

BOERHAVIA DIFFUSA EXTRACT AMELIORATES CARBON TETRACHLORIDE INDUCED OXIDATIVE STRESS: AN *IN VITRO* STUDY

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ABSTRACT

Aim: The aim of the present study was to evaluate the effect of *Boerhaviadiffusa* extract against carbon tetrachloride (CCl_4) – induced lipid peroxidation in liver of mice *in vitro*. **Methods:** Antioxidant activity of medicinally important plant *Boerhaviadiffusa* was determined by DPPH – radical scavenging activity. Liver homogenates were prepared and used for various treatments (CCl_4 and *Boerhaviadiffusa*) followed by analysis of lipid peroxidation and protein content by suitable method. **Results:** Carbon tetrachloride ($10 \mu\text{g/ml}$) to liver homogenates significantly increases H_2O_2 -induced lipid peroxidation *in vitro*. An addition of *Boerhaviadiffusa* aqueous extract ($25\text{--}150 \mu\text{g/ml}$) significantly ($p < 0.05$) reduced CCl_4 - induced lipid peroxidation in liver homogenates. The effect was concentration-dependent. The result was compared with standard drug Liv. 52. **Conclusion:** The study confirmed the ameliorative effect of the extract of *Boerhaviadiffusa* even higher than the Liv. 52, which may be attributed due to its antioxidative property.

KEY WORDS

Boerhaviadiffusa, Carbon tetrachloride, Lipid peroxidation, Liver.

Introduction

The liver is a vital organ present in vertebrates and some other animals. Liver damage may be caused by xenobiotics, alcohol consumption, malnutrition, infection, anaemia and medications [1]. Hepatotoxic chemicals cause the liver damages which are induced by lipid peroxidation and other oxidative damages [2, 3]. Antioxidative action plays an important role in protecting the liver against hepatotoxicants including CCl_4 - induced liver injury [4]. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically [5]. The liver injury induced by CCl_4 is the best characterized system for xenobiotic - induced hepatotoxicity and is commonly used models for screening of the anti-hepatotoxic and hepatoprotective activities of drugs [6, 7].

Boerhaviadiffusa Linn. (Nyctaginaceae), commonly known as 'Punarnava' in the Indian system of medicine is a perennial creeping herb found

throughout the waste land of India. *Boerhaviadiffusa* is a widely studied plant and has a long history of uses by the tribal people and in Ayurvedic and Unani medicines. The root and the whole plant of *Boerhaviadiffusa* are used in traditional medicine for the treatment of diabetes, stress, dyspepsia, abdominal pain, inflammation, jaundice, enlargement of spleen, heart diseases, bacterial infections, and impotence. Pharmacological studies have demonstrated that *Boerhaviadiffusa* known to possess anticonvulsant, diuretic, anti-inflammatory, antifibrinolytic, antibacterial, anthelmintic, anti-leprosy, antiasthmatic, antiurethritis, antilymphoproliferative, antimetastatic, immunosuppressive, antidiabetic, immunomodulation, anti-nociceptive, nephroprotective, antiurolithiatic and antioxidative activities [8]. Antioxidants play an important role in protecting the human body against damage from reactive oxygen species [8].

The purpose of the present study was to evaluate the ameliorative effect of *Boerhaviadiffusa* extract against CCl_4 - induced lipid peroxidation in liver of mice *in vitro*.

MATERIALS AND METHODS

Plant material

The whole plant of *Boerhaviadiffusa*(Punarnava) was obtained from Botanical garden of Gujarat University and was authenticated by the Botany Department, School of Sciences, Gujarat University, Ahmedabad, India.

Extract preparation

The extract was prepared according to World Health Organization protocol CG-06 with slight modifications [9]. 5 gm of shade dried powdered material of plant was extracted overnight by soaking method using 100 ml of 50% aqueous-ethanolic solvent. The content was filtered successively through ordinary and then Whatman filter paper No. 1. Extraction procedure was repeated. Both the fractions were pooled, dried and stored in dark bottle at 4°C . During the experiment known amount of dried extract was redissolved in double distilled water and used.

Chemicals

Analytical grade chemicals used in entire study were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India, Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Sigma-Aldrich, St. Louis, MO, USA. Olive oil was obtained from Figaro, Madrid, Spain.

