Antihepatotoxic role of Boerhaavia diffusa (Linn.) against antituberculosis drug rifampicin induced hepatotoxicity in male albino wistar rats

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ABSTRACT
Objective: To investigate the antihepatotoxic role of methanolic leaf extract of Boerhaavia diffusa on antituberculosis drug, Rifampicin induced liver injury in male albino rats.

Methods: In the present study, the methanolic leaf extract of Boerhaavia diffusa (50, 100 and 200 mg/kg body weight) was examined for its antihepatotoxic efficacy against rifampicin induced liver injury in rats. Thirty six healthy male albino rats (150-180 g weight) were chosen and divided in to six groups. Rifampicin and methanolic extract were given according to the experimental design. After 28 days of treatment, hepatic marker enzymes and liver histology were analyzed.

Results: Rifampicin induced liver damage showed significantly increased activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Gamaglutamyl transpeptidase (GGT), Lactate dehydrogenase (LDH) and bilirubin and decreased levels of protein in serum when compared with that of the control group. Treatment with Boerhaavia diffusa or silymarin could significantly restore to normal by a decrease in the ALT, AST, ALP, GGT and bilirubin whereas protein levels in serum increased when compared with rifampicin alone treated rats. The probable mechanism of hepatocuration by administration of methanolic leaf extract of Boerhaavia diffusa could minimize the activities of liver marker enzymes and preserve the normal histoarchitecture pattern of liver.

Conclusion: The present investigation suggests that the methanolic leaf extract of Boerhaavia diffusa has antihepatotoxic role against rifampicin induced hepatotoxicity.

KEYWORDS: Boerhaavia diffusa, bilirubin, hepatotoxicity, histology, rifampicin, silymarin.

1. INTRODUCTION
Liver is the most important organ in the body. It plays a pivotal role in regulating various physiological processes. It is also involved in several vital functions such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles and also is a frequent target of a number of toxicants. Liver diseases are mainly caused by substances like paracetamol, rifampicin, carbon tetrachloride, heavy metals, pesticides, virus and consumption of alcohol in excess quantity. Most of the hepatotoxic chemicals damage liver cells mainly by inhibiting the antioxidant enzymes. Liver diseases are worldwide problems, and conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects.

Hepatotoxicity denotes injury to the liver that is allied with diminished liver function. Numerous medical plants and their formulations are being used for liver disorders in ethnomedical practices and in traditional system of medicine in India. Conventional drugs used in the treatment of liver disease are often inadequate. It is therefore necessary to search for supplementation/alternative drugs for the treatment of hepatic damage caused by antitubercular drugs.

Tuberculosis is a leading public health problem worldwide, particularly in developing countries. About one third of world’s population has latent tuberculosis and approximately 9 million cases of active tuberculosis emerge annually resulting in 2–3 million deaths. Out of 1.86 billion people estimated to be infected with tuberculosis bacillus, an estimated 1.3 billion infected people were living in developing countries, such as India and China.

Patients on concurrent Rifampicin therapy have an increased incidence of hepatitis. This has been postulated due to Rifampicin-induced cytochrome P450 enzyme-induction, causing an increased production of the toxic metabolites from acetyl hydrazine (AcHz). Rifampicin also increases the metabolism of INH to isonicotinic acid and hydrazine, both of which are hepatotoxic. The plasma half life of AcHz (metabolite of INH) is shortened by Rifampicin and AcHz is quickly converted to its active metabolites by increasing the oxidative elimination rate of AcHz, which is related to the higher incidence of liver necrosis caused by INH and Rifampicin in combination. Rifampicin
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also interacts with antiretroviral drugs and affects the plasma levels of these drugs as well as risk of hepatotoxicity.\textsuperscript{11,12,13}

