PHARMACOLOGICAL STUDIES
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Introduction

Pharmacology can be defined as the study about the effects of chemical substances on the function of living systems. It was born in the mid-19th century as one of biomedical sciences based on principles of experimentation. Robert Boyle, laid the scientific foundations of chemistry in the middle of the 17th century when dealing with therapeutics to recommend concoctions of worms, dung, urine and the moss from a dead man’s skull (Rang et al., 2003).

The impetus for pharmacology came from the need to improve the outcome of therapeutic intervention by doctors those who were skilled at clinical observation and diagnosis but broadly ineffectual in treatment. Until 19th century, knowledge of the normal and abnormal functioning of body was too rudimentary to provide even and rough basis for understanding drug effects. The motivation for pharmacology came from clinical practice, but the science could only be built on the basis of secure foundations in physiology, pathology and chemistry.

Rudolf Buchheim created the first pharmacology institute in Estonia in 1847 (Rang et al., 2003). In its beginnings, pharmacology concerned itself exclusively with understanding the effects of natural substances, mainly of plant extracts. An early development in chemistry was the purification of active compounds from plants. Early pharmacologists focused most of their attention on such plant-derived drugs as morphine, atropine, digitaline, quinine, ephedrine and others.
Beginning in the 20\textsuperscript{th} century, the fresh wind of synthetic chemistry began to revolutionize the pharmaceutical industry and with it in the science of pharmacology. The enormous growth of synthetic chemistry in the first half of the 20\textsuperscript{th} century and the resurgence of natural product chemistry caused a dramatic revitalization of therapeutics. Each new drug class that emerged gave pharmacologists a new challenge, and it was then that pharmacology really established its identity and its status among biomedical sciences.

**Pharmacology today**

The boundaries of pharmacology, as with other biomedical disciplines, are not shapely defined not are they constant. Within the main subject fall a number of components as neuropharmacology, immunopharmacology and pharmacokinetics. Ion-channel pharmacology is likely to be a fertile source of future new drugs (Triggle, 1999; Clare et al., 2000). Recent trends on the fringe are subjects such as biotechnology pharmacogenetics, pharmacogenomics, pharmacoepidemiology and pharmaco economics (Nebert and Weber, 1990; Strom, 1994). Biotechnology refers mainly to the use of recombinant DNA technology for a variety of purposes. In relation to biomedical sphere, it includes the manufacture of therapeutic proteins, diagnostics, genotyping, production of transgenic animals, etc.

Pharmacogenetics is the study of genetic influences on response to drugs. Pharmacogenomics describes the use of genetic information to
guide the choice of drug therapy on an individual basis. It is based on the principle that the differences between individuals in their response to therapeutic drugs which can be predicted from their genetic makeup. Pharmacoepidemiology is the study of drug effects at the population level (Strom, 1994) whereas Pharmacoeconomics is a branch of health economics aims to quantify in economic terms the cost and benefit of drugs used therapeutically (Drummond et al., 1997).

**Phytopharmaceuticals**

Nowadays, the importance of medicinal plants in the treatment of a variety of human ailments has been immense. In today’s modern age of advancement in instrumentation technology, sophisticated techniques like NMR and MS, have markedly reduced the time and effort required for structural determination. The development of science of phytopharmaceuticals and the hopes for remedies in chronic diseases generated new enthusiasm in the research workers to develop herbal medicines (Kokate, 2004).

A considerable amount of work has been done to study the potentialities of herbal medicines and the modern science has accepted the potential of plant kingdom as a source of new biodynamic constituents. Nearly 50% of drugs used in medicine are of plant origin. The natural plant products often serve as chemical models or templates for the design and total synthesis of new drug entities. Numerous plants and polyherbal formulations are claimed to have hepatoprotective
activities. Nearly 150 phytoconstituents from 101 plants have been claimed to possess liver protecting activity (Handa et al., 1989; Doreswamy and Sharma, 1995). Only a small portion of the hepatoprotective plants as well as formulations used in traditional medicine are pharmacologically evaluated for their efficacy (Chattopadhyay et al., 1992; Saxena et al., 1993; Mitra and Sur, 1997; Ruckmani et al., 1998; Sane et al., Singh et al., 1991). The efficiency is not sufficient enough to use as effective drugs (Subramaniam, 1990). In almost all cases the mechanism of their hepatoprotective effects remains to be studied.