Animals

Healthy, adult, pathogen free, colony bred Swiss strain female albino mice (*Mus musculus*) weighing between 30-35 gm. (6-8 weeks old) used in the present study were obtained from Zydus Research Centre, Ahmedabad, India. The animals were housed under controlled conditions (temperature $25 \pm 2^\circ\text{C}$; relative humidity 50-55%; 12 h light/dark cycle) in the animal house of Zoology Department, Gujarat University, Ahmedabad, India. Animals of different experimental groups were caged separately and maximum of five animals per cage were maintained on certified pelleted rodent feed supplied by Amrut feeds, Pranav Agro Industries Limited, Pune, India and potable water *ad-libitum*. The experimental procedures were assessed and approved by "The

Committee for the Purpose of Control and Supervision of Experiments on Animals" (Reg-167/1999/CPCSEA), New Delhi, India. Guidelines for Care and Use of Animals in Scientific Research 1991 published by Indian National Sciences Academy, New Delhi, India were followed.

DPPH radical scavenging activity:

The free radical scavenging activity of plant extract of *Boerhaviadiffusa* was done according to the method as reported by Gyamfiet *al.* [10]. 50 μl of the plant extract in methanol containing 100 $\mu\text{g/ml}$ extract in each reaction was mixed with 1 ml of 0.1 mM 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) in methanol solution and 450 μl of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 μl) only was used as control of experiment. After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by recording the absorbance at 517 nm. BHT was used as standard.

The percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{\text{Absorbance of control}} \times 100$$

Experimental Design:

Animals were sacrificed by overdose of diethyl ether and liver was dissected out, blotted free of blood, quickly weighed and used for the experiments.

Lipid peroxidation:

The level of lipid peroxidation in the liver was measured by the method as described by Ohkawa *et al.* with slight modification [11]. This method is based on the formation of a red chromophore that absorbs light at 532 nm following the reaction of thiobarbituric acid (TBA) with products of lipid peroxidation like malondialdehyde (MDA) and others collectively called as thiobarbituric acid reactive substances (TBARS). The results were expressed as n moles MDA formed /mg protein /60 min.

The experiment was set as mentioned below.

Liver homogenate (10%) was prepared in 0.1 M phosphate buffered saline (pH 7.4).

Following sets of tubes were prepared.

(A) Control tubes containing 0.2 ml of homogenate only.

(B) Dimethyl sulfoxide (DMSO) control tubes containing 0.2 ml DMSO only.

(C) Toxin-treated tubes containing 0.2 ml of homogenate and CCl_4 (10 $\mu\text{g/ml}$) in DMSO (0.4% V/V). Concentration of 10 $\mu\text{g/ml}$ CCl_4 was based on our earlier studies [12].

(D) Antidote control tubes containing *Boerhaviadiffusa* extract (150 $\mu\text{g/ml}$) only.

(E) Set of tubes containing *Boerhaviadiffusa* extract of different concentrations (25-150 $\mu\text{g/ml}$) along with CCl_4 (10 $\mu\text{g/ml}$).

The final volume (1.0 ml) in each tube was adjusted with additional 0.1 M phosphate buffered saline. Reaction was initiated by addition of 10mM H_2O_2 and the mixture was incubated at 37°C for 30 min with occasional shaking [13].

Lipid peroxides (TBARS) were estimated by using 8.1% sodium dodecyl sulphate, 20% acetic acid and 1% thiobarbituric acid solution. The blank for each sample was prepared by substituting the TBA solution with distilled water. The solution was mixed and heated in a water bath at 95°C for 60 min. The tubes were immediately cooled and centrifuged at 1000 $\times$ g for 10 min. The absorbance of the resulting supernatant fraction was read at 532 nm against blank on a Systronics 118 UV-Vis spectrophotometer.

Protein content:

Protein content was estimated in the liver by the method of Lowry *et al.* using bovine serum albumin as a standard [14]. When protein reacts with phenol reagent of Folin Ciocalteu, a deep blue colour develops. The blue colour that develops is quantitatively proportional to the total protein, which was measured at 540 nm. The protein content was expressed as mg/100 mg tissue weight.

The experiment was set as mentioned below.

Liver homogenate was prepared by using 100 mg liver tissue in 5 ml of double distilled water. Reaction mixture contained 0.2 ml of homogenate and CCl_4 (10 $\mu\text{g/ml}$) in DMSO (0.4% V/V).

Following sets of tubes were prepared:

(A) Control tubes containing 0.2 ml of homogenate only.

