Discussion
5. DISCUSSION

The present study was performed to unveil the mystery behind the traditional use of *Cajanus indicus* in India for the treatment of hepatomegally with jaundice. A number of plants have been screened for the treatment of liver disorder, but so far not much work has been done for evaluating the hepatoprotective activity of *Cajanus indicus*. Eventually, the present endeavour is to develop a new drug scientifically, responsible for hepatoprotection from the above plant.

In our present study, some preliminary investigations were carried out on the crude extract of the leaves of *Cajanus indicus*. The extract was found to possess an antihepatotoxic activity against carbon tetrachloride induced toxicity in rodents. The whole experimental design including purification of the active principle was planned accordingly. Silymarin, isolated from *Silybum marianum* in late 1960's is now used as a hepatoprotective compound. Similarly, Ayurvedic drugs like Arogya-wardhani, Punarnavadi kwath and many other herbal formulations containing the aqueous extracts of medicinal plants, have been evaluated for their efficacy in the treatment of viral hepatitis by double-blind, controlled, randomized clinical trials. Studies on medicinal plants like *Phyllanthus amarus, Eclipta alba, Tephrosea purpurea, Ocimum sanctum, Picrorhiza kurroa, Phyllanthus niruri* and many others have been made and their hepatoprotective activity has been screened in CCl4 induced liver damage.

The seeds of *Cajanus indicus*, are the rich source of lenticels and consumed by Indian people in their day to day life. It is widely cultivated as an annual crop and is a rich source of vegetable protein. The protein designated CI-1, has been isolated from the leaves of *Cajanus indicus* by the present candidate. It has been found to possess marked hepatoprotective activity against hepatotoxic agents namely carbon tetrachloride, paracetamol, β-galactosamine HCl and ethanol.

Analysis of the purified protein by DEAE cellulose and SDS-PAGE followed by silver nitrate staining demonstrated that CI-1 is a single polypeptide with an apparent molecular mass of 40-42 kDa. Periodic acid staining and Anthrone test showed that this single polypeptide contains carbohydrate moiety. Iso-electric pl of the protein, CI-1 is 4.6. Toxicity level, LD50 of CI-1 was found to be 40.0 mg/Kg i.p. in Swiss albino mice and 55.6 mg/Kg i.p. in Charles Foster rats. In
subsequent hepatoprotective studies 1.5-6.0 mg/Kg i.p. was the dose selected in rodents. The protein CI-1 was found to be nontoxic in the above doses. The overall purification achieved was 30.11 fold with a yield of 6.4%.

Carbon tetrachloride was used as a model for systemic studies to evaluate the process of repair of damaged and disturbed liver function by CI-1. The mechanism of CCl₄-induced hepatic damage is well known. It is considered as a standard reference compound in hepatotoxic studies. Hepatic damages induced by CCl₄ are very severe, causing hepatic centrilobular necrosis, cirrhosis and massive infiltration which are not common clinically. Experimental studies in our laboratory reveal the development of ascitis in CCl₄ treated mice. Therefore, CCl₄ has been taken as a model for experimental liver damage to evaluate the therapeutic efficacy of the protein fraction isolated from Cajanus indicus. Biochemical evidence of hepatic injury often includes greatly elevated activities of transaminases, phosphatases, serum bilirubin and variety of other hepatic enzymes which are recognized as a sensitive marker of hepatic injury. As such the efficacy of a new drug should be reflected and assessed by the evaluation of those parameters. A significant rise in serum bilirubin and transaminases were observed in CCl₄ treated animals. Treatment with the active fraction caused an appreciable reduction in the concentration of SGOT, SGPT, ALP activities including serum bilirubin. It is known that CCl₄ toxicity is dependent on one of its highly reactive product the trichloromethyl free radical (•CCI₃) which binds covalently to neighbouring proteins and lipids, initiates lipid peroxidation causing severe membrane alterations. Transaminases leaks out through damaged membrane, elevating serum levels. Inhibition of CCl₄ bioactivation is either by decreasing the production of •CCI₃ (free radical) or by impairment of CCl₄ induced lipid peroxidation.