**Boerhaavia diffusa for human ailments**

*Boerhaavia diffusa* is used as a multipurpose medicinal herb. The each and every part of this plant has medicinal properties. It's leaves have been found to possess anti-diabetic (Pari and Satheesh, 2004a) and analgesic effects (Lima et al., 2000), reduces pain (Robineau, 1996; Lima et al., 2000) and used against snake venom (Robineau 1995). The diuretic (Misra and Tiwari, 1971), immunomodulatory (Sohni and Bhatt, 1996; Mungantiwar et al., 1999; Mehrota et al., 2002), antilymphoproliferative (Mehrotra et al., 2002; Tomoo Inoue and Jackson, 1999) antimicrobial activity (Abo and Ashidi, 1999) and hepatoprotective properties (Rajdawar, 1983; Chakraborti and Handa, 1989; Chandan et al., 1991; Rawat et al., 1997) have been attributed to the roots of *Boerhaavia diffusa*. The roots have also been used as an
anti-stress agents (Kirtikar and Basu, 1956; Dandiya, 1991; Chopra et al., 1996). The seeds are used as tonic and carminative preparations (Aslam, 1996). Inhibitory effect of *B. diffusa* on experimental metastasis by B16F10 melanoma cells in C57BL/6 Mice have been studied by Leyon et al. (2005). The alkaloidal fraction of the plant was showing some immunostimulatory activity *in vivo*, without any effect in *in vitro* systems (Mungantiwar et al., 1999). Ethanolic extract of the plant has well demonstrated to have anti-mitotic activity in the *in vitro* systems (Ene Obong and Madi, 1987). The tribal population in South Garhwal used the roots in the treatment of liver enlargement (Rajdawar, 1983). Liriodendrin isolated from the methanol of the roots of *B. diffusa* was found to exhibit significant calcium channel antagonistic activity (Lami et al., 1991).

The root extract of *Boerhaavia diffusa* appears to be a potent antifibrinolytic and anti-inflammatory agent in monkeys (Barthwal and Srivastava, 1991). The other pharmacological activities of this plant include kidney regeneration and nutrition (Mishra and Singh, 1980). An experimental evaluation of the possible teratogenic potential of *Boerhaavia diffusa* showed no teratogenic effect (Singh et al., 1991). Because of different uses in natural medicine, *B. diffusa* has played an important role in the herbal practitioner’s medicine, the chest of natural remedies.
Biological evaluation

When the estimation of potency of crude drug or its preparation is done by means of its effect on living organisms, it is known as bioassay. Bioassay plays a key role in the development of new drugs. Many different experimental designs have been proposed to maximize the efficiency reliability of bio assays (Laska and Meisneu, 1987). Bioassay methods are mainly of three types, viz, toxic, symptomatic and tissue methods. In toxic and symptomatic techniques the animals are used, whereas in tissue method, the effect of drug is observed on isolated organ or tissue (In vitro study).

Animal cell and tissue culture

The foundation of animal cell and tissue culture were laid at the beginning of the present century, when cell could be shown to survive and divide in vitro (Jolly, 1903). The actual beginning of animal cell and tissue culture was however, made by Ross Harrision (1907), by using frog as a source of tissue. Alexis Carrel (1912) used tissue and embryo extract as culture media. Similar to plants, for animal if the explants maintain its structure and function in culture, it is described as an organotypic culture irrespective of tissue from which it was derived and it is described as a histotypic culture.

The animal tissue culture is a technique in which the tissue/cells are taken out from an animal’s body and grown, and maintained in laboratory in a suitable medium. Animal cultures have many
applications including their use as modal systems for biochemical, physiological and pharmacological studies and the production of growth factors, blood factors, monoclonal antibodies, interferons, enzymes, vaccines and hormones.

At present, animal tissue culture is an indispensable tool, particularly in the field of medical sciences. Cells are more frequently being cultured in aggregates, as this provides a better model than monolayer culture for the tissue in vivo. This is particularly true for hepatocyte culture. The cells retain their hepatocyte specific characteristics and so their potential utility in drug metabolism and toxicity studies is much enhanced.

