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Research Article

ACUTE, SUB-CHRONIC AND CHRONIC TOXICITY STUDIES OF HYDRO ALCHOLIC EXTRACT OF BOERRHAVIA DIFFUSA ON EXPERIMENTAL ANIMALS

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A B S T R A C T

Despite Boerhaavia diffusa has many ethnomedicinal benefits, very few studies have described the potential toxicity. The aim of the present study was to evaluate the *in vivo* toxicity studies of hydroalcoholic extract of Boerhaavia diffusa. The acute, subchronic and chronic toxicity of hydroalcoholic extract of Boerhaavia diffusa was evaluated in experimental animals. For the acute toxicity study, a single dose administration of 2000 mg/kg was given by oral-gavage to swiss albino mice. The mice were observed for mortality and toxicity signs for 14 days. In the subchronic toxicity study the rats were administered orally at doses of 50, 100, and 200 & 400 mg/kg per day for 20 days. In addition to toxicity signs & mortality, biochemical analysis were carried out. Chronic toxicity studies include administration of HAEBD for 90 days at the dose of 200, 400 & 600mg/kg for the three groups except the normal control. Change in body weight, heamatological parameters & blood chemical values were determined. There were no acute, subchronic, chronic toxicity observed and our results indicate that this extract could be devoid of any toxic risk.

INTRODUCTION

Natural plants have been used to prevent and to treat various diseases for thousands of years. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. Herbal drugs or medicinal plants, their extracts and their isolated compounds have demonstrated spectrum of biological activities. One such plant, *Boerhaavia diffusa linn*, invites attention of the researchers worldwide for its pharmacological activities. The root, leaves, aerial parts or the whole plant of *B. diffusa* have been employed for the treatment of various disorders in the Ayurvedic herbal medicine. The roots are reputed to be diuretic and laxative and are given for the treatment of anasarca, ascites and jaundice.[1] Traditional use of any plant for medicinal purposes, guarantees the safety of plant & concern about the potential toxic effects resulting from the short-term and long-term use of such plants. Therefore, evaluating the toxicological effects of any medicinal plant extract intended to be used in animals is a crucial part of its assessment for potential toxic effects. The present study aimed to assess the adverse effects related to different doses and to evaluate the safety profile of the Hydroalcoholic extract from the aerial parts or whole plants of *Boerhaavia diffusa* in animals by determining acute, sub-chronic and chronic toxicity studies.

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Plant

Boerhaavia diffusa is a perennial creeping weed, commonly known as 'Punarnava' in the Indian system of medicine, found throughout the waste land of India.

Plant Extraction

200g of dried fine powder of *Boerhaavia diffusa* from aerial parts were successively extracted using Soxhlet apparatus by hot continuous percolation method. The powdered material were soaked in the extractor and macerated for 30h with

petroleum ether. There it was reflexed successfully with petroleum ether, chloroform, after that it was extracted with alcohol and water by continuous hot percolation method using Soxhlet apparatus for 40h separately. The hydro-alcoholic extract was filtered with Whatmann filter paper No. 40 and concentrated under vacuum using rotary flask evaporator under reduced pressure.

Preparation of stock solution:

Stock solutions of the hydroalcoholic extract of Boerhaavia diffusa (200 mg/mL) were prepared by weighing the powder and dissolving in 10% Carboxy Methyl cellulose (CMC). The solution was divided to aliquots and kept at −10°C, until used.

Experimental Animals

The experiments are carried out after getting the Institutional Animal Ethics Committee approval (App. No. G. Nalini / TNMGRMU /Ph.D / IAEC / KMCP /112 / 2014-15). The animals were housed at central animal house, K.M. College of Pharmacy, Madurai, Tamil Nadu, India under standard conditions of temperature (27±2°C), relative humidity (44–56%) and light and dark cycles of 10 and 14h respectively, for 1 week before and during the experiments. Animals were provided with standard diet (Hindustan Ltd. Bangalore, India), and water *ad libitum.* The food is withdrawn at 18–24h before the start of the experiment. All the experiments were performed in the morning according to the ethical guidelines for the care of the laboratory animals.[2]

MATERIALS & METHODS

Acute, sub-chronic & chronic toxicity studies of hydroalcoholic extract of Boerhaavia diffusa were evaluated on experimental animals. Body weight, organ weight determinations were carried out. Signs of behavior, mortality were observed. Heamatological parameter & biochemical examination were estimated.

