8. Summary & Conclusion

8.1 In vitro studies

1. The sample and standard drugs were screened for their in vitro antioxidant activity using ABTS and NBT assay methods. Among the drugs tested, catechin showed maximum activity with IC$_{50}$ value of 40.47 ± 0.73 µg / ml and 9.36 ± 0.21 µg / ml for ABTS and NBT assays respectively. Silymarin showed moderate antioxidant activity. LOLA and L-ornithin did not show antioxidant activity even when tested with 1000 µg / ml concentration.

2. In vitro hepatoprotective activity was studied using chang liver cells. Cells were pretreated with three dose levels of each drug and challenged with liver toxicants independently. MTT assay was used to estimate the percentage cell viability. Cells which are exposed only with toxicants showed a percentage viability of approximately 40%. Cells which are pretreated with drugs showed significant increase in percentage viability which ranged from 75 to 92 %. Catechin and silymarin showed better increase in percentage viability followed by LOLA, lecithin and L-ornithin respectively. Out of the three dose levels used for each drug, the best two dose levels were selected for further studies.

3. In vitro primary rat hepatocytes were isolated and used to study the alterations in biochemical parameters with respect to each toxicant and drug. Important liver enzymes like ASAT, ALAT, ALP and LDH were taken into consideration. All the toxicants used in the study caused an increase in the liver enzyme levels significantly. Hepatocytes pretreated with drugs showed significant restoration of the altered biochemical parameters towards normal. Catechin and silymarin were found to be better in restoring the biochemical parameters back to normal.

4. Alteration in the nuclear structure is one of the important aspects to study morphological changes involved in cell. To visualize nuclear morphology, nuclear staining was done using dye acridine orange and hoechst 33342. The study was carried out against chang liver cells. Nucleus in control cells was very much intact, round or oval in shape, without any condensation and membrane blebbing. Cytoplasm was normal with intact cell membrane and cytoplasm disintegration was not observed. In cells treated with toxicants
extensive damage was observed which showed typical apoptotic morphological changes of nuclear and cytoplasmic condensation, loss of cell volume, and nuclear fragmentation. While cells which are pre treated with drugs showed minimum changes in cell morphology.

5. Since mitochondrial damage is an important event leading to cell death and apoptosis, we studied the effect of the toxicants and drugs on liver mitochondria. To study this we isolated mitochondria from liver cells which are pretreated with drugs and challenged with toxicants individually. The isolated mitochondria was stained with JC-1 (5,5,6,6′-tetrachloro-1,1′-3,3′-tetra ethyl benzimidazolocarbocyanine iodide) dye. From the results obtained, damage caused to the inner mitochondrial membrane due to the liver toxicants was confirmed. Further pretreatment of liver cells with drugs was able to prevent to damage to mitochondrial membrane.

6. Bax and Bcl-2 are the two main genes involved in maintaining the mitochondrial integrity. Further P53 gene acts as a regulator and can control the Bax / Bcl2 expression pattern. Hence the mRNA expression of Bax, Bcl2 and P53 were studied. Liver cells were pretreated with drugs and challenged with toxicants individually. Total RNA was extracted from the cells and reverse transcriptase polymerase chain reaction (RT PCR) was performed with help of specific primers for Bax, Bcl2 and P53 respectively. GADPH primer was used as reference standard for RT PCR. Agarose gel electrophoresis was performed to observe the results after amplification. Ethidium bromide-stained DNA bands were quantified by Alpha Innotech software, USA.

7. From the RT PCR results we confirm that Bax levels were significantly up regulated because of toxicant treatment. The up regulation of Bax was only up to a maximum of 12 hrs. There was no significant change in the P53 levels as well as Bcl2 levels.