**Hepatoprotection**

Depending on the exposure situation, nature of host and toxic chemicals hepatotoxicity can be classified into two types:

1. Acute toxicity
2. Chronic toxicity

In toxicology, the term ‘acute’ may be used to indicate exposure to a single dose and also used to indicate the severity of toxicity. Acute toxicity may result in necrosis of the liver. In most circumstances this is demonstrated by elevated levels of serum transaminase which are two types. One is called as Asparate Amino Transferase (ASAT) and another is called as Alanine Amino Transferase (ALAT). It may be increased 10 to 100 fold depending up on the degree of damage. Similarly, many
enzymes are released from the hepatocytes as a result of variety of hepatocellular injury. These enzymes include Alkaline Phosphatase (ALP), Acid Phosphatase (AP), Lactic Dehydrogenase Isoenzymes (LDI), Isocitric Dehydrogenase (ICD), Sorbitol Dehydrogenase (SRD), Gamma-Glutamyl Trans Peptidase (GGTP), bilirubin etc. The term chronic is used to indicate either repeated exposure over an extended period of time or the presence of a hepatic response over an extended period of time.

Chronic toxicity may lead to cirrhosis. Cirrhosis can be defined as a disease condition presenting morphological alterations of the globular structure characterized by destruction and regeneration of parenchymal cells and increased connective tissues. Major morphological changes include granular or nodular appearance and are characterized by the presence of separate of collagen throughout the liver. The aggregated liver cells circumscribed by sheath life fibrous growth of collagenized connective tissues give the appearance of nodules of hepatic cirrhosis. Cirrhosis is characterized by yellow coloration of the liver with broad and dense bands of connective tissue dividing liver into nodules of varying size.

**Mechanism of hepatotoxicity**

One of the most clearly defined hypothesis relative to the action of hepatotoxic agents is the lipid peroxidative damage of cellular membranes (Recknagel and Glende, 1978). In this mechanism toxicants (for example CCl₄) are cleaved into free radicals (CCl₃). This occurs in
the endoplasmic reticulum, and is catalysed by the cytochrome P-450 mono oxygenase enzyme. The cytochrome P-450 system is encased in a phospholipids membrane rich in polyenoic fatty acid. In the aerobic environment, the free radicles (CCl₃) enter a hydrogen abstraction reaction to form an organic free radical of the fatty acid and chloroform. Hence, these polyenoic fatty acids are the most likely immediate targets for the initial lipid peroxidative attack to occur. In the aerobic environment of the hepatocyte, the organic fatty acid radical rearranges, yielding organic peroxy and hydroperoxy radicals. These radicals destroy the cytochrome P-450 hemoprotein, thus, comprising the mixed function oxygenase activity. The rapid decomposition of the endoplasmic reticulum and its function is direct result of this lipid peroxidative process. The lipid peroxides and hydroperoxides attack the lipids and possibly the proteins. Generation of organic radicals by the free radical (CCl₃) causes another chains or reactions. The organic radical themselves interact with other lipids, either in the same membrane or in other membranes after being diffused from the original site of formation. These interactions result in the formation of other organic free radicals and peroxy and hydroperoxy radicals. The entire process is propagated to continue the autocatalytic reactions until the membranes are destroyed and many functions are disrupted, culminating in the demise of hepatocytes.

Many hepatotoxic compounds have been reported to interfere with the hepatic cellular protein synthesis. They inhibit the incorporation of
amino acids in to hepatic proteins. These findings have led to other beliefs that inhibition of protein synthesis is the cause of hepatic necrosis.

Present study

Liver diseases or hepatotoxicity constitute a major medical problem of world wide population. Till this date, there is no effective medicine of hepatitis. Consequently, control of liver disease has become a major goal of modern medicine. As it is the function of hepatoprotective agents to interfere with these pathological processes by blocking their evolution and helping recovery, the development of new anti hepatotoxic drugs is the need of the hour. Since increase in the use of synthetic drugs in therapy leads to many side effects and undesirable hazards, there is a world wide trend to go back to natural resources. Therefore, in this study Boerhaavia diffusa leaf and root extracts were subjected to hepatoprotective activity. The effect of these drugs on drug metabolising enzymes in the liver toxicated by CCl₄ was studied. Silymarin (flavanoid) a known hepatoprotective drug obtained from the fruits of the Silybum mariamm (family Composite) was tested simultaneously for comparison.