Acute toxicity study

Healthy albino mice (25-30 g) were used in the acute toxicity study. The evaluation of acute toxicity study was carried out as per Organization of Economic Co-operation and Development (OECD) guidelines 423 (Acute toxic class method). The mice were fasted overnight and provided only water, after which the hydro-alcoholic extract of *Boerhaavia diffusa* Linn was administered by gastric intubation orally at a dose of 50mg/kg b.w. After this single administration, the animals were observed for signs of possible toxicity every 30 minutes after dosing for the first 24h and thereafter daily for a total of 14 days. All animals were weighed daily and monitored for any signs of toxicity and for mortality for up to 14 days. Additional signs of toxicity such as changes in body weight, skin and fur, eyes and mucus membranes, respiratory system, circulatory system, ANS and CNS, somatomotor activity and behaviour pattern were also recorded. If mortality observed in 2 to 3 animals in 14 days, then the dose was said to be toxic dose. But if mortality in one animal was observed, then the same dose was repeated again for confirmation. However, if mortality was not observed, the procedure was repeated for further higher doses such as 300 and 2,000mg/kg b.w Toxic symptoms are observed for 72 hrs including behavioral changes, locomotion, convulsions and mortality.[3, 4]

Subchronic toxicity study in rats

A subchronic repeated dose (20days) study in rats was conducted according to the OECD testing guidelines. Rats of both sexes were randomly distributed to five groups of six animals each. HAEBD was orally administered daily for 20 days in single doses of 50 mg/kg (group I), 100 mg/kg (group II), 200 mg/kg (group III), 400mg/kg.[5-7] The control rats (group IV) received only vehicle 0.5 ml of Tween 80. The body weight was recorded every 5 days. Along with food and water consumption, signs of toxicity and mortality were also recorded daily throughout the study period. Other signs of toxicity such as changes in body weight, skin and fur, eyes and mucus membranes, respiratory system, circulatory system, ANS and CNS, somatomotor activity and behaviour pattern were observed systematically and recorded for each animal. At the end of the experiment, blood samples were collected by puncturing retro orbital plexus after mild anesthesia for biochemical analysis. The collected blood sample was centrifuged within 5min of collection at 4000g for 10min to obtain plasma, which was analyzed for total cholesterol, total triglyceride, HDL-cholesterol levels, LDL-cholesterol, plasma glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and urea.

Chronic toxicity test

The study on chronic toxicity of HAEBD was carried out based on the OECD signs of toxicity the male and female rats were randomly divided in to four groups of six each. The extract was prepared at the concentration of 200mg/kg, 400mg/kg and 600mg/kg in distilled water. The extract was given orally to treated groups of rats at doses 200, 400, 600mg/kg body weight daily for 90 days while the control group received water vehicle. Toxic manifestation such as Body weight, signs of toxicity and mortality were observed daily. At the end of the study, all rats were fasted overnight and anesthetized for blood collection. Heparinized blood samples were taken for determining complete blood count, red blood cell count, platelet count and red cell indices. The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis. All rats were sacrificed after the blood collection. The internal organs and some tissues were weighed to determine relative organs weights.

Statistics

The results were expressed as Mean ± SEM. The data was evaluated using one way Anova followed by Newman- keuls multiple range test & differences below $p<0.05$ are considered as significant.

RESULTS

Acute toxicity studies

In acute toxicity studies, no mortality or morbidity were observed in animals throughout the 14 days period following the single oral administration of the extract **(Table 1)**.The animals did not show any changes in general appearance during the observation period. Morphological characteristics such as fur, skin, eyes and nose appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy or unusual behaviors such as self-mutilation, walking backward and so forth were observed. Gait and posture, reactivity to handling or sensory stimuli, grip strength was also normal. Thus it was reported no overt signs of acute toxicity or death were observed in mice

treated with a Hydroalcoholic extract of *Boerhaavia diffusa* up to the dose of 2000 mg/kg.

Subchronic toxicity

The results obtained for the effect of HAEBD on body weight, biochemical, liver parameters and haematological parameters in sub chronic toxicity study are presented in **Table 2 to 4.** HAEBD at doses of 50, 100, and 200, 400mg/kg administered orally daily for 20 days did not result in any mortality in the tested animals. No signs of observable toxicity were detected during the entire experimental period. Significant increase ($p<0.05$) in body weight in all the animals were observed. The effects of HAEBD on organ weight & macroscopical changes of the rats didn't shows much difference in all the groups. The effect of HAEBD on various biochemical parameters of experimental rats was observed and there was significant decrease ($p < 0.05$) in plasma glucose level in treated rats especially at higher dose (400mg/kg) compared to control rats. A significant decrease ($p<0.05$) in plasma total cholesterol (TC), triglyceride (TG) and LDL cholesterol levels and significant increase ($p< 0.05$) in HDL cholesterol levels were observed in all the treated animals when compared to control animals (Table 4) and AST, ALT and ALP levels were normal in the HAEBD treated animals (Table 5). The effect of HAEBD on haematological parameters were studied and significant increase ($p<0.01$) in haemoglobin content and RBC count and significant decrease in ($p<0.01$) in the WBC count in group treated 200 and 400mg/kg b.w of HAEBD compared to normal control group was observed. There was no significant change in the calcium level in all the treated animals compared to the control.