β-galactosamine induced hepatic damage has been extensively used as an experimental model because it histologically resembles viral hepatitis. Studies in our laboratory has been made to produce a true replica of hepatic damage found in clinical conditions with β-galactosamine. It brings about an increase in cell membrane permeability leading to enzyme leakage and eventually cell death. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of the cell membrane in liver.
Paracetamol or Acetaminophen, is a common household analgesic. However, acute overdose of paracetamol produces massive hepatic and centrilobular necrosis leading to hepatic failure, hence, it has been selected in our study. Hepatotoxicity by paracetamol is attributed to the formation of toxic metabolite NAPQI which bring about a cascade of events resulting in hepatocellular death. Alcoholic fatty liver on continuous consumption lead to serious liver disorder throughout the world and specially in India, due to chronic over indulgence of non-standardized country made liquor. Alteration in liver functions due to alcoholism range from fatty liver to hepatitis and ultimately development of cirrhosis. The accumulation of fat or fatty liver is an early event after ingestion of ethanol and is produced in the experimental animals in our laboratory. Acetaldehyde is produced which is the reactive and toxic substance that can form adducts with proteins and other compounds, leading to inhibition of a wide variety of enzymes and generation of immunogenic derivatives.

When the liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released in the blood stream. If the injury involves other organelles, such as mitochondria, then soluble enzymes will also be similarly released. Their estimations in the serum are useful quantitative markers of the extent and type of damage. It was interesting to observe that serum transaminases, lactate dehydrogenase, bilirubin were markedly increased in paracetamol, β-galactosamine and ethanol induced hepatotoxicity in mice. Alkaline phosphatase activity is, however, only slightly elevated. The plasma prothrombin time was severely prolonged and levels of serum total protein, albumin were markedly reduced. Treatment with the purified protein, CI-1 offered hepatoprotection as evidenced by the decreased levels of serum and tissue (liver) GOT, GPT, ALP activities. The present study indicates that CI-1 accelerates the synthesis of hepatic proteins such as clotting proteins, serum total protein and albumin in different models causing hepatic damage.

To confirm the hepatoprotective activity of CI-1 in vitro studies were also carried out using freshly isolated rat hepatocytes in β-galactosamine induced toxicity. A significant degree of protection by CI-1 was observed, although the exact mechanism of action is yet to be fully elucidated. Apparently, hepatotoxicants and hepatoprotectives molecules interact with the constituents of cellular and intracellular membranes and alter their functions. β-galactosamine
decreased the levels of GOT, GPT and ALP activities in isolated hepatocytes suspension, whereas, increased enzyme levels in the serum. CI-1 restored the levels of the enzymes to normal, both in isolated hepatocytes and serum. β-galactosamine caused leakage of enzymes from the cells due to altered membrane permeability, which results in decreased levels of GOT, GPT and ALP in hepatic cells and a raised level in serum. The restoration of the enzyme level to normal both in hepatocytes and in serum further suggests the action of CI-1 on the cell membrane like silymarin. The hepatoprotective activity of CI-1 was compared with a standard hepatoprotective agent, Silymarin.

The chief histological abnormalities include hepatic steatosis, hepatocytic necrosis in the central lobule, specially in the areas surrounding the central vein, marked degeneration and hydropic changes with occasional fat cysts. CI-1 treatment protected the hepatic tissue from necrosis, a very few inflammatory cells and cytoplasmic vacuolations were observed, which suggests that the hepatoprotective action may be due to its membrane stabilising effect on hepatic cells, accelerating regeneration of hepatocytes as in silymarin. Light microscopical findings were supported by the electron microscopical studies on the liver. The appearance of binucleated cells, increased number of mitochondria and augmented number of rough and smooth endoplasmic reticulum around the nucleus are the indication of cell division in CI-1 treated animals after hepatotoxicity.