Materials and methods

All the chemicals were purchased from Loba Fine Chemicals, Mumbai, India. MTT [3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide], collagenase, insulin, dexamethasone were
purchased from Sigma Chemical Co., St. Louis, MO, USA, Minimum Essential Medium of Eagle’s (EMEM) and Ham’s F 12 from Hi-Media Laboratories, Mumbai, India and Ecoline diagnostic kits from E. Merck, Mumbai, India. Silymarin (Reddy’s Laboratory, Hyderabad, India) was used as standard. Human liver derived HEp-G2 cell line was obtained from National Centre for Cell Science, Pune, India.

**Plant material and extraction**

*Boerhaavia diffusa*, entire plant was collected from the grounds of Government Arts College, Karur – 639 005, Tamil Nadu, India. The plant was identified and authenticated by comparing with standard specimen deposited in the Botanical Survey of India, Southern Circle, Coimbatore, India and preserved in the Department of Botany, Government Arts College, Karur for future reference. The roots and leaves were separated from the plant, shade dried and powdered. Each of the powder (250 g) was subjected to a single Soxhlet extraction using methanol (1 litre) for 24 h. The extracts were then concentrated to dryness under reduced pressure and controlled temperature to yield dark brown semisolids (yield 6.85% and 5.24% respectively, for leaf and root), which were preserved in refrigerated condition till further use.

10 mg of the methanolic extracts of the leaf and stem of *Boerhaavia diffusa* were dissolved in 1 ml DMSO (dimethyl sulphoxide) and the volume was made up to 10 ml with Ham’s F12/MEM to obtain stock solution of 1 mg/ml concentration and stored at -20°C till use.
Further dilutions were made to obtain different concentrations with respective media and used for in vitro investigations. A suspension of silymarin was also prepared (250 μg/ml) in a similar manner. The methanol extracts and the standard silymarin were suspended in sodium CMC (0.3%) in distilled water separately and used for in vivo investigations.

**In vitro pharmacological evaluation**

Both in vivo and in vitro test systems are used to assess hepatoprotective activity of herbal drugs. Detailed bio-chemical and other in vitro assays are required to determine the mechanism of action. Fresh hepatocyte preparations and primary cultured hepatocytes are used to study direct antihepatotoxic activity of drugs. Hepatocytes are treated with hepatotoxin and the effect of the plant drug on the same is evaluated. The activities of the transaminases released into the medium are determined (Hostettman and Lea 1987; Chrungo et al., 1997 b). An increase in the activities in the medium indicates liver damage.

In the present investigation the hepatoprotective effects of methanol extracts of leaf and root of *B. diffusa* were studied on carbon tetrachloride (CCl₄) induced hepatotoxicity on HEp-G2 cells (Ira et al., 1997) and in animal models (Rao et al., 1993). Biochemical parameters such as levels of aspartate amino transferase (ASAT), alanine amino transferase (ALAT), alkaline phpsptahte (ALP) triglycerides (TGL), total
proteins, albumin, total bilirubin and direct bilirubin were estimated to assess the hepatic function (Harper, 1961).

**CCl₄ induced *in vitro* hepatocytes injury**

CCl₄ induced hepatocytes injury assay was carried out. After an incubation of 24 h, the hepatocytes were exposed to the fresh medium containing CCl₄ (1%) along with/without various concentrations of the methanol extracts or the medium containing the same amount of DMSO used for extract alone (as control). After 60 min of CCl₄ challenge, concentrations of ASAT, ALAT, ALP, TGL, total proteins, albumin, total bilirubin and direct bilirubin in the medium were measured as an indication of hepatocytes necrosis using Ecoline diagnostic kits (Yoshinobu *et al.*, 1983).