Recently, a herbal drug has become an important material of global market, having both medicinal and economical implications. Medicinal plants have been known since ancient times throughout the world as a rich source of therapeutical substances for remediation and prevention of many diseases. Boerhaavia diffusa is a widely dispersed plant occurring throughout India, the Pacific and southern United States. Boerhaavia diffusa is commonly known by the name Punarnava in India. Punarnavine, an alkaloid isolated from this plant has been shown \textit{in vitro} to possess anti cancer, anti estrogenic, anti amoebic and immunomodulatory activities.\textsuperscript{14} The leaves of Boerhaavia diffusa are often used as a green vegetable in many parts of India and are very well known to possess medicinal properties. They are generally used for pain relief and to protect and improve eyesight. Extracts of \textit{Boerhaavia diffusa} leaves have been shown to possess antioxidant, hepatoprotective, diuretic and anti-diabetic properties in pharmacological models.\textsuperscript{15,16}

Among the herbal drugs, silymarin has been used as a dietary supplement for hepatoprotection for over 2000 years. Silymarin, commercially available as Milk Thistle, is an extract from the seeds of \textit{S. marianum}. Silybines (A and B isomers), isosilybines (A and B), silychristine and silydianine are active flavonoids found in silymarin extract. Silymarin has been shown to be safe in animal models, and no significant adverse reactions are reported in human studies.\textsuperscript{17} Rifampicin is a first line antituberculosis drug which effectively cure tuberculosis but liver cell injury is inevitable. Therefore, the present investigation was aimed to evaluate the antihepatotoxic efficacy of methanolic extract of \textit{Boerhaavia diffusa} on rifampicin induced hepatotoxicity in rats.

2. EXPERIMENTS

2.1. MATERIALS AND METHODS

2.1.1. Chemicals

Silymarin, as a standard drug was obtained from Alfa Aesar Johnson Matthey Company, Hyderabad. Animal feed pellets were procured from Brook Bond Lipton India Ltd., Hyderabad. All other chemicals and solvents used in the study were of analytical grade.

2.1.2. Collection and extraction of plant material

The collected leaves of \textit{Boerhaavia diffusa} were air dried and powdered. The powdered \textit{Boerhaavia diffusa} were kept in airtight containers in a deep freezer until the time of use. A sample containing 1000 g of \textit{Boerhaavia diffusa} was mixed with 4 L of methanol and stirred magnetically overnight (12 h) at 37º C. This was repeated three consecutive times. The residue was removed by filtration and the extract was evaporated to dryness at a lower temperature (<40º C) under reduced pressure in a rotary evaporator. The residual extract was dissolved in normal physiological saline and used in the study. The yield of the extracts was approximately 32.64 g. The suitable optimum dosage schedule were identified by administering the methanolic extract of \textit{Boerhaavia diffusa} at different dosages (50, 100, 200, 400 and 800 mg/kg body weight) in a day for twenty eight days. The optimum doses were selected as 50, 100 and 200 mg/kg body weight of the animals for twenty eight days respectively.

2.1.3. Preparation of dose for dried extracts and standard drug

The methanolic extracts were formulated as suspension in distilled water. The strength of the suspension was according to the dose administered and was expressed as weight of the dried extract.\textsuperscript{18} Silymarin at 25 mg/kg body weight was used as a reference standard drug for evaluating antihepatotoxic activity which was made into suspension in distilled water.

2.1.4. Procurement and rearing of experimental animals

Adult male albino rats (Wistar strain) were collected from Central Animal House, Rajah Muthiah Medical College, Annamalai University and were used for the present study. The rats were housed in polypropylene cages at room temperature (27 ±2 º C). The animals were randomized and separated into normal and experimental groups with body weight ranging from 150-180 g. The animals received a diet of standard pellets (Hindustan Lever Ltd., Bombay). Rats were provided free access to water \textit{ad libitum} and food through the tenure of acclimatization to the environment for a minimum period of two weeks prior to the commencement of experiment. The study was approved by the Institutional Animal Ethical Committee of Rajah Muthiah Medical College and Hospital (160/1999/CPCSEA, Proposal No. 962), Annamalai University, Annamalainagar, Tamil Nadu, India.