The herbal protein CI-1, was able to protect the liver against a wide range of hepatotoxins. The probable mechanism of protection could be by (i) inhibition of triglyceride accumulation in injured liver, an effect which would protect the liver from development of cell necrosis, (ii) inhibition of ethanol metabolising enzymes, (iii) prevention of binding of acetaldehyde to liver cell proteins (iv) prevention of GSH depletion and induction of glutathione transferase and reductase and (v) prevention of stimulation of cytochrome P450. However, the exact mechanism by which the herbal protein, CI-1 provides protection is still obscure.

Clinical trials of novel plant molecule, started with Silymarin and Phyllanthus amarus, for the treatment of liver disorders. Recent studies have reported that 50% ethanol extract of Picrorhiza kurroa leaves stimulate cell-mediated and humoral components of the immune system as well as phagocytosis in experimental animals. The augmentation of the
humoral response to SRBC by *Picrorhiza kurroa*, as evidenced by increase in number of plaque forming cell (PFC) and haemagglutination antibody titre in mice and rats\(^{33,56}\). Recently the mechanism of action of Silymarin, the immunomodulatory role has been postulated\(^{70-71}\).

Advancement on the mechanism of action of the Ayurvedic drugs is recent, but with unknown, unexplained and undeciphered mechanism, they are being considered potential drug for protection of dearranged hepatocellular function and in some of the autoimmune disorders.

Studies in our laboratory showed massive pneumonic changes, with infiltration of PMN neutrophils and inflammatory cells and a number of mononuclear phagocytes in lungs and liver of CCI\(_4\) treated mice, which were found to be completely removed, when the animals were treated with the purified protein CI-1. This lead us to further investigate the immunopotentiating effect of CI-1, which may be effective in preventing secondary infections of lungs and gastrointestinal tract, so common in acute and chronic infectious diseases, like viral hepatitis.

The present study on CI-1 showed (a) increased leucocyte and macrophage migration inhibition responses (b) enhanced paw thickness in delayed type of the hypersensitivity (DTH), a T-cell mediated reaction, (c) prevented systemic anaphylactic shock in 50% of the animal, (d) significant enhancement in IgG and IgM level and (e) increase in primary and secondary serum antibody titre against SRBC and BSA. Moreover, enhanced lymphopoiesis, increase in neutrophil phagocytic index, increase in bone marrow and thymus secretory proteins were also observed. These are suggestive of CI-1 induced, lymphokine mediated, augmentation of cell mediated immune (CMI) response. The upregulation of humoral immune response (HIR) to SRBC and BSA by CI-1 suggests enhanced responsiveness of macrophages, facilitating the presentation of antigen to T and B cells. However, it is not clear how CI-1 exerts its effect on CMI and HIR, but the immunomodulatory action of CI-1 is apparent from this study.

It should be mentioned here, some of the biochemical parameters like y-GTP (y-gutamyl transpeptidase) in CCI\(_4\) toxicity, GSH (Glutathione) in paracetamol model, acetaldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH) and lipid peroxidation in alcohol induced hepatotoxicity could not be studied due to some unavoidable reasons for better comparison. Further investigations are needed on the protein CI-1 for full characterisation, namely, determination of amino acid sequence and identification of the carbohydrate moiety.
The herbal protein, CI-1 isolated from *Cajanus indicus* deserves extensive clinical evaluation. The present study justifies the traditional and prolonged use of the plant in Ayurveda for the treatment of liver disorder particularly jaundice. Hence, as a new drug development, for protection of deranged hepatocellular function, imparting immunity, enhancement of body resistance against diseases and secondary infections, it will be a new addition with important scientific footing. The present work that has been carried out certainly justifies the continuation of investigations on the active principle of *Cajanus indicus* for its potential use in clinical medicine.