**Hepatoprotective effect in HEp-G2 cell line**

The screening of hepatoprotective activity was based on the protection of human liver derived HEp-G2 cells against CCl₄ induced damage (*Ira et al.*, 1997) determined by estimating mitochondrial synthesis using microculture Tetrazolium (MTT) assay (*Ke et al.*, 1999). HEp-G2 cells are routinely grown and subcultured as monolayers in DMEM supplemented with 10% newborn calf serum. The experiments in this investigation were conducted with cells that had been initially batch cultured for 10 days. At this stage, the cells were harvested and plated at approximately 30,000 cells/well in 96 well microtitre plates (Nunclon) and left to rest for 24 h at 37° C in a humified atmosphere of 5% CO₂.
The cells were then exposed to toxicant (medium containing 1% CCl₄) along with/without various concentrations of the methanolic extracts of leaf and root of *B. diffusa* on the medium containing same amount of DMSO used for extracts alone (as control). At the end of the period, cytotoxicity was assessed by estimating the viability of HEp-G2 cells by MTT reduction assay. After 1 h incubation, the test solution from each cell was removed by aspiration and replace with 50 μl of MTT prepared in MEM without phenol red (MEM-PR). The plates were gently shaken and incubated for 3 h at 37°C in a humified 5% CO₂ atmosphere. The supernatant was removed and 50 μl propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at 540 nm.

The effects of methanol extracts of leaf and root extracts of *Boerhaavia diffusa* on the CCl₄ exposed HEp-G2 cells showed a percentage of viability of 35%. The exposed cells when treated with different concentrations of the methanolic extracts of *Boerhaavia diffusa* showed a dose dependent increase in percentage ranged between 36 to 51% for the extracts of leaf and 42 to 62% for the root extracts at 200 – 1,000 μg/ml concentrations and was found to be less effective when compared to the standard (Figs. 4.1, 4.2 & 4.3; Table 4.1).
Table 4.1 Hepatoprotective activity of the methanolic extracts of leaves and roots of *Boerhaavia diffusa* on CCl₄ intoxicated HEp-G2 cells.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatment</th>
<th>Concentration (µg/ml)</th>
<th>Cell viability * (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>CCl₄</td>
<td>1%</td>
<td>25.4 ± 1.86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>42.4 ± 2.36&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>38.3 ± 1.46&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>34.4 ± 1.89&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>28.6 ± 1.22&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>27.5 ± 1.48&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>CCl₄ (1%) + Leaf Extract</td>
<td>1000</td>
<td>56.6 ± 1.45&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>52.3 ± 2.81&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>400</td>
<td>49.8 ± 1.92&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>42.6 ± 1.33&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>39.5 ± 1.14&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>CCl₄ (1%) + Root Extract</td>
<td>1000</td>
<td>56.6 ± 1.45&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>52.3 ± 2.81&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>400</td>
<td>49.8 ± 1.92&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td>300</td>
<td>42.6 ± 1.33&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>200</td>
<td>39.5 ± 1.14&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* (average of 6 replicates).

a = P < 0.001 when compared to control; b = P < 0.01, c = P<0.001 when compared to CCl₄ group.
**In vivo pharmacological evaluation**

The effects of methanol extracts of leaf and root extracts of *Boerhaavia diffusa* on CCl₄ intoxicated rats are recorded in Table 4.2. Intoxication of rats treated with CCl₄ significantly (P < 0.01 and P < 0.001) altered the biochemical parameters when compared with normal control rats. Treatment with methanol extracts of leaf and root of *B. diffusa* at 250 mg/kg body weight showed a significant (P<0.01 and P<0.001) decrease in ASAT, ALAT, ALP, total bilirubin, direct bilirubin and a significant (P<0.01 and P<0.001) elevation in the TGL, total proteins and albumin levels in serum when compared with CCl₄ treated rats.

