Original Article

In-vitro and *In-vivo* toxicity assessment of ethylacetate extract of *Boerhavia diffusa* Leaves

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Abstract

Objectives: The present study aimed to evaluate the *In-vitro* and *In-vivo* toxicity of the traditional medicinal plants in the various models.

Methods: Extraction was carried out in the coarsely grounded leaf material with hexane and ethylacetate. Various concentrations of the extracts was checked for *in-vitro* toxicity in *Allium cepa,* and human RBC cells. The *in-vivo* toxicity was assessed using Zebra fish models.

Results: The extract was not toxic after 48 hours of treatment in *in-vitro* models. The toxicity was observed in *in-vivo* zebra fish models. Hence, the dose of the *Boerhavia diffusa* leaves extract should be standardized when we use the extract for further studies.

Conclusion: Though the extract was not toxic to the *in-vitro* models but toxicity was observed in *in-vivo* models. Further studies have to be done to check the toxicity profile of the *B.diffusa* leaf extract.

Keywords: *B.diffusa* leaf extract, *in-vitro* models, *in-vivo* model.

Introduction:

The World Health Organization has estimated that more than 80 % of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs. Many medicinal plants have been discovered and used in traditional medicine practices since prehistoric times. Numerous phytochemicals with potential or established biological activity have been identified[1]. Since ancient times, medicinal plants played an important role in treatment of many diseases in different ways. Modern technology helps in the development of useful drugs from medicinal plants. The potential of higher plants as a source for new drugs is still largely unexplored. In the recent past, there has been a tremendous increase in the use of plant-based products in developing as well as developed countries resulting in an exponential growth of herbal products globally. A variety of phytochemicals are accumulated in plants accounting for their constitutive antimicrobial activities. Plants have great potential uses, especially as traditional medicine and pharmacopeia drugs. A large proportion of the world's population depends on traditional medicine because of the scarcity, high costs of orthodox medicine and unpleasant side effects[2]. Though,

medicinal plants are considered to be safe, they are not completely free of side effects or toxicity. Thus assessing the toxicity levels before the usage helps in better utilization of the medicinal plants[3]. The toxicity levels of medicinal plants vary according to their chemical composition. The toxic effect of the medicinal plant arises from the acute and chronic exposure to the plant extract or any other means. It can also be due to the intake of increased concentration the particular plant[4]. Hence complete study of the medicinal plant's beneficial and toxic effects must be done before handedly and the toxic concentration must be determined. One such highly used medicinal plant in Indian traditional medicine is *Boerhavia diffusa*. The whole plant fresh or dried is the source of the drug punarnava which is official in Indian Pharmacopoeia as a diuretic[5]. *Boerhavia diffusa* is an important herbal constituent of various ayurvedic formulations. It has been used in various formulations meant for inflammation, jaundice, asthma, rheumatism, nephrological disorders, ascites, anemia, and gynecological disorders. *Boerhavia diffusa* is a good source of nutritional supplements with 15 amino acids (6 essential) in the whole plant and 14 amino acids (7 essential) in the roots along with iso palmitate acetate, behenic acid, arachidic acid (6.3%), and saturated fatty acids (38%). The root also contains some of the vitamins C, B3, and B2 (44.80, 97.00 mg, and 22.00 mg) along with calcium (174.09 mg)[6]. The current study involves using *In-vitro* and *In-vivo* techniques to assess the toxicity levels of *Boerhavia diffusa* leaves.

Materials and Methods:

1. Boerhavia diffusa leaves extract preparation:

The Extraction is prepared using coarsely powdered leaves of *Boerhavia diffusa* and solvent. 25 gm of powdered leaf is taken and added to the 250 ml of Hexane in a conical flask and covered with a foil sheet. The contents were at room temperature for 72hrs and every 24hrs the conical flask was shaken for even mixing of solvent and leaf powder. After 72 hrs, hexane was filtered and 250 ml of Ethyl acetate was added and kept for resting again at room temperature for 72 hrs. After the resting period, the contents were kept in a water bath for solvent evaporation at 80°C to 100°C. The beaker was left on the water bath until complete evaporation of the solvent. 25 μg, 0.5 μg, 0.75 μg, 1mg / ml concentrations of *B.diffusa* leaf were chosen to perform the main toxicity tests and plant extracts of different concentrations.