(B) DMSO control tubes containing 0.2 ml DMSO only.

(F) Reaction mixture contained 0.2 ml of homogenate and CCl_4 (10 $\mu\text{g/ml}$) in DMSO (0.4% V/V). Concentration of CCl_4 (10 $\mu\text{g/ml}$) was based on our earlier studies [12].

(C) Antidote control tubes containing *Boerhaviadiffusa* extract (150 $\mu\text{g/ml}$) only.

(D) Set of tubes containing *Boerhaviadiffusa* extract of different concentrations (25-150 $\mu\text{g/ml}$) along with CCl_4 (10 $\mu\text{g/ml}$). The standard tube contains 0.2 ml of bovine serum albumin in (mg/ml) concentration substituted by liver homogenate.

The final volume (1.0 ml) in each tube was adjusted with distilled water [13].

Reaction mixture was incubated at 37°C for 30 min with occasional shaking. After incubation, 4.0 ml of alkaline copper sulphate solution was added and incubated at 37°C for 15 min and then 0.4 ml of FolinCiocalteu reagent was added and incubated for 30 min at room temperature. The absorbance of the resulting supernatant fraction was read at 540 nm against blank on a Systronics 118 UV-Vis spectrophotometer.

STATISTICAL ANALYSIS:

The results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis and linear regression analysis were performed using GraphPad Instat, software, version 5.0. The values were analysed by one way Analysis of Variance followed by Tukey multiple comparison test at a significance level of $p < 0.05$.

RESULTS

No significant difference was noted between different controls. Addition of 10 $\mu\text{g/ml}$ CCl_4 in liver homogenate caused significant increase in lipid peroxidation as compared to untreated control (Table 1). Results shown in Table 1 also indicate that concurrent addition of *Boerhaviadiffusa* extract (25-150 $\mu\text{g/ml}$) along with CCl_4 (10 $\mu\text{g/ml}$) in liver homogenate significantly retarded CCl_4 – induced lipid peroxidation. The effect was concentration – dependent ($R^2 = 0.9775$) with maximum effect at 150 $\mu\text{g/ml}$ concentration of *Boerhaviadiffusa* extract (Figure 1).

Addition of 10 $\mu\text{g/ml}$ CCl_4 caused significant ($p < 0.05$) decrease in protein content in liver homogenate as compared to untreated control (Table 1). Results shown in Table 1 indicate that concurrent addition of *Boerhaviadiffusa* extract (25-150 $\mu\text{g/ml}$) along with 10 $\mu\text{g/ml}$ CCl_4 significantly ameliorated CCl_4 – induced

reduction in protein content in a concentration – dependent manner ($R^2 = 0.9767$) (Figure 2). Maximum effect was obtained on addition of 150 $\mu\text{g/ml}$ extract; it was even higher than that of Liv. 52. An addition of only *Boerhaviadiffusa* extract (control) did not cause significant effect in protein content in liver homogenate.

The DPPH radical scavenging activity of the hydro-alcoholic extract of *Boerhaviadiffusa* is shown in Figure 3. It was observed that the DPPH free radical

scavenging activity was concentration – dependent ($R^2 = 0.9146$), with a maximum inhibition (79.99%) at a concentration of 250 $\mu\text{g/ml}$ for *Boerhaviadiffusa* extract. No difference in inhibition was noted with further increase in concentration of *Boerhaviadiffusa* extract. The values are also comparable with butyl hydroxy toluene (BHT) (97.10%) at the same concentration (Figure 1). The IC_{50} values was calculated. The IC_{50} values are 100 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ for *Boerhaviadiffusa* and BHT respectively.