Plant drugs are known to play a vital role in the management of liver diseases. The preventive action in liver damage induced by CCl₄ has been widely used as an indicator of the hepatoprotective activity of drugs or medicinal plant extracts, by *in vivo* and *in vitro* techniques (Kiso *et al.*, 1983, Allis *et al.*, 1990). CCl₄ produces an experimental liver damage, which histologically resembles that of viral hepatitis (James and Pickering, 1976). The present study reveals the hepatoprotective effect of *Boerhaavia diffusa* against CCl₄ induced toxicity in HEp-G2 cells and in animal models. HEp-G2 cells retain many of the morphological and biochemical characteristic of normal cells (Jover *et al.*, 1994). A reproducible micro plate screening assay based on the protection of cells of the human liver derived HEp-G2 cell line against toxic damage have been used (Ira *et al.*, 1997) for the rapid identification of the protective
activity of the plant extracts. Since most of the plant extracts are toxic above 1,000 µg/ml, the highest concentration was limited to 1,000 µg/ml in vitro studies. CCl₄ has been found to induce extensive liver damage within a period of 24 h following intra-peritoneal administration. Intoxication with CCl₄ in rat hepatocytes and in animals caused elevated serum levels of hepatospecific enzymes such as ASAT, ALAT, ALP as well as alterations in different liver parameters (Table 4.2). Treatment with the methanolic extract of *Boerhaava diffusa* exhibited significant restoration of the altered biochemical parameters towards normal in CCl₄ intoxicated rat hepatocytes and in rats. These results and the absence of mortality observed with extracts treated group are indicative of the potent hepatoprotective action of the plant. The maximum degree of protection was observed with the highest dose of extracts. Among the two extracts studied the root extract was found to be more effective. The investigation when carried out in human liver derived HEp-G2 cells against CCl₄ induced damage also confirmed the hepatoprotective nature of the methanolic extracts. The results from the present study also indicate good correlation between the in vivo and in vitro studies.

Colony bred Wistar strain albino rats (200 – 220 g) of either sex were used for the investigations. All the animals were maintained under standard husbandry conditions with food and water ad libitum. The experimental procedures were approved by the Institutional Ethical Committee (IEC). The animals were divided in to six groups consisting of six animals in each group. Liver damage was induced by
Table 4.2 Effect of treatment with methanolic extracts of *Boerhaavia diffusa* on the biochemical parameters of CCl₄ treated animals

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment</th>
<th>Dose</th>
<th>ASAT (U/L)</th>
<th>ALAT (U/L)</th>
<th>ALP (U/L)</th>
<th>TGL (mg/dL)</th>
<th>Total Protein (g/dL)</th>
<th>Albumin (g/L)</th>
<th>Total Bilirubin (mg/dL)</th>
<th>Direct Bilirubin (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>--</td>
<td>64.5 ± 2.43</td>
<td>24.9 ± 1.65</td>
<td>269.5 ± 11.43</td>
<td>72.5 ± 4.39</td>
<td>9.5 ±0.43</td>
<td>4.25 ± 0.16</td>
<td>0.562 ± 0.03</td>
<td>0.175 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>CCl₄ Treated</td>
<td>1/ml/kg bw</td>
<td>124.2 ± 7.43ᵇ</td>
<td>74.5 ± 2.56ᵃ</td>
<td>489.9 ± 18.43ᵇ</td>
<td>21.4 ± 1.43ᵃ</td>
<td>3.8 ± 0.25ᵃ</td>
<td>2.46 ± 0.05ᵃ</td>
<td>1.453 ± 0.06ᵃ</td>
<td>0.526 ± 0.06ᵃ</td>
</tr>
<tr>
<td>3</td>
<td>CCl₄ + Standard</td>
<td>1/ml/kg bw + 250 mg/kg</td>
<td>61.8 ± 2.32ᵃ</td>
<td>21.6 ± 1.02ᵇ</td>
<td>328.6 ± 11.66ᵃ</td>
<td>49.6 ± 2.6ᵇ</td>
<td>8.4 ± 0.52ᵇ</td>
<td>4.11 ± 0.16ᶜ</td>
<td>0.492 ± 0.06ᶜ</td>
<td>0.190 ± 0.01ᶜ</td>
</tr>
<tr>
<td>4</td>
<td>CCl₄ + Leaf Extract</td>
<td>1/ml/kg bw + 250 mg/kg</td>
<td>43.6 ± 1.24ᵃ</td>
<td>31.5 ± 1.45ᵃ</td>
<td>369.4 ± 12.56ᵇ</td>
<td>59.4 ± 1.82ᵇ</td>
<td>8.6 ± 0.32ᵇ</td>
<td>3.99 ± 0.12ᵇ</td>
<td>0.510 ± 0.09ᵇ</td>
<td>0.125 ± 0.04ᵇ</td>
</tr>
<tr>
<td>5</td>
<td>CCl₄ + Root Extract</td>
<td>1/ml/kg bw + 250 mg/kg</td>
<td>59.8 ± 2.34ᵇ</td>
<td>26.4 ± 1.05ᵇ</td>
<td>395.6 ± 11.69ᵇ</td>
<td>66.5 ± 1.43ᵇ</td>
<td>9.1 ± 0.65ᵇ</td>
<td>4.00 ± 0.08ᶜ</td>
<td>0.564 ± 0.04ᵇ</td>
<td>0.112 ± 0.03ᵇ</td>
</tr>
</tbody>
</table>

