Evaluation of hepatoprotective activity of *Boerhaavia diffusa* against carbon tetrachloride induced liver toxicity in albino rats

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ABSTRACT

**Background:** Modern allopathic medicine has very little to offer for the treatment of liver disorders in spite of consistent effort for new drug discovery. Hence, this study was conducted to elucidate the hepatoprotective activity of aqueous extract of traditional medicinal plant *Boerhaavia diffusa* against carbon tetrachloride (*CCl₄*) induced toxicity in male albino rats.

**Methods:** The hepatoprotective effect of the aqueous extracts of *B. diffusa* was evaluated by biochemical parameters such as serum alanine transferases (ALT), serum aspartate transferases (AST), alkaline phosphatase (ALP), total serum bilirubin, and serum protein, and confirmed by histopathology of liver. The toxicant *CCl₄* was used to induce hepatotoxicity and silymarin were used as control drug. The aqueous extracts of *B. diffusa* were administered at the doses of 250 mg/kg/day and 500 mg/kg/day orally for 4 days. One-way analysis of variance was used for the statistical analysis of data. A probability value of *p*<0.05 was considered as significant.

**Results:** Administration of *B. diffusa* at doses 250 and 500 mg/kg orally demonstrated hepatoprotective activity by preventing the increase of ALT, AST, ALP, and serum bilirubin and also confirmed by histopathology of the liver. The results were comparable to that of silymarin®.

**Conclusion:** The results of this study confirmed the hepatoprotective activity of aqueous extracts of *B. diffusa* at doses of 250 mg/kg and 500 mg/kg against *CCl₄* induced hepatotoxicity in rats. However, the dose adjustments may be necessary to optimize the similar hepatoprotective efficacy in clinical settings.

**Keywords:** Carbon tetrachloride, Hepatoprotective activity, *Boerhaavia diffusa*, Silymarin

INTRODUCTION

Liver is a vital organ with a wide range of functions like metabolism, protein synthesis and production of necessary substances for digestion. The liver receives a dual blood supply with about 20% of blood coming from the hepatic artery and 80% from the portal circulation. One of the primary functions of the liver includes processing and regulating the substances from the gastrointestinal tract before distributing to the different parts of the body. The common causes of liver disorders include excessive alcohol consumption, infective hepatitis, and drug toxicity. Modern allopathic medicine has very little to offer for the treatment of liver disorders in spite of consistent effort for new drug discovery. There are several herbal formulations used for their hepatoprotective activity in the traditional system of medicine in India and China. However, only a small proportion of these herbal preparations have been evaluated scientifically in animal and clinical studies. *Boerhaavia diffusa* is a perennial diffuse herb found as a weed in wasteland and road sides throughout the India. It is commonly known as Purnarnava in India and spreading Red hogweed in English. The roots and the whole plant are used as an Ayurvedic medicine in India and Unani medicine in Arab countries for the treatment of diabetes, jaundice and heart failure. *B. diffusa* is reported to possess anti-inflammatory, hepatoprotective and diuretic activity.

Pharmacognosy


There are very less scientific data regarding the identification and effectiveness of individual B. diffusa for the treatment of edema, ascites, and liver disorders. Both the anecdotal reports and scientific studies have found that the aqueous extracts of B. diffusa exhibited hepatoprotective activity against carbon tetrachloride (CCl₄) induced hepatotoxicity in animal models. The study was undertaken to evaluate the hepatoprotective activity of aqueous extracts of B. diffusa against CCl₄ induced hepatotoxicity in albino rats to validate folkloric claim with scientific data. Silymarin, an ayurvedic formulation, which has shown to be hepatoprotective in CCl₄, paracetamol and ethanol induced hepatotoxicity in various animal models and also in clinical studies is used as a standard control. Silymarin is a mixture of mainly three flavonolignans, viz, silybin, silidianin, and silychristine obtained from the seeds of “milk thistle” (Silybum marianum). Silymarin has been used medicinally to treat liver disorders, including acute and chronic viral hepatitis, toxin/drug-induced hepatitis, and cirrhosis and alcoholic liver diseases. In the recent past, there has been a paradigm shift toward evaluation of herbal preparations in liver disease models with the modern concept of randomized clinical trials to support safety and efficacy. Thus, this study is aimed at evaluation of B. diffusa for its hepatoprotective activity in animal models to compile data for standardization of the dose for its efficacy and safety to move forward toward clinical trials of the same.