Chronic toxicity

The results obtained for the effect of HAEBD on body weight, haematological parameters & biochemical values in chronic toxicity study are presented in Table 7-9

In chronic toxicity study for 90 days, the rats treated with HAEBD at all three doses had no signs of behavior changes and toxic signs. The treated groups revealed that there was slight increase in body weight. However, the result from animal health monitoring in the entire period of 90 days showed no sign of morbidity and diseases. In regard to hematological values, most of values in treated groups were normal in comparison with the control group. Significantly, slight difference in values were observed for RBC, MCV, MCHC and platelet in treatment group from that of control group but such values are within the normal ranges. Therefore, these results suggest that the extract did not cause hematological or immunological defects in rats. Furthermore, blood chemical examination was performed in order to evaluate any toxic effects on liver and kidney. In this study, the levels of blood chemical value showed minor changes which remained within the normal range. This study clearly explain that HAEBD given orally to wistar rats did not produce chronic toxicities.

Table 1 Acute toxicity studies of hydro-alcoholic extract of *B. diffusa* Linn on mice

Group(n=3)	**Dose (mg/kg)**	**Sign of Toxicity (ST/NB)**	**Mortality (D/S)**
Group I	0	0/3	0/3
Group II	300	0/3	0/3
Group III	2000	0/3	0/3

Note: ST- sign of toxicity; NB- normal behaviour; D- died; S- survived

Sub chronic Toxicies ofhydroalcokolic extract of B.diffusa Linn on rats

Table 2 Effects of HAEBD on body weight changes in rats

Treatment n=6) mg/kg b.w.	**Day 1**	**Day 5**	**Day 10**	**Day 20**
Control	185.10±6.5	188.45 ±6.20	191.08 ±6.40	196.3±6.58
HAEBD50	190.22 ±6.8	192.32 ±6.34	195.25 ±6.72	198.28±6.75*
HAEBD100	188.40 ±6.2	190.26 ±6.46	193.50±7.05	195.30±6.38*
HAEBD200	192.34 ±7.2	195.10±6.55	198.20 ±7.22**	200.42±7.20**
HAEBD400	186.65 ±6.1	189.4 ±6.44	191.64±6.32**	202.65±7.35**

Note: Values are expressed as mean ± SEM. Statistical analysis was carried out using one way ANOVA method where ** p <0.01;*p <0.05.

Table 3 Effect of HAEBD on kidney, heart, liver and brain of rats

Treatment (n=6) mg/kg b.w.	**Heart (g)**	**Kidney (g)**	**Liver(g)**	**Brain (g)**
Control	0.32 ± 0.02	0.63 ± 0.01	3.23 ± 0.02	0.64 ± 0.03
HAEBD 50	0.33 ± 0.01	0.78 ± 0.02	3.38 ± 0.01	0.65 ± 0.1
HAEBD100	0.34 ± 0.02	0.76 ± 0.03	3.34 ±0.01	0.66 ± 0.2
HAEBD200	0.33 ± 0.01	0.70 ± 0.03	3.28 ± 0.01	0.72 ± 0.06
HAEBD400	0.32 ± 0.01	0.72 ± 0.01	3.32 ± 0.02	0.74 ± 0.05

Note: Values are expressed as mean ± SEM. Statistical analysis was carried out using one way ANOVA method where ** p <0.01;*p <0.05.

Table 4 Effect of HAEBD on biochemical parameters

Treatment mg/kg b.w. (n=6)	**Glucose (mg/dl)**	**Cholesterol (mg/dl)**	**Triglyceride (mg/dl)**	**HDL (mg/dl)**	**LDL (mg/dl)**
Control	90.62 ± 0.65	38.60 ± 0.55	25.25 ± 0.50	136.20 ± 0.55	80.10 ± 1.80
HAEBD 50	88.50 ± 0.56	22.80 ± 0.20*	12.20 ± 0.22*	175.30 ± 0.70*	67.65 ± 1.10
HAEBD 100	85.40 ± 0.48	23.75 ± 0.24*	12.40 ± 0.28*	165.20 ± 0.82*	65.80 ± 1.22
HAEBD 200	86.20 ± 0.52**	30.20 ± 0.28	16.85 ± 0.35*	184.25 ± 0.88*	44.60 ± 1.05
HAEBD 400	84.22 ± 0.42**	29.75 ± 0.26	16.30 ± 0.32*	182.3 ± 0.85*	42.50 ± 0.95

Note: Values are expressed as mean ± SEM. Statistical analysis was carried out using one way ANOVA method where ** p <0.01;*p <0.05.