2. Allium cepa root tip assay:

Commercial small onion (*Allium cepa* L.) bulbs of good health were chosen. Young and same sized bulbs (2.5-3 g weight) were used for determination of different toxicity. The outer dead scales of the bulb were removed without damaging the root primordia to promote the growth of new roots. We use a group of 6 bulbs of *Allium cepa* for treatment. The scarped *Allium cepa* were germinated in the borosil bottles containing tap water for 24 hrs in the dark. Roots of *Allium cepa* have been treated with a series of concentration 0.25 μg, 0.5 μg, 0.75 μg and 1mg/ml and left to grow for 96 hrs. The extract was changed for every 24 hrs in order to avoid contamination. After 96 hrs, the roots of *Allium cepa* were observed. The number and length roots that were developed were observed and noted[7].

3. The Allium cepa root tip fixation:

For *Allium cepa* root fixation on slides, the *Allium cepa* root exposed to different concentrations of *B.diffusa* were viewed and then one by one the root tips were cut and rested in the watch glass containing Distilled

water. Using forceps, the tips were shifted to the microscopic slides and dried for a second and then 1-2 drops of Acetocarmine stain was added to each slide and kept for a few seconds. After which the slides were exposed to heat using Ethanol lamp for the stain to get dry and then the slides were covered with cover slip and squashed immediately. Finally, the total number of cells in each concentration and Different cell cycle phases were observed and counted using the Compound microscope[7].

4. Hemolytic assay:

Hemolytic activity of any compounds is an indicator of general toxicity test which differentiates the normal and healthy cells. 3ml of blood sample was collected from the healthy individual and RBC suspension was prepared from the whole blood by centrifugation at 2000 rpm for 15 minutes. After centrifugation, a clear supernatant of RBC was obtained. 200 µl of different concentrations of *B.diffusa* leaf extract were taken separately in different eppendorf tubes and 9ml of saline was mixed. 500 µl of RBC and 500 µl of 0.25 µg, 0.5µg, 0.75 µg, 1mg/ml of *B.diffusa* leaf extract was added. For positive control, 500 µl of RBC and 500 µl of DMSO were added. For negative control, 500 µl of RBC and 500 µl of Saline were taken. The tubes were incubated for 2 hrs with gentle intermittent shaking. After incubation, the tubes were centrifuged at High-speed centrifuge at 1000 rpm for 10 minutes. (shown in figure 9). The pellets were clearly observed and OD values were recorded using Photo calorimeter[8].

5. Zebrafish embryo toxicity assay:

Maintenance of zebrafish: Zebrafish were obtained from the existing breeding stock at the local aquarium in Chennai, Tamil Nadu, and kept in a proper aeration system to maintain the oxygen and water quality required for a healthy environment. The temperature of the tank system was generally maintained at 28 ± 0.5°C using the submersible heater (55W) and the lighting conditions were 14:10 hours. Water was dechlorinated either by reverse osmosis purification system or it can be dechlorinated by aging the water for at least 48 hours. Ideally, the pH of the water should be 7.0 to 8.0 in the tank when necessary, adjusting pH with sodium bicarbonate. It is recommended to remove and add 10% of the dechlorinated water daily. Next step is to disinfect and clean the tank by using laboratory disinfectant (10% sodium hypochlorite) in order to avoid algal growth and fish waste accumulation. Once the tanks have been cleaned, fill the tanks with fresh water.

