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Toxicity Studies on Leaf Extracts of *Alternanthera brasiliana* (L.) Kuntze and *Alternanthera bettzickiana* (Regel) Voss

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ABSTRACT

Herbal medicine is the source for the search of many novel therapeutic compounds in developing countries. Before used as medicine, drugs from plant origin must be ensured as safe. **Objective:** The present work focused to study the *in vivo* and *in vitro* toxicity effects of hydroethanolic leaf extracts of *Alternanthera brasiliana* (*A. brasiliana*) and *Alternanthera bettzickiana* (*A. bettzickiana*). **Methods:** Sub-acute toxicity studies of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* were carried out *in vivo* on mice. 11 groups of albino mice were treated with five different doses of the hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* orally for 14 days. General appearance and behavior were observed for 14 consecutive days. Effect on hematological parameters and histopathological changes were also monitored. Cytotoxicity was assessed by observing its toxicity *in vitro* against DLA cell line by using MTT assay. **Results:** Sub-acute toxicity studies results showed that up to the tested dose of 2000 mg/kg bwt in both extract treatments, throughout the 14 days of treatment, the extract does not produced any toxicity symptoms. MTT assay indicated that both leaf extracts exhibited significant concentration-dependent *in vitro* cytotoxic activity against DLA cell line. Hydroethanolic leaf extract of *A. bettzickiana* exhibited more potent cytotoxic effects on DLA cells than *A. brasiliana* extract. **Conclusion:** No toxicity related symptoms were observed in different doses of leaf extract treated mice groups. So the LD_{50} values of the tested leaf extracts were more than 2000 mg/kg bwt.

INTRODUCTION

Cancer is considered to be one of the most dreaded diseases. Uncontrolled cell proliferation or metastasis of abnormal cells in the body results in cancer (Kaufman and Chabner, 1996). Cancer is found to be one of the major factor causing mortality. More than one-third of the world's population is affected by cancer. It accounts for more than 20% of all deaths. Causes of cancer include tobacco, viral infection, chemicals, radiation, environmental factors, and dietary factors (Lemkebthomas *et al*., 2008). At present, the common treatment strategies in cancer are the use of chemotherapeutic agents, surgery, and radiation. These treatments are not fully effective and cause many side effects. Hence, there is a great interest to develop safe and low cost anticancer agents from natural sources (Moongkarndi *et al*., 2004).

Since 1950, a vast number of plant-derived agents are used to treat cancer (Kinzler and Vogelstein, 2002). Plants contain a large profile of secondary metabolites that are mainly responsible for their cytotoxic activity. In the development of anti-cancer agents, the isolation of vincristine and vinblastine from vinca and podophyllotoxins from Podophyllumhexandrum are considered as milestones (Newman *et al*., 2003). Various traditional herbal medicines are used by a majority of the people in developing countries to treat a number of diseases and ailments (Liu, 2011). Herbal based medications are often considered to be safe as they are natural and free from side effects (Lopes *et al*., 2000). The increase in the popularity of herbal remedies and the limited number of scientific works on their safety and efficacy, toxicity and adverse effects related to herbal remedies are widely recognized (Saad *et al*., 2006). There are growing evidence that support the toxicity of herbal medicines towards their users. Though various studies of the pharmacological potential of medicinal plants have been carried out in the past,

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works investigating their potential toxicities are very limited (Wojcikowski *et al*., 2004).

No drug should be used clinically without its clinical trials and toxicity studies (Anisuzzaman *et al*., 2001). Sub-acute oral toxicity studies of herbal medicines are essential to identify the safety and the determination of dose level that could be used subsequently. It also helps in the investigation of the therapeutic index of drugs and xenobiotics (Rang *et al*., 2001).

A. brasiliana and *A. bettzickiana* were herbaceous plants belonging to the family Amaranthaceae. Amaranthaceae family consists of 64 genera and about 800 species, represented by herbs and shrubs. This cosmopolitan family is most abundant in tropical regions of Africa, India, and America (Hussien, 2005). The leaves of *A. brasiliana* are most widely used for therapeutic applications. The phytochemical constituents reported in this plant are flavonol glycosides (3-O-robinobioside derivatives of kaempferol and quercetin), vitamins (riboflavin and niacin), betacianin and steroids such as β-sitosterol (Kumar *et al*., 2011). The whole plant of *A. bettzickiana* was useful in nourishing and purifying the blood, as a soft laxative, as antipyretic, as a galactagogue and also had wound healing property. Studies in the liver of ovariectomized mice showed the potential of *A. bettzickiana* in improving superoxide dismutase and catalase activities (Suphanthip *et al*., 2013).

