This observation is well correlated with histological changes in hepatic parenchyma described as cloudy swelling which is an early indicator of degenerative changes and later necrosis. As administration of \textit{A.indica} increased glutathione content and reduced hepatic necrosis it can be said that the hepatoprotective activity of the drug may be due to inhibition of cytochrome P$_{450}$ and consequent glucuronidation; also there is a depression in hepatic degeneration, activation of the functions of reticulo-endothelial system and inhibition of protein biosynthesis.

In choleretic study, a significant increase in the bile flow rate was observed (69.64\%) when compared to the control group. Reports of Subramoniam et al.$^{163}$ and Asha$^{269}$ are in concurrence with the present results. There are other results also establishing the said effect.$^{190,196,322,323}$

In liver regeneration study also a marked increase in the macromolecular constituents like RNA, DNA, protein and cholesterol could be observed after partial hepatectomy in drug-treated animals when compared to sham operated and untreated ones. There was a significant hike in the levels of all the above said parameters at the 36$^{\text{th}}$ hour. Later another hike was observed at the 120$^{\text{th}}$ hour. Similar effect was observed for the standard drug silymarin also. After the 120$^{\text{th}}$ hour all the results were found
gradually decreasing till they reached the levels of the sham operated ones. These results are supported by the previous reports of Srivastava et al.\textsuperscript{204,205}

When the rat is subjected to partial hepatectomy, a compensatory hyperplasia occurs in the remnant liver, which is initially consistent with prompt increase in macromolecular levels.\textsuperscript{324} The increase in the level of macromolecular constituents like DNA, RNA, protein and cholesterol is associated with the growth of remnant liver. Results of the present study indicate that \textit{A. indica} enhances DNA, RNA, protein and cholesterol levels like that of silymarin in regenerating rat liver after partial hepatectomy.

Recent studies on the process of cell division show the presence of several stages which can be termed as sub-phases or check points\textsuperscript{325} suggesting that the classical designation of four phases (G\textsubscript{1}, S, G\textsubscript{2}, M) may only serve as organizing principles. When quiescent state cells are triggered to enter into G\textsubscript{1}/S phase by mitogens, a complex series of molecular events occur which culminates in DNA synthesis. G\textsubscript{1} events require many hours and they appear sequentially terminating in DNA synthesis. In regenerating liver RNA synthesis is also induced and coincides with the DNA synthesis.\textsuperscript{205,326-327} Both DNA and RNA synthesis are further enhanced in regenerating liver by \textit{A. indica} and the effect is observed between 36 and 120 hours. These results suggest that \textit{A.indica} accelerates liver regeneration following partial hepatectomy by enhancing macromolecular synthesis and this effect occurs in
the early phase of regeneration. Similar results were obtained for silymarin also.\textsuperscript{205-206}

Phytochemical investigations of the most effective extract of \textit{A. indica}, as evidenced in the study could confirm the presence of some of the active constituents (5 compounds) present in it. They were identified as:-

1. Kaur-en-18-oic acid
2. 3, 4, 5 – trihydroxy benzoic acid (gallic acid)
3. 16\(\alpha\), 17- dihydroxy – ent – kauran 19 – oic acid
4. 3, 3\(^1\), 4\(^1\), 5, 7 – pentahydroxy flavone (quercetin)
5. 4,4',5,5',6,6'hexahydro diphenic acid 2,6,2',6'-dilactone (ellagic acid)

There are reports showing that hepatoprotective plant principles possess antioxidant activity also.\textsuperscript{328,329} In the present study also the ethanol extract has shown significant antioxidant activity. Ethanol extract of \textit{A.indica} leaves produced 50% inhibition in lipid peroxidation at a concentration of 800\(\mu\)g/ml. Similarly it could scavenge (\(\text{IC}_{50}\)) superoxide and hydroxyl radicals in the concentrations of 83\(\mu\)g/ml and 190 \(\mu\)g/ml respectively. Hence the activity can be believed to be due to the active constituents present (isolated) in it, chiefly flavonols, polyphenols (tannins) and kauranes.