2.1.5. Acute oral toxicity studies

The acute oral toxicity study of the extracts was carried as per the OECD (420) guidelines. Administration of the extracts stepwise up to the dose 2000 mg/kg body weight caused no considerable signs of toxicity in the tested animals. 1/10\textsuperscript{th} of the dose was selected for examination of antihepatotoxic activity of the methanolic leaf extract of \textit{Boerhaavia diffusa}.\textsuperscript{19}

2.1.6. Experimental design

The animals were divided into 7 groups of 6 rats each. Group 1 : Control rats given physiological saline solution at 10 mL/kg body wt. Group 2: Rats given rifampicin (1 g/kg body wt./p o) for one day only. Group 3: Rats given rifampicin + \textit{Boerhaavia diffusa} (50 mg/kg body wt. / p o) Group 4: Rats given rifampicin + \textit{Boerhaavia diffusa} (100 mg/kg body wt / p o) Group 5: Rats given rifampicin + \textit{Boerhaavia diffusa} (200 mg/kg body wt / p o) Group 6: Rats given rifampicin + silymarin (25 mg/kg body wt / p o) Group 7: Rats given \textit{Boerhaavia diffusa} (200 mg/kg body wt / p o) alone

At the end of the experimental period for 24 h after last treatment the
animals were killed by cervical decapitation. The liver tissues were excised immediately and washed with chilled physiological saline.

2.1.7. Collection of blood
Blood was collected by cardiac puncture under mild ether anesthesia and serum was separated by centrifugation at 3000 rpm for 15 min to determine the biochemical parameters.

2.1.8. Biochemical analysis
Blood samples were taken in the centrifuge tube with rubber caps labeled and centrifuged at 3000 rpm for 15 minutes. Serum biochemical parameters such as transaminases (AST and ALT), ALP, GGT, LDH, bilirubin and protein were estimated according to standard methods respectively.20-25

2.1.9. Histopathological studies
All rats were sacrificed by cervical dislocation and then midline laparotomy was performed. Dissected liver specimens of each rat in all groups were fixed in 10% buffered formaldehyde for 24 hours and embedded in paraffin after 16 h of alcohol process. Five µm thick sections were obtained from the paraffin blocks and stained with hematoxylin and eosin. Each slide was examined under a light microscope.26

3. Statistical analysis
Statistical analysis was done by analysis of variance (ANOVA) and the groups were compared by Duncan’s multiple range test (DMRT). The level of statistical significance was set at p < 0.05.27

4. RESULTS AND DISCUSSION
Liver is a vital metabolic organ, which also has secretory and excretory functions. It has a paramount importance in the body because of its vital functions like detoxification of endogenous and exogenous substances like xenobiotics, viral infections, chronic alcoholism, bile secretion, etc. Exposure to all the above challenges the liver and is overpowered, resulting in liver failure. All over the world the mortality and morbidity of liver diseases increases year by year. Nearly 20,000 deaths and 2,50,000 new cases are found each year.29-30 Jaundice may result from various diseases or conditions that affect the liver, like Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, Hepatitis E, Autoimmune hepatitis, Liver cirrhosis, liver cancer and Hemolytic anaemia.31,32 Serum enzymes serve as markers or fairly specific indicators of the liver status. Commonly elevated level of liver enzymes in the serum is an indication of liver cells damage and even liver cell death.33 Elevated levels of serum marker enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in the liver.34 Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cells. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure.35 Estimating the activities of serum and tissue marker enzymes like ALP, ACP, AST, ALT and LDH can make assessment of liver function. When liver and kidney cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage.36 Serum GGT is a sensitive marker enzyme widely used as a laboratory test for the hepatobiliary diseases.37

In the present investigation rats treated with rifampicin developed significant hepatic damage which was observed through a substantial increase in the concentration of AST, ALT, ALP, GGT, LDH and bilirubin whereas a decrease in the level of protein. Oral administration of rats with methanolic extracts of Boerhaavia diffusa (50, 100 and 200 mg/kg body wt.) and silymarin after the challenge of rifampicin produced an alleviation of the hepatic injury to a considerable extent which was reflected by the ability of the methanolic extracts of Boerhaavia diffusa to lower the elevated serum enzymes levels resulting from the administration of rifampicin alone (Tables 1 and 2). Similarly after treatments with Bauhinia variegata and silymarin against isonized and rifampicin induced hepatotoxicity, the levels of hepatic marker enzymes were nearly normal or only slightly elevated, indicating protection against liver damage. Bauhinia variegata has increased the levels of total proteins and albumin in the serum, which