In our preliminary study, phytochemical constituents and *in vitro* antioxidant potentials of *A. brasiliana* and *A. bettzickiana* were carried out (Kasthuri and Ramesh, 2018). The current work, sub-acute toxicity study is formulated to find out the safe dose level and LD_{50} values of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* in swiss albino mice model. The leaf extracts were also screened *in vitro* for cytotoxic activity against DLA cell line using MTT assay.

MATERIALS AND METHODS

Plant materials and extraction

The healthy leaves of *A. brasiliana* and *A. bettzickiana* were collected from SKM Siddha and Ayurvedha, Erode. The plants were identified and authenticated at Botanical Survey of India, Coimbatore with voucher number BSI/SRC/5/23/2015/Tech/100 for *Alternanthera brasiliana* (L). Kuntze – AMARANTHACEAE and BSI/SRC/5/23/2015/Tech/101 for *Alternanthera bettzickiana* (Regel) Voss – AMARANTHACEAE. The leaves of *A. brasiliana* and *A. bettzickiana* were separately washed, shade dried and was coarsely powdered using a mechanical grinder. The shade dried coarsely powdered leaf samples of (500 g) *A. brasiliana* and *A. bettzickiana* were extracted with hydroethanol by using hot continuous percolation process (Soxhlet). The leaf extracts were concentrated by using a rotary vacuum evaporator (Buchi) at 50°C, dried in a vacuum dessicator and stored at –20°C till further use.

Sub-acute toxicity studies

The *in vivo* sub-acute toxicity studies of *Alternanthera* leaf extracts were conducted according to OECD guideline 423 which stipulate the use of only three animals (Jonsson *et al*., 2013). Male swiss albino mice weighing (25 ± 2) g were used to observe the sub-acute toxicity. Swiss albino mice deprived of food for 18 hrs, were administered with different doses of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* ranging from 100 to 2000 mg/kg bwt, orally once a day for 14 days. 11 groups of mice were used to study the acute toxicity and each group consisting of 3 mice as per the Institutional Animal Ethical Committee (IAEC) Proposal No: SVCOP/IAEC/013/2016-17 dt 10.03.2017.

EXPERIMENTAL GROUPS

Group I: Normal control mice

Group II: Mice fed orally with 100 mg/kg bwt of *A. brasiliana* leaf extract for 14 days.

Group III: Mice fed orally with 250 mg/kg bwt of *A. brasiliana* leaf extract for 14 days.

Group IV: Mice fed orally with 500 mg/kg bwt of *A. brasiliana* leaf extract for 14 days.

Group V: Mice fed orally with 1000 mg/kg bwt of *A. brasiliana* leaf extract for 14 days.

Group VI: Mice fed orally with 2000 mg/kg bwt of *A. brasiliana* leaf extract for 14 days.

Group VII: Mice fed orally with 100 mg/kg bwt of *A. bettzickiana* leaf extract for 14 days.

Group VIII: Mice fed orally with 250 mg/kg bwt of *A. bettzickiana* leaf extract for 14 days.

Group IX: Mice fed orally with 500 mg/kg bwt of *A. bettzickiana* leaf extract for 14 days.

Group X: Mice fed orally with 1000 mg/kg bwt of *A. bettzickiana* leaf extract for 14 days.

Group XI: Mice fed orally with 2000 mg/kg bwt of *A. bettzickiana* leaf extract for 14 days.

On the evaluation of acute toxicity, all groups of mice treated separately with hydroethanolic leaf extracts of *A. brasiliana and A. bettzickiana* were monitored during the entire study period for the signs and symptoms of toxicity and/or mortality, behavioral alterations, food and water intake and changes in body weight. Blood samples were collected after 24 hrs of the last dose of hydroethanolic leaf extracts for the analysis of hematological parameters. On day 14, the mice in each group were sacrificed by cervical dislocation, a small portion of the liver sample was fixed in 10% formalin for the analysis of histopathological architecture.

Estimation of hematological profile

The hematological parameters such as hemoglobin, PCV, WBC, RBC, and platelets were estimated. The whole blood sample was analyzed using SYSMEX Xs – 800i automatic hematology analyzer.