8. Since we observed that the expression of Bax mRNA increased only up to 12 hrs of toxicant treatment we studied flow cytometric analysis to determine the percentage of apoptotic cells before and after toxicant challenge. The percentage of cell death via apoptotic pathway is less than 15%. Hence apoptotic cell death is to a lesser extent. Major pathway of cell damage is due to necrosis (almost 80-85%) which is proved by elevated enzyme levels in hepatocytes after toxicant challenge.
8.2 In vivo studies

1. Estimation of hepatic superoxide dismutase (SOD) and catalase (CAT) levels were done using rat models. SOD and CAT levels in normal animals were found to be 0.6125 ± 0.0042 and 3.478 ± 0.048 units/mg of tissue respectively. Toxicant treated animals showed significant decrease in SOD and CAT levels. Animals which were pretreated with selected drugs showed restoration of SOD and CAT levels back to normal. Among the selected drugs catechin and silymarin showed better activity in restoration of enzyme levels on comparison with other drugs. Catechin at a higher concentration of 100 mg/kg.bt.wt showed SOD levels in range of 0.5445 ± 0.0046 to 0.5807 ± 0.0045, which is significantly increased in comparison with toxicant treated animals.

2. Important liver biochemical parameters such as ASAT, ALAT, ALP and LDH were estimated to confirm the effect of toxicants on the liver as well as to check the hepatoprotective potential of the selected drug samples against each toxicant individually. Normal animals ASAT, ALAT, ALP and LDH levels were found to be 66.60 ± 2.20, 27.51 ± 1.14, 325.3 ± 4.84 and 333 ± 6.39 U/L respectively. Toxicant treated animals showed elevated levels of all the enzymes. All the values of toxicant treated groups showed significant increase when compared with that of the normal animals. All the four liver enzymes namely ASAT, ALAT, ALP and LDH levels were maintained normal upon pretreatment with catechin, lecithin, LOLA, L-ornithin and silymarin respectively. Among the selected drugs, catechin (100 mg/kg.bt.wt) followed by silymarin (100 mg/kg.bt.wt) showed better activity in comparison with other drugs.

3. Histopathology of liver tissue was performed and compared between groups to understand the pattern of liver injury. Histopathology of normal liver sample showed intact histological structures of hepatic lobules. There was no evidence of hepatocytes injury observed in the normal control group of animals. Histopathology of toxicant treated liver showed damage to hepatocytes with hepatocellular vacuolization, focal hepatic necrosis and congestion of hepatic sinusoids. This damage to the hepatocytes clearly demonstrates the necrotic pattern of cell death. Pretreatment with selected drugs did not show much damage to the hepatocytes, confirming their hepatoprotective ability.
4. At the end of the study, DNA was extracted from excised liver of animals from every group. The Extracted DNA samples were analyzed using agarose gel electrophoresis technique. Normal group showed intact single band of DNA, without any fragments. Toxicant treated samples showed necrotic pattern of cell death, with DNA smear. There was no DNA fragments observed after toxicant treatment. Upon pretreatment with selected drugs, showed intact DNA, which confirms the hepatoprotective activity of the selected drugs. If there is any DNA damage observed in the form of fragments, then P53 expression should have been up regulated to induce apoptosis. Since only a smear of DNA was observed after toxicant challenge, proves that P53 protein is not involved directly. This finding substantiates our \textit{in vitro} results, that the pathway of toxicant is indeed P53 independent.

5. Hence we propose that apoptotic signals induce liver cell death initially after toxic insult, but due to the massive damage to the mitochondria, and ATP depletion, necrosis dominates the apoptotic pathway of cell death. Apoptosis occurs upto a maximum of 15 % as proved by the flow cytometry analysis. Massive necrosis is proved by histopathology, elevated enzyme levels and agarose gel electrophoresis studies.

Hence we propose that the hepatoprotective ability of the selected drugs such as silymarin, catechin is mainly by controlling the over expression of Bax levels. Drug pretreatment did not have much effect on the expression of P53 and bcl-2 levels. We believe that since the expression of Bax was maintained in normal range, the mitochondrial integrity was intact, which is clearly seen in mitochondrial staining experiments, this again confirms the hepatoprotective property of the selected drugs. Since P53 is not involved directly we believe the involvement of P53 independent pathway such as p19\textsuperscript{ARF} induced apoptosis. Hence drug pretreatment involves certain signals which controls the over expression of Bax and thus prevents the translocation of Bax from cytoplasm to the mitochondrial membrane. Further studies using knockout models will give us more understanding regarding the mechanism of action.