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>LDH (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73.47±5.59</td>
<td>44.39±3.38</td>
<td>166.53±12.68</td>
<td>372.54±28.37</td>
</tr>
<tr>
<td>Rifampicin (1g /kg)</td>
<td>142.72±10.87</td>
<td>157.65±12.01</td>
<td>318.37±24.25</td>
<td>864.46±65.82</td>
</tr>
<tr>
<td>Rifampicin + Boerhaavia diffusa (50 mg/kg)</td>
<td>128.67±9.80</td>
<td>142.20±10.83</td>
<td>282.18±21.48</td>
<td>735.26±55.98</td>
</tr>
<tr>
<td>Rifampicin + Boerhaavia diffusa (100 mg/kg)</td>
<td>102.34±7.79</td>
<td>116.28±8.85</td>
<td>235.67±17.95</td>
<td>586.88±44.69</td>
</tr>
<tr>
<td>Rifampicin + Boerhaavia diffusa (200 mg/kg)</td>
<td>81.53±6.21</td>
<td>95.76±7.29</td>
<td>188.15±14.32</td>
<td>414.28±31.54</td>
</tr>
<tr>
<td>Rifampicin + Silymarin (25 mg/kg)</td>
<td>89.41±6.81</td>
<td>108.54±8.27</td>
<td>216.76±16.51</td>
<td>483.43±38.81</td>
</tr>
<tr>
<td>Boerhaavia diffusa (200 mg/kg) alone</td>
<td>72.99±5.57</td>
<td>44.14±3.35</td>
<td>163.47±12.47</td>
<td>366.92±27.94</td>
</tr>
</tbody>
</table>

All the values are mean ± SD of six observations, Values which are not sharing common superscript differ significantly at 5% level (P < 0.05), Duncan Multiple Range Test (DMRT)
indicates hepatoprotective activity. Stimulation of protein synthesis has been advanced as a contributory hepatoprotective mechanism which accelerates the regeneration process and the production of liver cells.39 Administration of ethanolic leaf extract of Plectranthus amboinicus to antituberculosis drug treated rats showed decrease the activities of transaminase and alkaline phosphatase when compared with that of the antituberculosis drug treated rats.39

ALP and ACP are ubiquitous in nature. Their primary role of extra cellular phosphatases is to provide inorganic phosphate for cell growth by the hydrolysis of external phosphate esters which do not penetrate the cytoplasmic membrane40 and also ALP is the prototype of these enzymes that reflect the pathological alteration in biliary flow.41 Oral administration of Terminalia arjuna bark extract lowered the elevated serum enzyme level.42

Proteins are important building blocks of all cells and tissues. They are important for body growth, development and health. They form the structural part of most organs and make up enzymes and hormones that regulate body functions.43 A low protein level may reflect overproduction of globulins, such as seen in multiple myeloma or autoimmune diseases, or underproduction of albumin, such as that occur with cirrhosis, or selective loss of albumin from the circulation.44

Among different ailment, jaundice is the commonest ailments affecting the citizens of the world countries. Jaundice is the yellowish staining of the skin and sclera (the whites of the eyes) that is caused by high levels of the chemical bilirubin in the blood. The color of the skin and sclera vary depending on the level of bilirubin. When the bilirubin level is mildly elevated, they are yellowish. When the bilirubin level is high, they tend to be brown.

The liver not only synthesizes the protein for its needs but produces numerous export proteins. Among the latter, serum albumin is the most important one.45,46 The decrease in total serum protein observed in CCl4 treated rats may be associated with the decrease in the number of hepatocytes which in turn, might have led to the decreased hepatic capacity to synthesize protein.47 Administration of aqueous and ethanolic leaf extracts of Cassia italica to CCl4 treated rats showed decreased in the protein level to near normal when compared with CCl4 alone treated rats.48 On administration of Terminalia arjuna bark extract the LDH level was found to be decreased and this was similar to that of the normal rats. The decrease of LDH activity in serum, liver and kidney thereby decreased the endogenous glucose production.42