In vitro cytotoxicity - MTT assay

MTT assay was employed to study the i*n vitro* cytotoxicity of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana.*

MTT assay

MTT[3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] assay has been used widely for testing the *in vitro* chemosensitivity of tumor cell lines. The assay is based on the principle that only metabolically active cells can reduce the water-soluble MTT salt to its formazan product. The dead cells do not interfere with MTT absorbance levels (Mosmann, 1983).

In living cells, the mitochondrial enzyme succinate dehydrogenase reduces the yellow colored water-soluble substrate

MTT into an insoluble purple colored formazan product which can be measured spectrophotometrically. MTT assay depends on the mitochondrial activity per cell and number of cells present. MTT reduction can take place only in metabolically active cells and the level of activity is a measure of the viability of the cells (Wilson and John, 2000).

Cell line

The DLA cell line was purchased from amala cancer research institute, Thrissur, kerala and it was grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Weekly, the maintenance cultures were passaged and the culture medium was changed twice per week.

Cell treatment procedure

Single cell suspensions were made by detaching the monolayer cells with trypsin-ethylene diamine tetra acetic acid (EDTA). Using a hemocytometer, the number of viable cells were counted and are diluted with the medium containing 5% FBS to attain a final density of 1×10^5 cells/ml. Cell suspension was seeded into 96-well plates at a plating density of 10,000 cells/well and incubated for 24 hrs at 37°C, 5% CO_2, 95% air and 100% relative humidity to allow for cell attachment. After incubation, the medium on cells was removed and treated with different concentrations (12.5, 25, 50, 100 and 200 µg/ml) of *A. brasiliana* and *A. bettzickiana* leaf extracts. The cells were further incubated at 37°C in a 5% CO_2 incubator for 5 days. To each well, 30 µl of MTT (stock solution of 5 mg/mL in phosphate-buffered saline) was added and the plates were incubated for 4 hrs at 37°C. The medium was carefully removed by aspiration and the MTT crystals were dissolved into formazan by the addition of 50 µL of DMSO to each well. The absorbance, proportional to the amount of MTT reduced, was measured at 570 nm using a microplate reader (Versamax). By using a linear regression equation, the LD_{50} values were calculated with the concentration of *Alternanthera* leaf extracts that resulted in a 50% reduction in absorbance when compared to that of untreated cells. LD_{50} values are expressed as the mean of three independent triplicate assays and values were expressed as mean of quadruplicate assays.

Statistical analysis

Results of the current study were expressed as mean ± SD. The statistical significance ($p < 0.05$) between experimental groups were determined using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test using SPSS version 17.0.

RESULTS AND DISCUSSION

For the prevention and treatment of various ailments that affect humans, there was a continuous search for new leads from plants and traditional foods. Medicinal plants constitute a realistic and promising anticancer therapeutics (Yan-Wei *et al*., 2009). Agents from phytotherapeutic products are many times, mistakenly believed to be safe as they are natural (Gesler, 1992). But there are evidence that these products contain several bioactive principles that had the potential to cause adverse toxic effects (Bent and Ko, 2004). Paracelsus, known as the father of toxicology stated that "All substances are poisons; there is none which is not a poison. It is the right dose that differentiates a remedy from poison" (Hunter, 2008).

Sub-acute oral toxicity studies

Appropriate animal is used as models in toxicity studies. These animal models are commonly used to assess the potential health risks in humans (Schulz *et al*., 2001). Determination of sub-acute oral toxicity is the first step in the screening and evaluation of toxic potentials of pharmacological compounds (Akhila *et al*., 2007). The assessment of the toxic nature of plant extracts is useful to define the intrinsic toxicity of the plants and the effects of an acute overdose. It was also indispensable to consider a treatment as safe. Mice are sensitive to toxic components present in plants. The dosing of the plant extracts in increasing amounts helps to evaluate the toxicity limits (Parra *et al*., 2001).

Sub-acute toxicity studies using animal models provide important preliminary data that helps to select natural remedies with potential health benefits for future work (Rosenthal and Brown, 2007). Toxicity effects of natural remedies in animals and humans are analyzed using some physiological parameters like behavior, body weight, food intake, biochemical, hematological and histological analysis (Ahmad *et al*., 2013).