Table 5 Effect of HAEBD on AST, ALT, ALP, TP and albumin in rats

Treatment mg/kg b.w. (n=6)	**AST (IU/l)**	**ALT (IU/l)**	**ALP (IU/l)**	**TP (g/l)**	**Albumin (g/l)**
Control	323.4 ± 10.47	68.4 ± 3.19	255.60 ± 8.78	68.86 ± 3.30	35.11 ± 2.33
HAEBD 50	315.4 ± 9.81**	66.6 ± 2.16**	263.00 ± 2.74**	69.33 ± 2.30	36.25 ± 2.60
HAEBD100	318.2 ± 7.47**	64.0 ± 3.02**	257.60 ± 6.98**	79.12 ± 2.80	34.25 ± 3.00
HAEBD200	309.0 ± 7.70	59.5 ± 2.85	262.00 ± 5.50	69.20 ± 3.30	36.22 ± 2.80
HAEBD 400	319.0 ± 8.99	61.4 ± 3.54	269.44 ± 4.40	70.00 ± 2.60	35.45 ± 2.70

Note: Values are expressed as mean ± SEM. Statistical analysis was carried out using one way ANOVA method where ** p <0.01;*p <0.05.

Table 6 Effect of HAEBD on Haematological parameters

Treatment mg/kg b.w. (n=6)	**Haemoglobin (mg/dl)**	**RBC (10^6 /mm^3)**	**WBC (10^6/mm^3)**	**Calcium (mg/dl)**
Control	11.2± 0.20	9.06± 0.02	11.3± 0.03	9.05 ±0.02
HAEBD50	12.6 ± 0.20*	9.57 ± 0.04*	8.4 ± 0.03*	9.05 ± 0.02
HAEBD100	12.4 ± 0.12*	9.32 ± 0.02*	7.2 ± 0.30*	9.00 ± 0.30
HAEBD200	10.8 ± 0.20*	8.16 ± 0.12*	10.2 ± 0.02*	9.68 ± 0.03
HAEBD400	11.6 ± 0.30*	8.55 ± 0.45*	9.6 ± 0.12*	9.42 ± 0.22

Note: Values are expressed as mean ± S.E.M. Statistical analysis carried out using one way ANOVA method where *p < 0.05.

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Table 7 Effect of HAEBD on the body weight

Treatment (n=6) (Dose in mg.kg^{-1})	**Day 0**	**Day 90**
Control	215.65 ± 6.45	267.70 ± 7.90
HAEBD @ 200	219.35 ± 6.40	272.20 ± 8.20
HAEBD @ 400	222.80 ± 6.56	280.20 ± 8.30
HAEBD @ 600	218.22 ± 6.80	275.35 ± 7.52

Note: Values are expresses as mean ± S.E.M. Not significant from normal control

Table 8 Effect of HAEBD on Hematological Parameters

S. No	Parameters	Control	Drug Concentration (mg/kg)		
			200	400	600
1	White blood cells($X10^3/\mu l$)	8.20 ± 0.30	8.15 ± 0.20	8.18 ± 0.13	8.26 ± 0.16
2	Hemoglobin(g/dl)	10.10 ± 0.14	9.80 ± 0.26	9.40 ± 0.30	9.60 ± 0.20
3	Mean corpuscular volume	60.35 ± 0.40	59.20 ± 0.55	59.72 ± 0.36	58.58 ± 0.20
4	Mean corpuscular hemoglobin conc.(g/dl)	34.60 ± 0.44	33.10 ± 0.30	33.20 ± 0.12	32.82 ± 0.86
5	Platelet($X10^5/\mu l$)	5.70 ± 0.23	5.15 ± 0.15	5.46 ± 0.20	5.70 ± 0.14
6	Red blood cell($X10^6/\mu l$)	3.70 ± 0.26	4.10 ± 0.25	4.65 ± 0.10	4.82± 0.42

Note: Values are expressed as mean ± S.E.M. No significant difference from normal control