Histology is an essential tool of biology, medicine and veterinary medicine. Histology is the study of their microscopic structures of cells and tissues of plants and animals. It is often carried out by examining a thin slice of tissue under a light microscope or an electron microscope. In order to distinguish different biological structures more easily and accurately histological stains are often used to add colours to enhance the colours of certain types of biological structures differently from other types of structures that may be located next to and/or in contact with each other. In the present study, the histological examination of the liver sections revealed that the normal liver architecture (Fig. 1a) was disturbed by the administration of antituberculosis drug, rifampicin which showed necrosis, ruptured hepatocytes, space formation, vacuolization, fatty accumulation, loss of cell boundaries and enlargement of hepatocytes (Fig. 1b). In the sections obtained from the rats treated with methanolic leaf extracts of Boerhaavia diffusa and standard drug, silymarin showed gradually minimized aforementioned damage (fig. I C,D,E,F) and normal cellular architecture was retained to some extent, thereby confirming the antihepatotoxic efficacy of the Boerhaavia diffusa extract. Administration of Boerhaavia diffusa extract alone (200 mg/ kg body weight) showed normal histoarchitecture of liver (Fig. 1G). Administration of Boerhaavia diffusa extract (200 mg/ kg body weight) to rifampicin treated rats revealed maximum hepatocurative effect. The results obtained indicate that the plant Boerhaavia diffusa extract is useful in the treatment of drug induced liver related complications. Similarly

**Table 2 – Serum GGT, Bilirubin and Protein levels in control and experimental groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>GGT (U/L) ± SD</th>
<th>Protein (mg/dL) ± SD</th>
<th>Bilirubin (mg/dL) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.26±1.31</td>
<td>7.68±0.58</td>
<td>0.54±0.04</td>
</tr>
<tr>
<td>Rifampicin (1g /kg)</td>
<td>39.47±3.01</td>
<td>5.32±0.40</td>
<td>2.29±0.17</td>
</tr>
<tr>
<td>Rifampicin + Boerhaavia diffusa (50 mg/kg)</td>
<td>34.65±2.64</td>
<td>6.10±0.46</td>
<td>1.86±0.14</td>
</tr>
<tr>
<td>Rifampicin + Boerhaavia diffusa (100 mg/kg)</td>
<td>26.82±2.04</td>
<td>6.82±0.52</td>
<td>1.32±0.10</td>
</tr>
<tr>
<td>Rifampicin + Boerhaavia diffusa (200 mg/kg)</td>
<td>20.16±1.53</td>
<td>7.47±0.56</td>
<td>0.73±0.05</td>
</tr>
<tr>
<td>Rifampicin + Silymarin (25 mg/kg)</td>
<td>22.94±1.74</td>
<td>7.06±0.53</td>
<td>0.94±0.07</td>
</tr>
<tr>
<td>Boerhaavia diffusa(200 mg/kg) alone</td>
<td>17.14±1.30</td>
<td>7.70±0.45</td>
<td>0.53±0.04</td>
</tr>
</tbody>
</table>

*All the values are mean ± SD of six observations, Values which are not sharing common superscript differ significantly at 5% level (P < 0.05), Duncan Multiple Range Test (DMRT)*
administration of *Cichroium intybus* (Linn.) root extract against carbon tetrachloride treated rats showed that regeneration of hepatocytes conform the hepatoprotective activity. The rats treated with ethanolic extract of *Ixora pavetta* against isonizid and rifampicin...
showed a sign of protection has it was a evident for the absence of necrosis with regeneration changes at central vein in the liver tissue.44

5. CONCLUSION
It is concluded that administration of methanolic extracts of Boerhaavia diffusa showed recovery against the toxic effects of rifampicin. The antihepatotoxic efficacy of the drugs was further confirmed by the histopathological examinations of the liver sections, which reveal that the normal liver shape was disturbed by hepatotoxic intoxication. In the liver sections of the rats treated with methanolic extracts of Boerhaavia diffusa and intoxicated with rifampicin the normal cellular shape was retained when compared to silymarin, thereby confirming the antihepatotoxic efficacy of the extracts of Boerhaavia diffusa. The antihepatotoxic activity of Boerhaavia diffusa could be due to the presence of flavonoids, which have antihepatotoxic properties. These results indicate that it is worth undertaking further studies on possible usefulness of the extracts of the leaves of Boerhaavia diffusa in hepatotoxicity.

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