ᵃ = P<0.001 when compared to normal group;  
bᵇ = P<0.001; ᶜ = P<0.01; ᵈ = P<0.05 when compared to CCl₄ treated group  
bw = body weight; U/L = Unit per Litre; mg/dL = milligram per deciliter; g/dL = gram per deciliter; g/L = gram per litre.
administration of CCl₄ (1 ml/kg body weight) intraperitoneally a day prior to the treatment. Group I received the vehicle (Sodium CMC, 0.3%) and served as control and was not treated with the toxicant. The second group served as CCl₄ treated control and received the vehicle (Sodium CMC, 0.35). Groups III, IV and V received a suspension of the methanolic extracts of leaf and root of Boerhaavia diffusa at 250 mg/kg body weight respectively. Group VI received the standard Silymarin (Dr. Reddy's Lab, Hyderabad, India) at 250 mg/kg body weight. After 24 h of intoxication, the animals received these treatments by oral route for a period of six days. On the 9th day, blood was collected in sterile centrifuge tubes and allowed to clot. Serum was prepared and used for the estimation of ASAT, ALAT, ALP, total glycerides, total proteins, albumin, total bilirubin and direct bilirubin using Ecoline diagnostic kits (Figs. 4.4 to 4.12).

Results and Discussion

Table shows the various enzyme levels for normal, CCl₄ intoxicated, standard (Silymarin treated) and leaf extract and root extract treated groups. Elevation of serum enzyme levels in the CCl₄ intoxicated group when compared with normal group ensured the damage of liver by CCl₄. Also elevation of serum transaminase (ASAT and ALAT) level in the CCl₄ intoxicated group of animals revealed the necrosis type of lesion produced in the liver and the elevation in the level of alkaline phosphotase revealed the cholestasis type of lesion.
produced in the liver. The standard drug silymarin treated group showed significant reduction in the enzyme levels when compared to the intoxicated group indicating the model employed for the study was appropriate and the data thus obtained could be tested for the significance of the hepatoprotective activity of the test drugs.

From the Table 4.2 it can be observed that the leaf extract and root extract treated showed a significant reduction in enzyme levels as compared with the CCl₄ intoxicated group thus indicating their effectiveness against hepatotoxicants. The hepatoprotective activity of these drugs is in fact comparable to that of silymarin, the standard drug used.

Analysis of further results of various biochemical parameters was confirmed by the histo-pathological studies. Multiple section studies on the liver of the normal group showed the (grouped treated with vehicle alone) preservation of normal globular architecture of the liver. Hepatocytes were arranged in cell cords. Central vein, portal tract and sinusoids appeared normal and no specific lesion was seen. The biopsy study of the liver intoxicated with CCl₄ indicated the distortion of lobular architecture of the organ. The hepatocytes showed marked fatty changes. Also, a mild perportal inflammatory infiltrate of lymphocytes was also observed. It was observed that the CCl₄ intoxicated liver treated with the standard drug silymarin showed preservation of normal lobular architecture. There was no evidence of hepatocyte necrosis, cirrhosis or malignant change while the group
treated with the test drug leaf extract showed a moderate degree of fatty change (searosis). But no hepatocyte necrosis or cirrhosis was found. The result thus clearly revealed the usefulness of root extract as hepatoprotective agent.

The biopsy study of the liver treated with the test drug root extract indicated the preservation of hepatic architecture and it was seen that most of the hepatocytes appeared normal and were recovered from the fatty changes caused by CCl₄. No bridging necrosis, cirrhosis or neoplastic change was found. The results revealed that the test drug root extract showed marked protective action of the liver against hepatotoxins and the potency was as comparable with that of the standard drug silymarin.