Breeding condition: Male and female zebrafish recognition is the initial step for successful breeding. Usually, females can be easily distinguished from males because of their bigger underbelly. Zebrafish initiate breeding at the onset of white light. Fertilized eggs can be obtained either through in-tank breeding or pair wise breeding. For this study pairwise breeding technique was utilized. Pairwise breeding is usually initiated after feeding. After sex determination, male and female were kept in separate tank under optimum conditions for at least 8 h or until night before breeding. Fishes were assembled in breeding tanks set-up in our lab (1) simple meshed tank (2) pebbles and aquatic plants for analyzing better breeding efficacy. Three male and one female zebrafish was transferred to two breeding tanks and left undisturbed overnight at dark. Next morning shortly after the onset of white light, allow the spawning to occur undisturbed for 30 min to 1 hour or notice the visibility of sufficient embryos is laid down at the bottom of the tank. Return the fishes to their tanks and

collected the embryo by pipette suction, wash the embryos thrice with tap water. Finally, the total number of eggs produced from both breeding tanks were compared and illustrated in visual section. Eggs free of macroscopically discernible symptoms of infection and disease were picked and maintained in embryo medium at 28 ± 0.5°C until experiment.

Preparation of Embryo Medium: The stock solution for E. medium was prepared by adding 3.65 gm of NaCl, 0.1625 gm of KCl, 0.55 gm of $CaCl_2$, 1.0125 g of $MgCl_2$ and 100 µl of 1% of methyl blue. Then, 20 ml stock solution was measured and added to the 230 ml distilled water to make Working solution (E. medium).

Fish embryo toxicity assay: 0.25 µg, 0.5 µg, 0.75 µg and 1mg/ml concentrations of *Boerhavia diffusa* leaves extract were taken in separate Eppendorf tubes. 200 µl extract was pipetted out and was added to 9.0 ml of E medium making 9.2 ml of samples. 9.2 ml sample was divided into three equal parts and poured into three different petri dishes. 7 eggs were added to each petri dish. For every concentration the same procedure was followed. Triplicates are put for each concentration. For the positive control 7 eggs were exposed to E. Medium and for negative control 7 eggs were exposed to a mixture of E. Medium and 200 µl of DMSO. For every 24 hrs, the medium was changed to avert the contamination up to 72 hrs. From 24 hrs to 96 hrs, the development stages of embryos were observed using the compound microscope[9].

Results:

***1. Allium cepa* root tip assay:**

Allium cepa roots grown in different concentrations of *Boerhavia diffusa* leaves extract were observed for morphological changes like number of roots and length of roots. After which the root tips were fixed on a microscopic slide and were using Acetocarmine. The number of cells and different cell cycle phases were observed using a compound microscope. Table 1 and figure 2, 3 describe the above mentioned results.

Table 1: *Allium cepa* roots tips exposed to different concentrations of *Boerhavia diffusa* leaves extract

Concentration	Number of roots in *Allium cepa*	Length of the roots	Total number of cells	Number of specific cell division	Zone measurement
Positive control-H2O	15 roots	7.4cm	325	Interphase-2 Prophase-19 Metaphase-0 Anaphase-1 Telophase-0	Wide width-19 Length-117
Negative control-1 ml of DMSO and H2O	13 roots	2.5cm	152	Interphase-22 Prophase-5 Metaphase-1 Anaphase-0 Telophase-0	Wide width-28 Length-118
1mg /ml	8 roots	1.7cm	307	Interphase-35 Prophase-16 Metaphase-1 Anaphase-1 Telophase-1	Wide width-28 Length-117
0.75mg /ml	10 roots	0.9cm	256	Interphase-36 Prophase-20 Metaphase-1 Anaphase-3 Telophase-0	Wide width-26 Length-118
0.5mg /ml	11 roots	1.1cm	260	Interphase-75 Prophase-5 Metaphase-1 Anaphase-1 Telophase-1	Wide width-16 Length-117
0.25mg or 250µg/ml	13 roots	1.7cm	305	Interphase-32 Prophase-10 Metaphase-1 Anaphase-0 Telophase-1	Wide width-17 Length-118