General appearance and behavioral observations

The clinical signs and symptoms exerted by drugs on vital body organs are considered as principal observations among toxicity indicators (Subramanion *et al*., 2011). On the 14 days treatment with the different doses of *Alternanthera* leaf extract, the mice in all groups were survived throughout the entire study period. No treatment-related toxic symptoms or mortality were observed after oral administration of various doses of *A. brasiliana* and *A. bettzickiana* leaf extracts. None of these mice had shown any abnormal behavioral responses in any dose range. There was no change in behavior, body weight, temperature, food intake and water consumption, skin effects, fur coating, eyes, mucus membranes and respiratory activities when compared to control group (Table 1).

No major differences were observed between control and different doses of *Alternanthera* leaf extract treated groups. However, sedation and drowsiness were observed in 1000 and 2000 mg/kg bwt treated groups of the two studied leaf extracts. Any pharmaceutical drug or compound with the oral LD_{50} higher than 1000 mg/kg bwt could be considered safe and low toxic. Oral LD_{50} values for sub-acute toxicity as per OECD are as follows: <5mg/kg bw - very toxic, >5<50mg/kg bw - toxic, > 50 < 500 mg/kg bwt - harmful and > 500 < 2000 mg/kg bwt - no label (Walum, 1998).

Following the administration of different doses of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana*, there was no noticeable change in food and water intake. This showed that the oral administration of leaf extracts did not induce any suppression in appetite and had no deleterious effect on food and water intake. This indicated that the metabolism of carbohydrate, protein, and fat are not affected (Klaassen, 2001).

Table 1: General appearance and behavioral observations for control and *A. brasiliana* and *A. bettzickiana* leaf extracts treated groups.

Observation	Body Wt	Temp	Food intake	Breathing	Change in skin	Drowsiness	Sedation	Coma	Alive/Death
Group I (Control)	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group II	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group III	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group IV	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group V	Normal	Normal	Normal	Normal	No effect	Present	Observed	Not present	Alive
Group VI	Normal	Normal	Normal	Normal	No effect	Present	Observed	Not present	Alive
Group VII	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group VIII	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group IX	Normal	Normal	Normal	Normal	No effect	Not present	Not present	Not present	Alive
Group X	Normal	Normal	Normal	Normal	No effect	Present	Observed	Not present	Alive
Group XI	Normal	Normal	Normal	Normal	No effect	Present	Observed	Not present	Alive

No mortalities were observed after the administration of leaf extracts up to the administered dose level of 2000 mg/kg bwt. This showed that the leaf extracts had a negligible level of toxicity when administered orally to mice. Similar results were found for oral dose administration of P. longifolia extracts in mice (Nair *et al*., 2009). As there were no observable changes in the general appearance and behavior of control and extract treated groups, the hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* were found to be safe at the dose level of up to 2000 mg/kg bwt. Therefore, the LD_{50} value for both leaf extracts were considered to be >2000 mg/kg bwt. This indicated that the oral administration of *Alternanthera* leaf extracts could be considered relatively safe.

Effect on hematological parameters

In animal toxicity studies, analysis of blood parameters were important to report any alterations and also to evaluate the relative risk effects on the hematopoietic system when interpreting those findings to human beings (Jothy *et al*., 2011). The mechanisms of toxicity of a therapeutic agent could be studied by analyzing the biochemical profile and examining its major toxic effects on tissues like liver (Yamthe *et al*., 2015). Estimation of blood profile parameters like haemoglobin, total red blood cell (RBC), white blood cell (WBC), packed cell volume (PCV) and platelet counts are an important indexes in evaluating the physiological and pathological status of man and animals (Schalm *et al*., 1975). Intake of some toxic plants results in an alteration in the normal level of these parameters (Ajagbonna *et al*., 1999). Studies on blood profile provides important information about abnormalities in metabolic processes and the response of the body towards injury or lesion, deprivation and stress (Bosco *et al*., 2014). Therefore, the haematological parameters can serve as an index to study the extent of the toxic effect of plant extracts (Raza *et al*., 2002). When the data are translated from animal studies, the changes in haematological status have higher predictive value for human toxicity (Olson *et al*., 2000). For the diagnosis of the root cause of any disease, the level of haematological index was the important factor. Changes in blood profiles may be due to changes in cellular integrity, cellular membrane permeability or due to exposure to toxic chemicals (Hoffbrand and Pettit, 1997).