Thus, the results of biochemical enzymatic analysis and the biopsy studies on the liver intoxicated by CCl₄ showed the efficacy of Boerhaavia diffusa leaf and root extracts as hepatoprotective agents. Hepatoprotective ability of the plant constituents has been correlated with their anti-lipoperoxidant property or with the elevation in reduced glutathione (GSH) or other cellular antioxidant levels (Kiso et al., 1984; Chander et al., 1992). It is also known that free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. Naturally there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effect (Finkel and Holbrook, 2000; Nose,
But additional burden of free radicals either from environment or produced with in the body, can alter the free radicals and anti free radical balance leading to oxidative stress which may result in tissue injury and subsequent disease (Sies, 1991; Davies, 1995; De Groot and Rauen, 1998; Dringen, 2000). Thus, the antioxidant status in human reflects the dynamic balance between the antioxidant defense and the pro-oxidant conditions and has been suggested as a useful tool in estimating the risk of oxidative damage (Papas, 1999; Polidori et al., 2001).

Flavonoids offer protection against human disease through their antioxidant action. The multiple properties of these phytochemicals have made them more attractive as they can modulate various aspects of diseases like lipid peroxidation involved in atherogenesis, thrombosis, carcinogenesis, hepatotoxicity and a variety of disease conditions. Concurrently the free radical scavenging properties of flavonoid compounds are associated with their ability to form stable radicals after their interaction with free radicals. Flavonoids that can scavenge radicals effectively usually give rise to semiquinone free radical in alkaline solution. The semiquinone free radical may react with a second free radical, acquiring a stable quinine structure (Pietta, 2000).

Flavonoids act as antioxidants not only by free radical scavenging, but also by the metal chelation and by inhibition of enzymes like NADH oxidase in human mitochondria succinoxidase...
and NADH oxidase and also inhibit microsomal cytochrome P-450 (Fe$^{2+}$) dependent enzymatic reactions by their metal chelating activity. The electron and H$^+$ donating capacity of flavonoids seem to contribute to the termination of lipid peroxidation chain reaction based on their reducing power. Griffiths (1998) observed that flavonoids are metabolized in vivo as phenolic acids. Limasset et al. (1999) analysed several phenolic acids, which are attributed to the metabolites of flavonoids and are known to present anti-oxidative and hereby hepatoprotective properties (Frankel and Meyer, 2000).
Fig. 4.1 Hepatoprotective effect of Boerhaavia diffusa leaf and root extract in *in vitro* HEp-G2 Cell line

a - Control
b - CCl$_4$ intoxicated
c - CCl$_4$ + leaf extract
d - CCl$_4$ + root extract
Fig. 4.2 Hepatoprotective activity of leaf extract of *Boerhaavia diffusa* on CCl₄ intoxicated *in vitro* HEp-G2 cell line cultures.

LE1 – 1000, LE2 – 500, LE3 – 400, LE4 – 300, LE5 – 200

Fig. 4.3 Hepatoprotective activity of root extract of *Boerhaavia diffusa* on CCl₄ intoxicated *in vitro* HEp-G2 cell line cultures.

LE1 – 1000, LE2 – 500, LE3 – 400, LE4 – 300, LE5 – 200
Fig. 4.4 Hepatoprotective effect of *Boerhaavia diffusa* leaf and root extract in *in vivo* rat.

a - Control
b - CCl$_4$ intoxicated
c - CCl$_4$ + leaf extract
d - CCl$_4$ + root extract
**Fig. 4.5** Activity of Aspartate aminotransferase (ASAT) in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw)

**Fig. 4.6** Activity of Alanine aminotransferase (ALAT) in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw)
Fig. 4.7  Activity of Alkaline phosphastase (ALP) in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw)

Fig. 4.8  The amount of Triglycerides (TGL) in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw)
Fig. 4.9 The amount of Total protein in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw)

Fig. 4.10 The amount of Albumin in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw)
**Fig. 4.11** The amount of Total bilirubin in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw).

**Fig. 4.12** The amount of Direct bilirubin in the CCl₄ (1ml/kg bw) intoxicated rat treated with *Boerhaavia diffusa* leaf extract (LE) (250 mg/kg bw) and root extract (RE) (250 mg/kg bw).