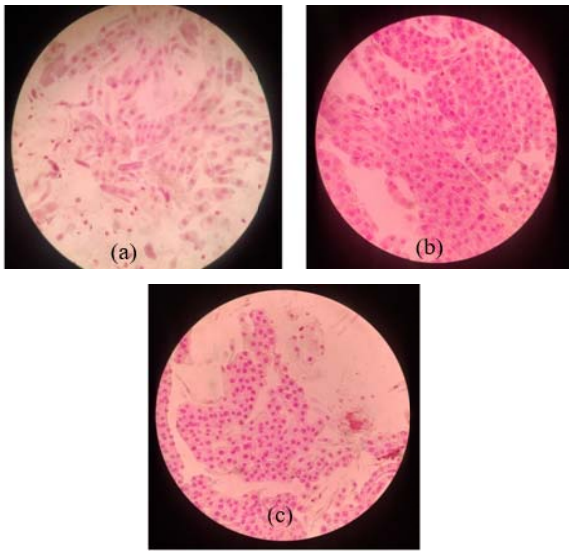


Figure 2: Microscopic view of control *Allium cepa* roots tips (a) positive control - H_2O (b) negative control – 1ml of DMSO and H_2O (c) higher concentration control – Higher concentration of 2ml DMSO and H_2O

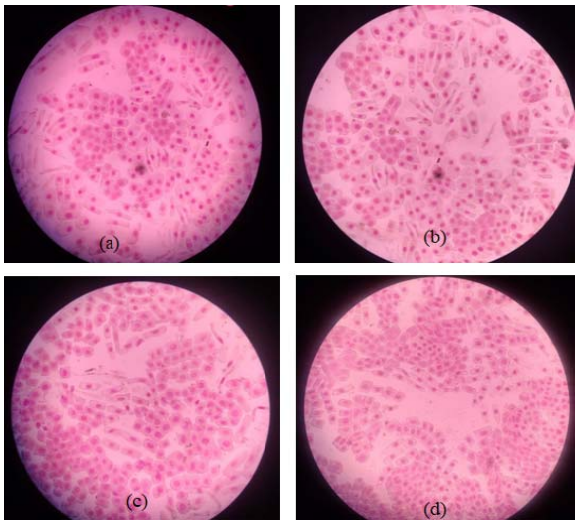


Figure 3 : Microscopic view of *Allium cepa* roots tips exposed to different concentrations of *Boerhavia diffusa* leaves extract.

Macroscopic analysis showed that at higher concentration root growth inhibited. This phenomenon was observed in all concentration after 96 hrs exposure compared to 24 hrs exposure indicating prolonged exposure of *B.diffusa* leaf extract found to be toxic. In microscopic analysis the total number of the cells and cell cycle phases varied depending upon the concentrations.

2. Hemolytic assay with red blood cells of human:

Human RBC cells isolated from whole blood were treated with different concentrations of *B. diffusa* leaf extract and the absorbance produced was measured using Photo calorimeter. Table 3 shows various concentrations used and their corresponding absorbance values.

Concentration	% of hemolysis
1 mg or 1000 µg/ml	10%
0.75 mg or 750 µg/ml	9%
0.5 mg or 500 µg/ml	7.4%
0.25 mg or 250 µg/ml	0.9%
DMSO and plant extract	0.12%
RBC and Distilled water (Positive control)	100%

Table 3: OD values of RBC cells treated with different concentrations of *B. diffusa* leaf extract

3. Fish embryo toxicity assay:

The embryos of Zebrafish were left in series of concentration of B.diffusa leaf extract for 96 hrs in order to analyze the Toxicity levels of the leaves of *Boerhavia diffusa.* Figure 4 a and b shows the immediate Fertile eggs after breeding and shows the unfertile egg.