Environmental pollutants or toxic stress results in physiological changes that affect the hematological parameters of animals (Jain *et al*., 2009). In the present study, all the tested hematological parameters (hemoglobin, packed cell volume, white blood cell, red blood cell and platelets count) were within normal limits when compared to the control group. No significant differences ($P > 0.05$) between *A. brasiliana* and *A. bettzickiana* extract treated mice groups and control were found (Table 2).

The level of hemoglobin was found to be 14.6g% in the control group. In *A. brasiliana and A. bettzickiana* extract treated groups, the level was found to vary between 14.0g% to 14.7g%. Control group showed a packed cell volume of 43.9%, whereas *A. brasiliana* and *A. bettzickiana* extract treated groups showed a maximum level of 44% and a minimum level of 41.7%. The RBC count was found to be 5.6 10^{12}/µl in control group, whereas the different doses of *A. brasiliana and A. bettzickiana* leaf extract treated groups showed RBC count that varies between 5.3 10^{12}/µl to 5.8 10^{12}/µl. The symptoms of anemia occur due to a decrease in the level of hemoglobin (Ojezele *et al*., 2013). Packed cell volume (PCV) was denoted as the proportion of blood volume that was occupied by red blood cells. Along with hemoglobin concentration, white blood cell count and platelet count, an integral part of a person's complete blood count was PCV (Purves *et al*., 2004). The detection of PCV is a simple and reliable method for detecting the presence or absence of anemia or polycythemia.

There was no significant difference in RBC profile following treatment with different doses of leaf extracts of *A. brasiliana and A. bettzickiana*. This result indicated that the administration of *Alternanthera* leaf extracts does not affect erythropoiesis, had no effect on morphology or does not induce osmotic fragility of red blood cells (Odeyemi *et al*., 2009). The level of packed cell volume was not altered significantly in the different group of mice studied. The above results showed the non-toxic nature of the *A. brasiliana and A. bettzickiana* extracts which does not induce any anemia and no significant difference in the level of hemoglobin, RBC and packed cell volume in different doses of leaf extract treated mice.

The level of WBC was found to be 7.5 10^3/µl in control group and the level was found to vary between 7 to 7.6 10^3/µl in different doses of *Alternanthera* leaf extract treated mice groups. The first line of cellular defense that responds to inflammation, infectious agents or tissue injury is WBC. In sub-acute toxicity studies, an increase in the level of WBC results from the effect of herbal extracts in inducing the immune response of treated animals (Tousson *et al*., 2011). On the other hand, a significant

decrease in WBC count indicated a decrease in the production of leukocytes called leukopenia, a condition that reflects the less ability of the body to fight off infections. However, in the present work, the hematological analysis revealed that the estimated total WBC count after administration of various doses of *Alternanthera* leaf extracts was not significantly changed when compared to the control. This result may confirm that the hydroethanol leaf extracts used in this study do not possess any chemicals capable of inducing leukocytosis or leukopenia (Weingand *et al*., 1996).

Table 2: Hematological profile of control and different doses of *A. brasiliana* and *A. bettzickiana* extract treated groups.

Groups	Hb (g %)	PCV (%)	RBC (1012/µl)	WBC (103/µl)	Platelets (109/µl)
Group I (Control)	14.6 ± 0.11	43.9 ± 0.34	5.6 ± 0.15	7.5 ± 0.10	6.2 ± 0.05
Group II (100 mg/kg bw)	14.6 ± 0.06^{ns}	43.7 ± 0.1^{ns}	5.7 ± 0.1^{ns}	7.5 ± 0.21^{ns}	6.2 ± 0.06^{ns}
Group III (250 mg/kg bw)	14.6 ± 0.17^{ns}	43.8 ± 0.31^{ns}	5.8 ± 0.35^{ns}	7.5 ± 0.3^{ns}	6.3 ± 0.15^{ns}
Group IV (500 mg/kg bw)	14.7 ± 0.25^{ns}	43.9 ± 0.06^{ns}	5.8 ± 0.15^{ns}	7.6 ± 0.25^{ns}	6.4 ± 0.25^{ns}
Group V (1000 mg/kg bw)	14.6 ± 0.32^{ns}	43.7 ± 0.40^{ns}	5.7 ± 0.15^{ns}	7.5 ± 0.20^{ns}	6.4 ± 0.06^{ns}
Group VI (2000 mg/kg bw)	14.0 ± 0.21^{ns}	41.7 ± 0.25^{ns}	5.3 ± 0.1^{ns}	7.0 ± 0.1^{ns}	6.2 ± 0.10^{ns}
Group VII (100 mg/kg bw)	14.6 ± 0.10^{ns}	43.8 ± 0.30^{ns}	5.8 ± 0.00^{ns}	7.4 ± 0.05^{ns}	6.3 ± 0.05^{ns}
GroupVIII (250 mg/kg bw)	14.6 ± 0.11^{ns}	43.9 ± 0.34^{ns}	5.8 ± 0.00^{ns}	7.4 ± 0.11^{ns}	6.4 ± 0.00^{ns}
Group IX (500 mg/kg bw)	14.6 ± 0.05^{ns}	44 ± 0.17^{ns}	5.8 ± 0.05^{ns}	7.56 ± 0.05^{ns}	6.5 ± 0.10^{ns}
Group X (1000 mg/kg bw)	14.5 ± 0.10^{ns}	43.5 ± 0.30^{ns}	5.7 ± 0.05^{ns}	7.3 ± 0.05^{ns}	6.4 ± 0.05^{ns}
Group XI (2000 mg/kg bw)	14.0 ± 0.21^{ns}	42.00 ± 0.60^{ns}	5.4 ± 0.05^{ns}	7.1 ± 0.05^{ns}	6.2 ± 0.05^{ns}