Herbal drugs play a major role in the treatment of hepatic disorders. In the absence of reliable hepatoprotective drugs in modern medicine, a number of medicinal plants and their formulations are used to cure hepatic disorders in traditional systems of medicine in India.

METHODS

Plant material and preparation of the extract

B. diffusa Linn roots were collected after the plant was identified and authenticated by Dr. B. B. Joshi at the Department of Rashashastra, Ayurvedic Medical College, Heggeri, Hubli, Karnataka, India. The roots were shade dried and powdered. The powdered materials of B. diffusa were subjected to successive solvent extraction using Soxhlet apparatus and refluxed successively with petroleum ether, chloroform, and methanol. The extracts were filtered and concentrated in vacuum using rotary flash evaporator. The crude extract was administered to the animals as aqueous solutions.

Chemicals

CCl₄ was purchased from Qualigens Fine Chemicals, Mumbai. The liver function kits alanine transferases (ALT), aspartate transaminases (AST), alkaline phosphatase (ALP), total serum proteins and total serum bilirubin were purchased from Bayer Diagnostics India Limited: silymarin was received as a gift from Micro Labs Limited, Goa, India.

Animals

Male albino rats weighing between 150 and 200 g bred in Central Animal House, Karnataka Institute of Medical Sciences (KIMS), Hubli, Karnataka, India were procured and used for the study. Animals were maintained in standard laboratory conditions with 12 hrs of daylight at 25°C±5. The animals were acclimatized to in-house conditions and were fed a commercial pellet diet (Hindustan Lever Limited, Mumbai, India) and water ad libitum. Experimental protocol was undertaken in accordance with the ethical guidelines, and the permission of the institutional animal ethics committee was obtained. All the animals received care according to the guidelines of CPCSEA/IAEC code 780. The study was approved by the Institute Animal Ethics Committee (KIMS), Hubli, Karnataka, India.

CCl₄ induced hepatotoxicity model

Hepatoprotective activity was evaluated by the method described by Gupta and Dixit. The rats were divided into five groups of six rats each.

1. Group I served as normal and was administered only vehicle (distilled water) of 1 ml/kg body weight (b.w) for 4 days
2. Group II served as toxin control and was administered vehicle on the 1st and 4th day and vehicle + CCl₄ at a dose of 2 ml/kg intraperitoneally (i.p) as 1:1 mixture with olive oil on the 2nd and 3rd day respectively
3. Group III received silymarin at a dose of 50 mg/kg b.w on the 1st and 4th day and silymarin (50 mg/kg b.w.) + CCl₄ at a dose of 2 ml/kg i.p as 1:1 mixture with olive oil on the 2nd and 3rd day respectively
4. Groups IV received aqueous extract of B. diffusa at a dose of 250 mg/kg b.w. on the 1st and 4th day and aqueous extract of B. diffusa at 250 mg/kg b.w. + CCl₄ at a dose of 2 ml/kg i.p as 1:1 mixture with olive oil on the 2nd and 3rd day respectively
5. Groups V received aqueous extract of B. diffusa at a dose of 500 mg/kg b.w. on the 1st and 4th day and aqueous extract of B. diffusa at 500 mg/kg b.w. + CCl₄ at a dose of 2 ml/kg i.p as 1:1 mixture with olive oil on the 2nd and 3rd day respectively.

Assessment of hepatoprotective activity

Biochemical tests

On the 5th day, the animals were anesthetized and blood was collected from the retro-orbital plexus. Serum was separated after coagulating at 37°C for 30 mins and centrifuging at
2000 rpm for 15 mins, and estimated for serum ALT, serum AST, ALP and total serum proteins and total serum bilirubin using kits supplied by Bayer diagnostics India, Limited.\(^\text{10}\)

Histopathology of liver

After collection of blood for biochemical estimation, the rats were sacrificed, and the liver was carefully dissected, cleaned of extraneous tissue, and fixed in 10% formalin for at least 24 hrs. Then, the paraffin sections were prepared (automatic tissue processor, auto-technique) and cut into 5 µm thick sections, using a rotary microtome. The sections were stained with hematoxylin-eosin dye and studied for histopathological changes. The hepatoprotective activity was confirmed through histopathological studies on liver of rats.\(^\text{11}\)

Statistical analysis

All the results were expressed as mean±standard error of the mean. One-way analysis of variance was used for the statistical analysis of data. Dunnett’s multiple comparisons tests were used for determining the significance. A p<0.05 was considered as significant.\(^\text{12}\)