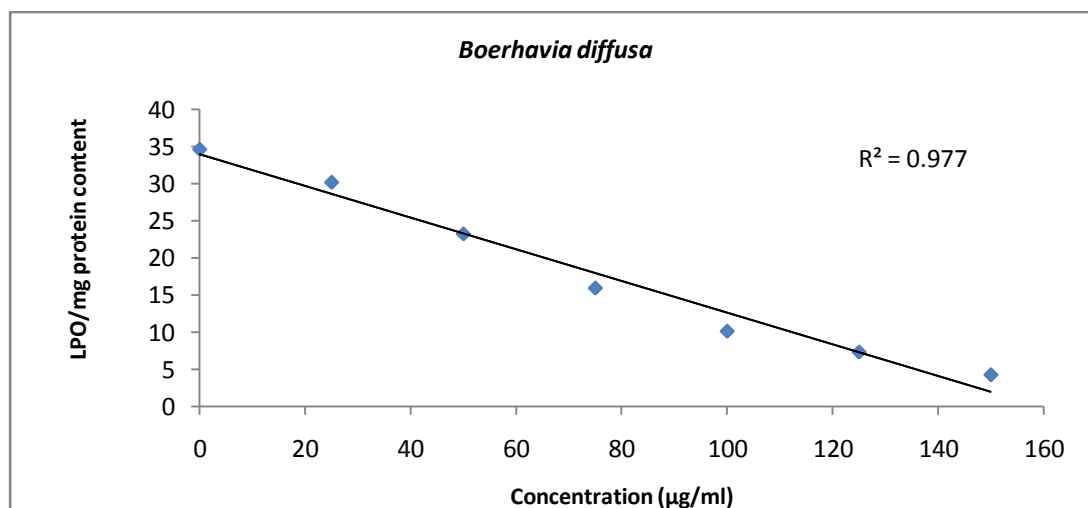


Figure 1: Linear regression curve showing dose-dependent effect of *Boerhaviadiffusa* extract on CCl_4 – induced lipid peroxidation.

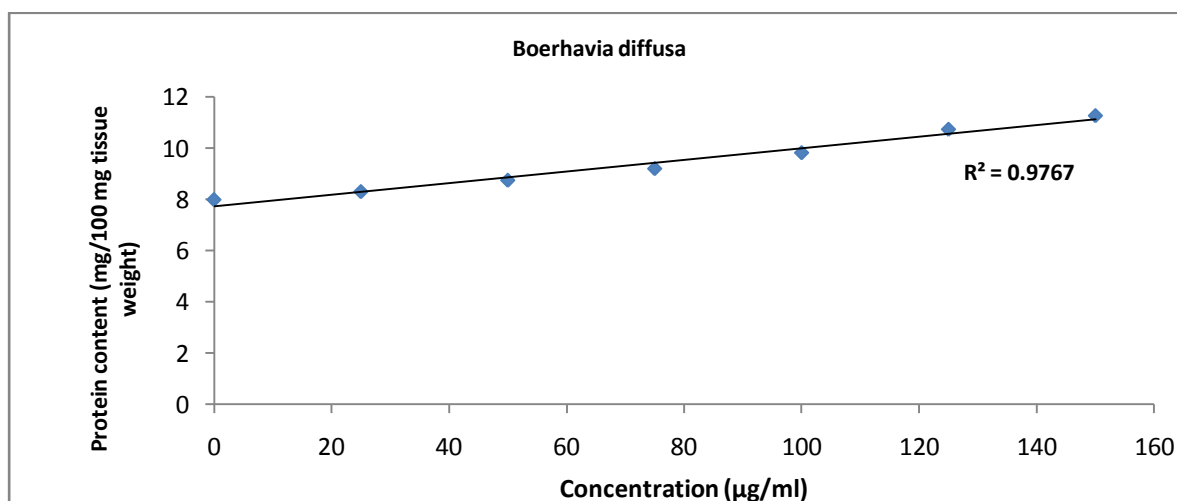


Figure 2: Linear regression curve showing dose-dependent effect of *Boerhaviadiffusa* extract on CCl_4 - induced changes in protein content.