The highest antioxidant activity was established for the synthetic antioxidants like BHT and BHA; slightly lower but still high, antioxidant
activity was shown by a homogenous group that included fisetin, kaemferol, galangin, quercetin, robinetin, morin and kaemferide, which are flavonols with a free hydroxyl group at the C-3 position. This suggests that the flavonol C-3 hydroxyl group is responsible for the high inhibition of β carotene oxidation in the heterogenous system. According to Chung et al, flavonols with a free 3-OH group has a high antioxidant potential than its substituted derivative. As shown by Russo and co-workers, on the basis of semiempirical calculations, radicals formed by H· removal from hydroxyls at C-3 and C- 4' may be involved in the antioxidant properties of quercetin. Also the ability to inhibit β carotene oxidation by flavonoids depends primarily on the free hydroxyls at C-3 and double bond between C-2 and C-3. According to Rice-Evans, flavanoids, which are derivatives of benzo-γ-pyrone, are wide spread in nature having the property of inhibiting reactive oxygen and nitrogen species. The presence of an ortho hydroxylation on the B-ring of the flavonoid molecule, the number of free OH groups, C2-C3 double bond in the C-ring and ortho presence of a 3-hydroxyl group are usually listed as conditions of antioxidant and anti free radical activities.

Polyphenols (tannins) are also proved to have antioxidant activity. According to Ho et al, polyphenol extracts prepared from various Chinese Tea containing gallic acid along with (-) epicatechin, (-) epigallo catechin etc produced a very significant antioxidant property.
In the present study quercetin (flavonol), gallic acid and ellagic acid (polyphenols) have been isolated from the extract. Hence the antioxidant property of the extract, may be due to the presence of these compounds.

Oxidative stress due to oxygen and various radical species is associated with the induction of DNA-single and double strand breaks and is considered to be a first step in several human degenerative diseases, cancer and aging. Naturally occurring antioxidants are being extensively used for their ability to protect DNA against such injury. According to Festa et al\textsuperscript{339}, ellagic acid produced a marked reduction of $\text{H}_2\text{O}_2$ and Bleomycin induced DNA damage. Wada et al\textsuperscript{340} have explained the oxygen radical absorbance capacity of ellagic acid (47-90 mg/g), which is higher than other polyphenols and anthocyanins.

Recent reports about antioxidant activity of gallic acid was published by Conca et al\textsuperscript{341} where antioxidant activity of gallic acid and its component phenols from mango puree are explained.

According to the published reports, gallic acid\textsuperscript{342}, ellagic acid\textsuperscript{343-344} and quercetin\textsuperscript{149} possess hepatoprotective activity also. Kauranes are diterpenes, which are anti inflammatory in nature and are reported to have hepatoprotective activity\textsuperscript{313}.

Atomic absorption spectroscopy revealed the presence of zinc in the leaves of \textit{Acalypha indica} (16 ppm), which is noteworthy. This finding is
consistent with the hypothesis that hepatic lipid peroxidation plays an important role in the aetiology of hepatic fibrogenesis and that Zinc mitigates this process. Dhawan et al\textsuperscript{108} have described the protective effect of Zinc treatment on the activities of various drug metabolizing enzymes, reduced glutathione content and extent of lipid peroxidation in liver of male rats subjected to long term CCl\textsubscript{4} toxicity and concluded that Zinc supplementation considerably attenuated the liver injury induced by chronic CCl\textsubscript{4} treatment to rats.

Thus it is seen that \textit{A.indica} leaf has got marked hepatoprotective activity as evidenced in albino rats and the activity is mediated through decreased production of free radical derivatives, and anti-oxidant activity exerted by the phytoconstituents present in it; also it prevents cellular leakage and there by protect the integrity of the cell membrane in liver.