RESULTS

Biochemical tests

The activity levels of serum ALT, AST, ALP, total serum bilirubin and total serum protein were taken as indices for hepatotoxicity induced by CCI\(_4\) in Group II. In the CCI\(_4\) treated Group II, ALT, AST, ALP, total serum bilirubin were increased significantly (p<0.001) to 60.76±4.95 U/L/mins/mg protein, 105.23±8.02 U/L/mins/mg protein, 30.16±2.31 KA units, 1.88±0.21 mg/dL respectively, whereas these values were showed 22.36±1.75 U/L/mins/mg protein, 67.50±4.08 U/L/mins/mg protein, 10.76±0.84 KA units, 0.25±0.11 mg/dL 127.9 U/L in control Group I, respectively. The total serum proteins in CCI\(_4\) treated Group II decreased significantly (p<0.001) to 4.16±0.20 g/dL from 6.93±0.26 g/dL in control Group I. Administration with aqueous extract of \(B. \text{diffusa}\) at a dose of 250 mg/kg b.w in Group IV showed statistically significant improvement (p<0.05) in the serum ALT, AST, ALP, total serum bilirubin levels to 1.42±0.12 mg/dL which are comparable to control Group I and silymarin Group III. The total serum proteins in Group V increased significantly (p<0.05) to 5.93±0.33 g/dL from 4.16±0.20 g/dL in CCI\(_4\) treated Group II and comparable to 6.93±0.26 g/dL in Group I, 6.91±0.24 g/dL in silymarin treated Group III and 6.18±0.26 g/dL in \(B. \text{diffusa}\) at 250 mg/kg treated Group IV. Silymarin used as standard positive control in group III showed a statistically significant (p<0.05) improvement compared with CCI\(_4\) administered Group II of biochemical values of ALT 25.26±2.04, AST 70.16±4.91, ALP 13.21±1.07, total serum bilirubin of 0.62±0.07 and total serum protein of 6.91±0.24 demonstrated that it is an effective hepatoprotective agent (Table 1).

Histopathological studies of rat liver

The typical architecture of liver tissue was observed with a central vein (CV) from which chords of hepatocytes were radiating. The portal triad was seen consisting of the hepatic artery, portal vein (PV) and bile duct. The Zone 1 constituted surrounding areas of the hepatic artery while Zone 2 and Zone 3 were situated to further periphery. The area around the CV is called centrizonal or centrilobular area (Figure 1).

The comparison of the liver section of rats treated with CCI\(_4\) in Group II showed a high degree of damage characterized by cell vacuolation, pyknotic and degenerated nuclei and wall of bile capillaries. The normal architecture of the liver was lost. The intralobular vein was badly damaged with wide spaces at some sinusoids. CCI\(_4\) administration produced extensive hemorrhagic necrosis of the liver which was more pronounced in the centrizonal (Zone 3) region with infiltration of inflammatory cells. Apart from fatty changes, hepatocytes showed hydriptic changes and the sinusoids were congested (Figure 2).

Silymarin used as a standard control in Group III at the doses of 50 mg/kg along with CCI\(_4\) showed only mild to moderate necrosis, with multiple tiny foci of liver cell injury (Figure 3).

Figure 1: Normal control Group I. Normal histological architecture of rat liver with central vein and radiating chords of hepatocytes.
The hepatic cells of rats treated with aqueous extract of *B. diffusa* extract at 250 mg/kg b.w and CCl₄ in Group IV were radially arranged. The vacuolation was present, similar to that of normal. The intralobular vein was normal in structure, but the wall was damaged and no pyknosis in the nucleus. The recovery was comparable to that with silymarin in Group III, a standard hepatoprotective agent (Figure 4).

Liver sections of rats that were administered aqueous extract of *B. diffusa* at 500 mg/kg b.w along with CCl₄ in Group V exhibited a high degree of protection to the liver by the absence of necrosis with few fatty vacuoles and normal arrangement of hepatocytes (Figure 5).