Table 9 Effect of HAEBD on Blood Chemical Values

S. No	Parameters	Control	Drug Concentration (in mg/kg)		
			200	400	600
1	Glucose(mg/dl)	145.56 ± 8.52	143.30 ± 2.85	148.40 ± 0.40	153.80 ± 3.90
2	BUN (mg/dl)	32.40 ± 1.40	18.16 ± 0.80	19.50 ± 0.36	20.45 ± 0.20
3	Creatinine (mg/dl)	0.42 ± 0.06	0.46 ± 0.05	0.48 ± 0.07	0.39 ± 0.02
4	Total protein (g/dl)	5.50 ± 0.20	5.45 ± 0.28	5.64 ± 0.32	5.86 ± 0.10
5	Albumin (g/dl)	3.72 ± 0.16	3.70 ± 0.43	3.68 ± 0.24	3.85 ± 0.18
6	Total bilirubin (mg/dl)	0.18 ± 0.10	0.16 ± 0.14	0.15 ± 0.15	0.14 ± 0.10
7	AST(u/i)	142.30 ± 10.80	130.34 ±16.42	136.60 ±18.40	142.40 ± 17.30
8	ALT(u/i)	83.40 ± 4.26	76.5 ± 3.50	74.40 ± 3.65	77.80 ± 3.66
9	ALP(u/i)	71.40 ± 2.66	61.40 ± 2.40	61.70 ± 2.43	63.43 ± 2.46

Note: Values are expresses as mean ± S.E.M. Not significantly different from normal control

DISCUSSION

The evaluation of sub-chronic and chronic dosing in experimental animals may be more relevant in determining the overall toxicity of the plant preparation. The highest overall concordance of toxicity in animals in comparison with humans is with hematological, gastrointestinal, and cardiovascular adverse effects whiles certain adverse effects in humans, especially hypersensitivity and idiosyncratic reactions, are poorly correlated with toxicity observed in animals.[8]

In the present study, where the acute toxicity study of HAEBD was carried out as per OECD-423 guidelines, No mortality was observed in both the animals of control group as well as animals treated with a maximum dose of 2000 $mg.kg^{-1}$. Hence, $1/10^{th}$ of 2000 $mg.kg^{-1}$ i.e. 200 $mg.kg^{-1}$ of dose was selected as a minimum dose for sub-acutetoxicity study.[9] The results of sub-acute toxicity study shows that there was no significant change in animal behaviour due to the absence of toxicity. The animals treated with HAEBD showed normal growth pattern and body weight compared with control rats treated with normal saline. So the changes in body weight can be used as an indicator of adverse effects of drugs and chemicals.[10-12] The changes in enzymes like ALP, AST and ALT levels show liver impairment, due to toxicity.[13] Serum cholesterol and proteins mainly regulated via synthesis in the liver and increase or decrease in serum concentrations of constituents suggest liver toxicity. The results of the present study were assessed after 20 days of administration of HAEBD, and it was found that HAEBD at all concentrations do not produce liver damage.

Analysis of blood parameters is likely to risk evaluation as the change in hematological system has a higher predictive value for human toxicity, when data are translated from animal studies.[8] After 20 days of treatment, there were no significant changes in the haematological parameters between control and treated groups. No significant changes in the levels of WBC, RBC were observed between control and test groups following repeated administration of HAEBD. Interestingly, significant increase in the levels of hemoglobin was found in treatment with HAEBD with a higher dose of 400 $mg.kg^{-1}$. The possible reason could be that one of the constituents HAEBD may increase absorption of iron.

The overall results suggest that HAEBD are non toxic to the haematopoietic and leucopoietic system. The haematopoietic and leucopoietic systems are the most sensitive targets for toxic compounds and an important index of physiological and pathological status in man and animal.[14] Therefore, it is possible to assume that the extract is non haematotoxic.

In chronic toxicity study for 90 days, the doses of 200, 400 and 600 mg/kg/day crude extract were chosen for the experiment, which are equivalent to 2.83-33.72 times of the normal human dose (crude plant material, 3–9 g/day for adults.[15] In the aspect of general behaviors, the rats treated with HAEBD at all three doses had no signs of behavior changes and toxic signs. The increase of body weight may have resulted from physiological changes in rats such as metabolism, food and water intake. However, the result from animal health monitoring in the entire period of 90 days showed no sign of morbidity and diseases. The albino wistar rats were healthy as shown by the normal appearance of general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes, and normal change in skin and fur.

RBC, MCV, MCHC, and platelet values are within the normal ranges. These variations may have resulted from normal variation among animal groups.[16, 17] Therefore, these results suggest that the extract did not cause hematological or immunological defects in rats.

In this study, the levels of these blood chemical values were remained within the normal range.[18-20]The above observations clearly establish the non-toxicity of HAEBD at a concentration of 1000$mg.kg^{-1}$.

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