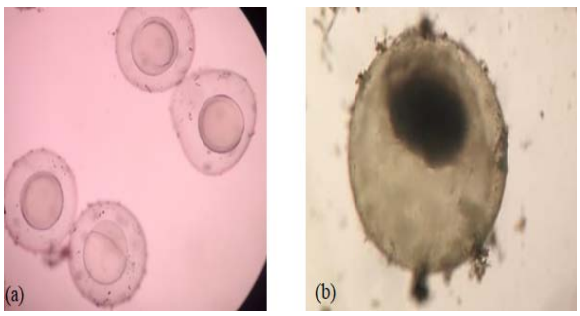


Figure 4: Microscopic view (a) fertilized and (b) unfertilized eggs of zebrafish

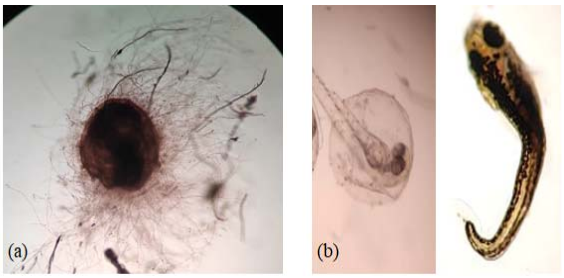
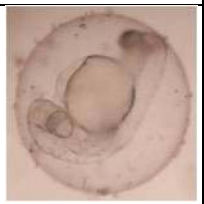
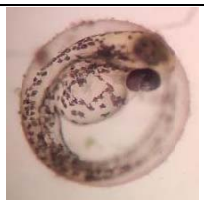
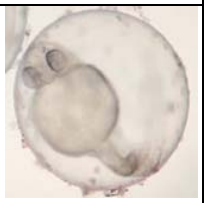
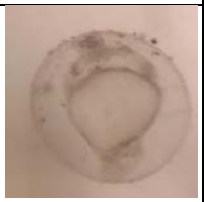
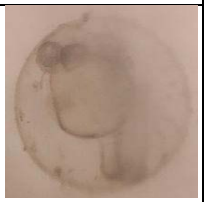
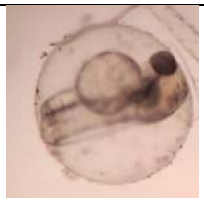
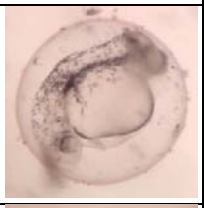
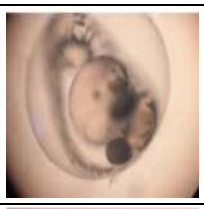
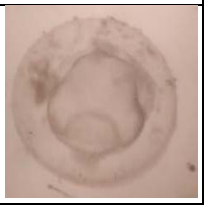
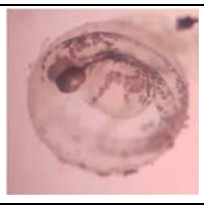


Figure 5 : (a) Fungal contamination around egg
(b) Abnormal embryos

In 24 hrs, the body structure was observed which had a normal healthy conditioned embryo with eyes, body and tail developed. In next 48hrs, heartbeat was observed and pigmentation of eyes in very living embryos were observed and then the heart beat along with the blood circulation throughout the body was also observed in 72 hrs. Finally, the complete structure of

Table 4: Zebrafish embryos exposed to different concentrations of *B.diffusa* leaf extract

Conc. Of samples	In 24hrs	In 48hrs	In 72hrs	In 96hrs (Dorsal and lateral view)	Total live (21)
Positive control-H2O					15
Negative control-1mlof DMSO and H2O					12
1mg or 1001mg or 1000µg/ml 0µg/ml		--	--	--	0
0.75mg or 750 µg/ml			--	--	0
0.5mg or 500µg/ml			--	--	3
0.25mg or 250µg/ml			--	--	6

zebrafish is observed from all living embryos after 96 hrs. Table 4 explains the morphological view of embryos of Zebrafish and the total live rate of embryos induced by B. diffusa leaf extract.

From this *in-vivo* study it is concluded that the leaves of *Boerhavia diffusa* in the series concentration of 0.25,0.5,0.75 μg/ml and 1mg/ml are slightly toxic compared to *In-vitro* conditions. It is seen that prolonged exposure for more than 72 hrs has inhibited embryo growth in all concentrations.1mg and 0.75 mg concentrations were found to be toxic for zebrafish embryos. Hence, the dose of the *Boerhavia diffusa* leaves extract should be adjusted accordingly when we use the extract for further studies.

Discussion:

The term medicinal plants include various types of plants used in herbal medicine. Since the prehistoric times, many medicinal plants have been discovered and used in traditional medicine practices for treating many diseases[10]. Numerous phytochemicals with potential or established biological activity have been identified. *Boerhavia Diffusa* is an important herbal constituent of various ayurvedic formulations. It has been used in various formulations meant for inflammation, jaundice, asthma, rheumatism, nephrological disorders, ascites, anemia, and gynecological disorders[6]. In this study, *In-vitro* and *In-vivo* Toxicity *B.diffusa* leaf extract was assessed.