**DISCUSSION**

The liver plays an important role in providing protection from exposure to foreign substances by detoxification and elimination. Excessive exposure of the liver to environmental toxins, alcohol, drug overdose, and other commonly used therapeutic agents such as paracetamol, rifampicin and bicalutamide can cause hepatotoxicity. The traditional herbal treatments for liver diseases have reached a new dimension with the support of modern evidence-based medicine in clinical studies. CCl₄ is commonly used for induction of experimental liver toxicity. Its metabolites such as trichloromethyl radical (CCl₃) and trichloromethyl peroxy radical (CCl₃O₂) are involved in the pathogenesis of liver toxicity. The activated free radical binds covalently to the macromolecules and induces peroxidative degradation of membrane lipids of endoplasmic reticulum which are rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides which in turn gives product such as malondialdehyde (MDA) that cause damage the membranes. The increased liver MDA contents in rats treated with CCl₄ only suggest that the natural antioxidant defense mechanism to scavenge excessive free radicals has been compromised. This results in changes in structures of the endoplasmic reticulum and membranes of other organelles, loss of metabolic enzyme activation, reduction of protein synthesis and loss of glucose-6-phosphatase activation, leading to liver injury and elevated levels of serum transaminases, ALP, and bilirubin. Increased serum bilirubin level could also be looked upon as a compensatory/retaliatory phenomenon in response to cellular peroxidative changes. This is because bilirubin functions *in vivo* as a powerful antioxidant, anti-mutagen, and an endogenous tissue protector. Serum ALT, serum AST and ALP were found to be significantly elevated after CCl₄ administration (Table 1, Figure 1) though the rise in bilirubin level was not to the same extent

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (IU/L/min/mg protein)</th>
<th>AST (IU/L/min/mg protein)</th>
<th>ALP (KU units/dL)</th>
<th>Total serum bilirubin (mg/dL)</th>
<th>Total serum protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups I</td>
<td>22.36±1.75</td>
<td>67.50±4.08</td>
<td>10.76±0.84</td>
<td>0.25±0.11</td>
<td>6.93±0.26</td>
</tr>
<tr>
<td>Groups II</td>
<td>60.76±4.95**</td>
<td>105.23±8.02**</td>
<td>30.16±2.31**</td>
<td>1.88±0.21*</td>
<td>4.16±0.20*</td>
</tr>
<tr>
<td>Groups III</td>
<td>35.26±2.04*</td>
<td>80.16±4.91*</td>
<td>23.21±1.07*</td>
<td>0.62±0.07</td>
<td>6.91±0.24*</td>
</tr>
<tr>
<td>Groups IV</td>
<td>37.36±3.04*</td>
<td>84.46±5.80*</td>
<td>22.11±0.56*</td>
<td>1.48±0.11*</td>
<td>6.18±0.26*</td>
</tr>
<tr>
<td>Groups V</td>
<td>36.46±1.04*</td>
<td>82.36±3.64*</td>
<td>21.10±0.66*</td>
<td>1.42±0.12*</td>
<td>5.93±0.33*</td>
</tr>
</tbody>
</table>

Values are mean±SEM, n=6, *p<0.05 compared with Group II; **p<0.001 compared to Group I, ALT: Alanine aminotransferases, AST: Aspartate aminotransferases, ALP: Alkaline phosphatases, SEM: Standard error of mean.

Figure 2: Carbon tetrachloride (CCl₄) induced hepatotoxicity. CCl₄ induced hepatotoxicity showing extensive areas of confluent necrosis and also showing fatty changes and hydropic degeneration. H and E stain, ×100. PV: Portal vein, BD: Bile duct.

Figure 3: Group III of silymarin (50 mg/kg) and carbon tetrachloride treated group showing partial centronzonal protection. H and E stain 40 and ×100.
as ALT, AST, and ALP. This could be explained by the fact that bilirubin reaches peak serum level in the 2nd hr after CCl₄ administration and probably declines afterwards. Blood collection in the present study was 48 hrs after CCl₄ administration, and the serum bilirubin levels would have been on the decline. Administration of CCl₄ causes centrilobular necrosis (around the CV in the liver), fatty changes, hydropic degeneration and hepatocytes with pyknotic nucleus and leakage of liver marker enzymes such as ALT, AST, ALP, and bilirubin and a decrease in serum total protein (Figure 2).