Values are expressed as mean ± SD of three mice in each group.
Statistical comparison: Group II, III, IV, V,VI, VII, VIII,IX, X and XI vs. Group I.
ns - not significant at 5 % ($p < 0.05$).

The platelet count was found to be 6.2 10^9/µL in the control group. This count alternate between 6.2 10^9/µL to 6.5 10^9/µL in the different doses of *Alternanthera* leaf extract treated groups. Thrombocytopenia is a condition that reflects abnormally low levels of platelets in circulation. Either a decrease in production or an increase in the destruction of platelets results in thrombocytopenia (Tousson *et al*., 2011). Platelet destruction may result from the administration of some drugs that provoke platelet antibodies, resulting in thrombocytopenia (Weingand *et al*., 1996). An abnormal increase in the number of circulating platelets is known as thrombocytosis (Aajibade *et al*., 2012). However, in this study, mice treated with different doses of *Alternanthera* leaf extracts showed nonsignificant alteration in platelets count when compared to control mice. This results showed that the hydroethanolic leaf extracts of *Alternanthera* does not affect platelet levels as it exhibited no significant effect in inducing neither thrombocytopenia nor thrombocytosis.

One of the most sensitive targets of toxic compounds in humans and animals are the hematopoietic system that serves as an important index for reflecting physiological and pathological condition. The various hematological parameters estimated in this study showed no significant difference ($P > 0.05$) in the *Alternanthera* extract treated groups when compared to control group. The inertness of the *Alternanthera* leaf extracts on this organ was evident from the above hematological analysis. Hence, the selected *Alternanthera* leaf extracts may not have any harmful effects on bone marrow function. This justifies the fact that at all doses of *A. brasiliana and A. bettzickiana* used in this study does not induce any significant alterations in hematological profile, making it safe for therapeutic applications.

Liver histopathology

Histopathological investigations were carried out to find out any damage and changes in the liver morphology in control mice and in mice groups following treatment with *Alternanthera* leaf extracts (Figure 1 and 2). Sub-acute toxicity studies on different doses of *A. brasiliana and A. bettzickiana* leaf extracts showed no discrete pathological changes in the liver tissue of swiss albino mice in doses below 1000 mg/kg b.wt. Group-I, normal control groups showed the normal architecture of the central vein, hepatocytes, sinusoids and vacuoles. Group-II, group-III, group-IV, group-VII, group-VIII and group-IX mice showed normal architecture compared with control group in which hepatocytes are distinct and relatively normal, no fatty changes, dilation of blood vessels and necrosis were observed. But treatment with *Alternanthera* leaf extracts at the concentration of 1000 and 2000 mg/kg of bwt, the treated mice (group-V, group-VI, group-X and group-XI) showed some damages like mild inflammation of hepatocytes, mild periportal inflammation, expanded vacuoles and mild alteration in the central vein structure.

Hydroethanolic extracts of both leaves to the dose range of below 1000 mg/kg bwt does not produced any histopathological changes in the liver architecture of treated mice. But the mice groups treated with leaf extracts of 1000 mg and 2000 mg/kg bwt showed very mild alteration of liver architecture. Being minor, the observed changes are not necessarily an indicator of hepatic damage as a result of the selected leaf extract treatment.