Allium cepa root tips assay has been used for decades for the assessment of toxicants. *Allium cepa* assay is a sensitive assay to determine the anti-mitotic, cytotoxic and genotoxic effects of the chemical substance and herbal preparations[11]. In the *In-vitro* condition, the study focused on microscopic and macroscopic analysis of *Boerhavia diffusa* leaves extract with *Allium cepa* root tip assay where the study resulted in higher percentage root growth inhibition was observed in all sites after 96 hrs exposure compared to 24 hrs exposure and the total number of the cells may vary depending upon the concentrations. Mitotic cell division was clearly observed in all the series of concentrations of 0.25, 0.5, 0.75 μg/ml and 1mg/ml. Hence the leaf extract was found to be non-toxic in this *in-vitro* condition.

Hemolysis is caused by the breakdown of the RBC, causing release of hemoglobin and resulting in the discoloration of the plasma. The degree of hemolysis is described as the percent of free hemoglobin in relation to the total hemoglobin with appropriate correction for the haematocrit[12]. Hemolytic assay was also conducted by exposing the isolated human red blood cells with different concentrations of *Boerhavia diffusa* leaf extract. This also suggested that *B.diffusa* leaf extract was found to be non-toxic *in-vitro*.

Zebra fish and other aquarium fish species have distinct advantages as models for biomedical research including much lower husbandry costs than mammals. Oviparous species including the zebra fish have external fertilization and development, facilitating access for observation and manipulation of developing embryos [13]. In this *in-vivo* study, Embryos of Zebrafish were used to test the *Boerhavia diffusa* leaves extract to view the toxicity experimentally. It is concluded that the leaves of Boerhavia diffusa in the series concentration of 0.25,0.5,0.75 μg/ml and 1mg/ml are slightly toxic compared to *In-vitro* condition. Exposure of 1mg concentration for more than 24 hrs showed some toxic effect on the embryo which concludes prolonged exposure can cause toxic effects. Previous

study has found that aqueous extract of *B.diffusa* leaf extract was non-toxic in albino rats but our study has showed that *B.diffusa* leaf extract is toxic to zebra fish embryo. This can be explained by the difference in extract preparation. Previous study was done with aqueous extract while our study was performed using ethyl acetate extract. Ethyl acetate extract compound could be a reason behind the toxicity. Thus further studies on toxicity of *B.diffusa* is required[14]. Hence, the dose of the *Boerhavia diffusa* leaves extract should be standardized accordingly when we use the extract for further studies.

Conclusion:

The study focused on *In-vitro* and *In-vivo* toxicity assessment of medicinal plants. In vivo study conducted on macroscopic analysis showed higher percentage root growth inhibition was observed in all sites after 96 hrs exposure compared to 24 hrs exposure. The total number of the cells may vary depending upon the concentrations. All *Boerhavia diffusa* leaves extract concentrations showed the mitotic cell division clearly. Hence, its non-toxic in macroscopic analysis. The Hemolytic assay resulted as non-toxic. In vivo study result concluded that life of embryos depends on the concentration. In the Macroscopic study, the variance is not so toxic in 1mg/ml. Even though compared to the positive and negative control, the macroscopic samples are not much toxic. In *in-vivo* condition; the microscopic analysis of *B.diffusa* leaves extracted alone induced embryos of the Zebrafish Model (*Danio rerio*) to analyze the Toxicity. It is concluded that the leaves of *B.diffusa* in the series concentration of 0.25, 0.5, 0.75 µg / ml and 1mg/ml are slightly toxic compared to *In-vitro* condition. Due to the more sensitivity of the embryos, 1mg concentration was observed in 24 hrs which indicated that prolonged exposure showed toxic effects on embryos of the zebrafish. Hence, the dose of the *B.diffusa* leaves extract should be adjusted accordingly when we use the extract for further studies. The toxicity effect varies from system to system.

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