The herbal drugs or polyherbal combinations are traditionally used in the treatment of liver diseases caused by viral hepatitis, alcohol, toxic drugs, and plant toxins. Silymarin from *Silybum marianum*, andrographolide from *Andrographis paniculata*, Curcumin from *Curcuma longa*, Picroside and kutkoside from *Picrorrhiza kuruoa*, phyllanthin and hypophyllanthin from *Phyllanthus niruri*, glycyrrhizin from *Glycyrrhiza glabra* are traditionally studied for their chemical and biological profile and hepatoprotective efficacy in animal models.¹⁶⁻¹⁸ These herbal preparations show hepatoprotection due to the antioxidant effect, but other effects like immunomodulatory, antiviral, anti-inflammatory, antifibrotic, membrane stabilizing and antiproteozaal activities are also documented. The flavonoids present in *B. diffusa* may probably prevent the accumulation of excessive free radicals and protect the liver against CCl₄ intoxication. We have tried to evaluate the hepatoprotective activity of *B. diffusa* against CCl₄ induced hepatotoxicity in the rat model in this study by assessing biochemical markers and histopathological methods. *B. diffusa* can have an important role in the future of treatment of various liver disorders. *S. marianum* or silymarin, a plant secondary metabolite, is a complex mixture of four flavonolignan isomers, namely, silybin (60-70%), silychristin (20%), silidianin (10%), and isosilybin (5%). Silymarin is used as a standard control for hepatoprotective activity in CCl₄ induced hepatotoxicity in our study. Silymarin has been reported to have antioxidant, anti-inflammatory, immunomodulatory, and liver-regenerating properties. The elevated levels of liver enzymes such as aspartate and ALT, AST, ALP and total serum bilirubin found in CCl₄ induced liver injuries are reduced significantly by its use of silymarin both in animal and clinical studies.¹⁹

Administration of aqueous extracts of *B. diffusa* showed significant hepatoprotective activity at 250 mg/kg and 500 mg/kg, which were comparable to the standard control silymarin at 50 mg/kg. The hepatoprotective effects were more pronounced with a higher dose of 500 mg/kg of aqueous extract of *B. diffusa*. The increased serum levels of ALT, AST, ALP and total serum bilirubin in CCl₄ treated animals might be due to the leakage of enzymes into the serum. The significant decrease in the serum levels of the ALT, AST, ALP and total bilirubin in *B. diffusa* aqueous extract administered animals might be due to decreased leakage from the liver cells. This suggests that the aqueous extract of *B. diffusa* was able to repair the probable hepatic injury and/or restore the cellular permeability; thus reducing the toxic effect of CCl₄ on the liver tissue. The qualitative phytochemical investigations on the aqueous extract of *B. diffusa* also showed positive for flavonoids that possess antioxidant properties and were found to be useful in the treatment of liver diseases. Since formation of free radicals by cytochrome P 450 after metabolism of CCl₄ has been implicated in lipid peroxidation mediated hepatocyte injury, the hepatoprotective property of *B. diffusa* can be ascribed to its inhibitory effect on the microsomal enzymes and hence that generation of free radicals is bound to be limited. *B. diffusa* is also claimed to act as a free radical scavenger thereby preventing lipid peroxidation by its antioxidant property and a stimulatory effect on hepatic regeneration.²⁰ It is also assumed that the hepatoprotective effect of *B. diffusa* aqueous extract may be related to glutathione (GSH) - mediated detoxification. *B. diffusa* is reported to enhance GSH status in cells and thereby afforded protection to hepatic cells from toxic damage. There are
still unexplained mechanisms of *B. diffusa*, which may be assumed to be involved in the protection of liver from CCl4 induced toxicity.1,3

The present study had a few limitations as the safety profile of *B. diffusa* was not studied, and the hepatoprotective drugs were given as pretreatment. Further, the pharmacokinetic studies of *B. diffusa* are largely unknown. An elaborate investigation is needed to explore the pharmacokinetic profile of *B. diffusa* and also for isolation and structure determination of the active hepatoprotective principle(s) ingredients.

It can be concluded from the present study that the aqueous extract of *B. diffusa* possesses hepatoprotective activity against CCl4 induced hepatotoxicity in the rat model. Aqueous extract of *B. diffusa* have demonstrated hepatoprotective activity based on biochemical parameters ALT, AST, ALP and total bilirubin levels, and also by histopathological studies by preserving the normal architecture of liver tissue to a large extent. Therefore, the study scientifically supports the further investigation for exploration of this aqueous extract of *B. diffusa* in animal and clinical studies before its introduction into medicine for treatment of liver disorders.

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**Conflict of interest:** Declared  
**Ethical approval:** The study was approved by the Institutional Animal Ethics Committee of Medical Sciences (IAEC)

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