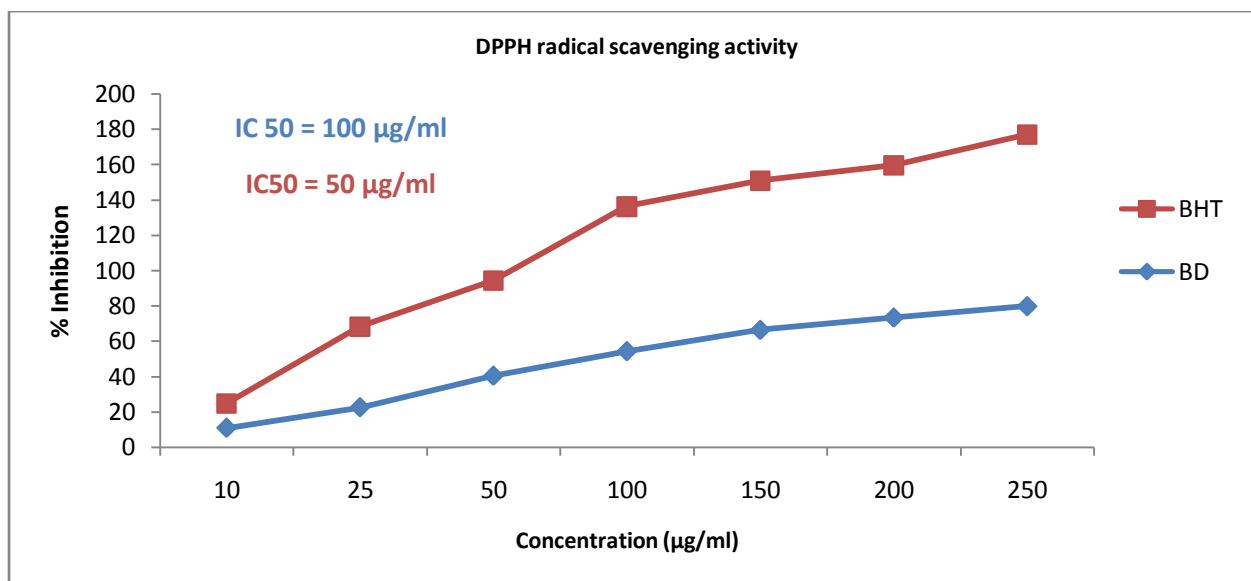


Figure 3: DPPH radical scavenging activity of the *Boerhaviadiffusa* extract

Table 1:

DISCUSSION

Carbon tetrachloride is used extensively in experimental models to induce oxidative stress in rats [15, 16]. Oxidative stress plays a crucial role in the development of carbon tetrachloride (CCl_4) - induced hepatotoxicity [17], and a correlation between oxidative stress and lipid peroxidation has been reported [18]. Lipid peroxidation is significantly

increased and protein content was significantly decreased in liver homogenate treated with CCl_4 (10 µg/ml) alone as compared to the controls (Table 1). Numerous studies noted that CCl_4 is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P450, generating a highly reactive carbon-centred trichloromethyl radical, leading to initiating a chain of lipid peroxidation and

thereby causing liver fibrosis [19, 20]. It is a well-known hepatotoxin that catabolizes radical induced lipid peroxidation, damage the membranes of liver cells and organelles and causes swelling and necrosis of hepatocytes. Carbon tetrachloride can induce liver damage through the formation of reactive free radicals that can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts; through the induction of hypomethylated ribosomal RNA, resulting in inhibition of protein synthesis. These injuries are mediated through the formation of reactive intermediates such as trichloromethyl ($\cdot\text{CCl}_3$) free radicals and ROS [21].

Concurrent addition of *Boerhaviadiffusa* (25-150 $\mu\text{g/ml}$) extract along with CCl_4 (10 $\mu\text{g/ml}$) caused significantly ($p < 0.05$) decreased lipid peroxidation as compared to CCl_4 alone treated liver homogenates. The effect was concentration – dependent ($R^2 = 0.9775$). Similarly addition of *Boerhaviadiffusa* (25-150 $\mu\text{g/ml}$) extract along with CCl_4 (10 $\mu\text{g/ml}$) significantly ameliorated protein content in liver homogenates. This may be due to free radical scavenging activity of the *Boerhaviadiffusa* (whole plant) extract.

Boerhaviadiffusa (79.99 %) showed potent DPPH radical scavenging activity (Figure 3). The *Boerhaviadiffusa* leaves are rich in alkaloids and sterols including ursolic acid, hypoxanthine-9-L-arabinofuranoside, punarnavine 1 and 2, myricyl alcohol, myristic acid and quinolizidine alkaloids [22]. The main chemical ingredients of this plant include alkaloids (punarnavine), rotenoids (boeravinones A to J) and flavones [23]. These compounds may be responsible for the antioxidant activity of *Boerhaviadiffusa*, which may be attributed to its protective action on lipid peroxidation and to the enhancing effect on cellular antioxidant defence contributing to the protection against oxidative damage induced by CCl_4 .

CONCLUSION

It is concluded that *Boerhaviadiffusa* extract significantly ameliorates CCl_4 – induced lipid peroxidation and protein content in liver homogenate even comparatively higher than the standard

polyherbal drug Liv. 52, in a concentration – dependent manner. *Boerhaviadiffusa* extract provide significant protection to the liver homogenate and prove its hepatoprotective activity.

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