The results of sub-acute toxicity studies on the hydroethanolic leaf extracts of *A. brasiliana and A. bettzickiana* revealed that up to the administered dose level of 2000 mg/kg bwt, the extracts do not induce any changes in general appearance

and behavior. Hematological parameters were not significantly altered. The histopathological investigations also showed that the liver architecture was normal in the dose level of below 1000 mg/kg bwt, but both plant leaf extracts at the concentration of 1000 and 2000 mg/kg bwt showed only minor alteration in the liver architecture. So, the hydroethanolic leaf extracts of *A. brasiliana and A. bettzickiana* up to the dose of 2000 mg/kg bwt were found to be safe.

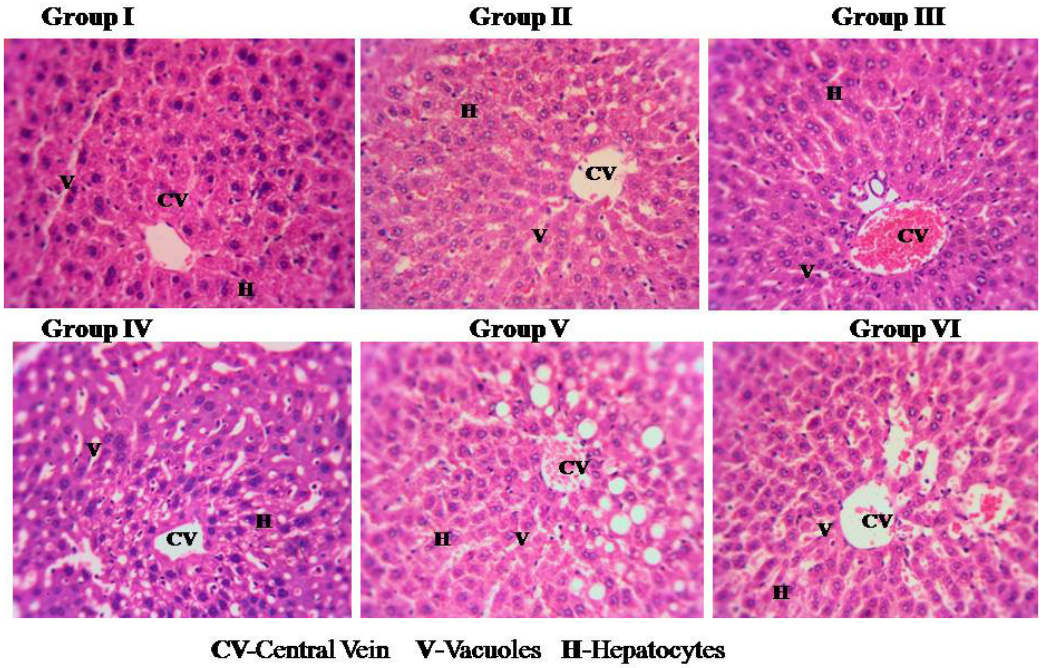

Fig. 1: Liver histopathology pattern of *A. brasiliana* leaf extract treated groups.

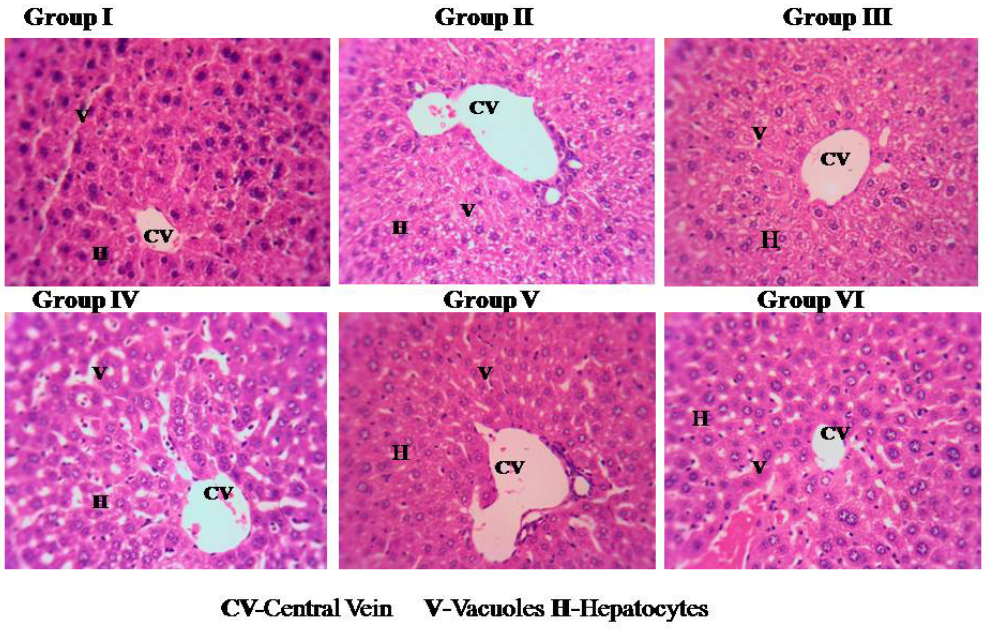

Fig. 2: Liver histopathology pattern of *A. bettzickiana* leaf extract treated groups.

***In vitro* cytotoxic activity**

Cell lines are used as models as they provide a large number of consistent cells for prolonged use. Cell lines are used to provide reliable experimental results as they maintain most cellular characters (Bretagnol *et al*., 2008). *In vitro* cytotoxicity screening studies provide important data that helps in the selection of plant extracts with potential antitumor properties for further studies (Cardellina *et al*., 1999).

In this study, the *in vitro* cytotoxic effects of hydroethanolic leaf extracts of *A. brasiliana and A. bettzickiana* versus DLA cell line was determined by MTT assay (Table 3). It was observed that there was an increase in cytotoxicity with an increase in the concentration of both leaf extracts. At 12.5 µg/ml concentration, *A. brasiliana* and *A. bettzickiana* produced 2.66% and 3.14% cell death, whereas at high concentration (200 µg/ml), 50.42% and 53.51% cell death were observed with an IC_{50} value of 192.20 µg/ml and 157.25 µg/ml respectively. This result revealed that *A. bettzickiana* extract was more potent than *A. brasiliana* extract in causing toxicity and death of DLA cell line.

Table 3: *In vitro* cytotoxic effects of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* versus DLA cell line.

Conc (µg/ml)	% Cell Inhibition	
	A. brasiliana	*A. bettzickiana*
12.5	2.66	3.14
25	14.15	15.11
50	20.56	22.61
100	35.91	38.27
200	50.42	53.51

IC_{50} Values: *A. brasiliana*: 192.20 µg/ml. *A. bettzickiana*: 157.25 µg/ml.

In the management and control of cancer, natural products identified from plants have played a major role. In a 2000 based worldwide sales, products from medicinal plants constitute 14 of top 35 drugs (Butlet, 2004). With more than 2,70,000 higher plants existing on this planet, only a small portion of plants has been studied phytochemically. So, it is evident that plants can serve as a source of potential bioactive compounds for the development of new 'leads' to combat cancer (Shoeb, 2006). The natural products from plants such as alkaloids, flavonoids, terpenes, phenols, etc., had received wide attention because of their diverse pharmacological properties including cytotoxic and cancer chemopreventive potentials (Babu *et al*., 2002). Our previous work revealed the presence of secondary metabolites in the hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana*. Those secondary metabolites present in *Alternanthera* may be responsible for their cytotoxic activity on DLA cell line.

CONCLUSION

For the control and treatment of many ailments, there has been a growing interest in the study of therapeutic potentials of natural products derived from plants. In the present work, the results of *in vivo* sub-acute toxicity study clearly showed the non-toxic nature of hydroethanolic leaf extracts of *A. brasiliana* and *A. bettzickiana* up to the tested dose level of 2000 mg/kg bwt. The LD_{50} values of both leaf extracts were considered to be more than 2000 mg/kg bwt. The *in vitro* MTT assay revealed the toxic potential of the leaf extracts on DLA tumor cells. It was concluded that further antitumor studies in *in vivo* animal models are necessary in order to establish the potential of *A. brasiliana* and *A. bettzickiana* as a source for new anticancer medicine.

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AUTHORS' CONTRIBUTIONS

Mrs. Kasthuri O R contributed in performing the experiment, data compilation and wrote the first draft of the manuscript. Dr. Ramesh B involved in corrections in the manuscript and overall management of the study.

CONFLICT OF INTEREST

We, the authors confirm that we have no conflict of interest in this article content.

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