Research Article

Cytotoxic Effects Of The Phytochemical (+)-α-Viniferin In Swiss Albino Mice

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ABSTRACT

A good quantity of (+)-α-viniferin is consumed by people in northeast India through phytomedicines. Anticancer, antifungal and anthelminth activities of the phytoproduct are established, however, cytotoxic effect in consumers, if any, is not known. Therefore, *in vivo* cytotoxic potential of (+)-α-viniferin was evaluated by exposing the Swiss albino mice to 1mg, 2mg and 5mg (+)-α-viniferin/kg body weight (b.w.) per day continuously for 14 days. Mice exposed to 1mg (+)-α-viniferin showed a significant increase in platelet count, serum creatinine, and alkaline phosphatase activity, whereas, alanine transaminase and aspartate transaminase were recorded to be significantly high at the exposure dose of 2 mg. Total WBC count, revealed to increased significantly when mice exposed to 5 mg (+)-α-viniferin. A highly significant increase in lymphocyte count in the spleen was also observed in the mice exposed to 2 mg per kg b.w. Ultrastructural observations revealed distortion of surface topography of liver and intestinal lumen, vacuolization of liver cells and disintegration of intestinal microvilli in the phytochemical exposed mice. An increase in proinflammatory cytokines like interleukin-1β, Interleukin-6 and Tumour Necrosis Factor alpha were also recorded in the treated mice. Thus, (+)-α-viniferin revealed to be toxic to animals if consumed 2 mg/kg b.w. continuously for 14 days.

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INTRODUCTION:

Herbal medicines are used by various traditional societies throughout the globe because of easy availability, low cost, shortage of primary medico-veterinary services and the common notion that "natural products are safe to consume". Though the popularity of traditional medicine is quite high in developing countries like India (about 70% population consume herbal medicine), the extent of consumption of the same is not negligible in developed country like United States of America, where about 17% population use natural products each year to cure different ailments.[1] Consumption of phyto-products having hundreds of unknown ingredients, either in the form of indigenous medicine or as dietary supplements at high doses for long period of time may cause adverse effect on health in the form of allergic reactions, hepatic, renal, dermal, nephrological and reproductive abnormalities culminating to death of the consumer.[2,3,4,5,6] (+)-α-viniferin (AVF) is a polyphenolic phytoalaxin produced by several species of *Carex* (anthelmintic, antidiabetic), *Caragana* (anticancer, antigynaecological problem), *Dryobalanops* (pain killer, antiscabies, anticancer) etc. used in traditional healing system.[7,8,9,10,11,12,13] The compound possesses diverse biological and pharmacological activities like antioxidant, anticancer, antifungal and anthelmintic properties.[14,15,16,17] The phytochemical is found to be proapoptotic to several cancer cell lines.[18] AVF is also revealed to be toxic to zoospores of the fungus *Plasmopora viticola* as it restricts the development of the diseases.[19] A recent report showed that the compound exerts its anthelmintic effect through inhibition of (an unique anaerobic energy metabolism related enzyme) fumarate reductase activity in the cestode leading to paralysis and death of the parasite.[20]

AVF is a major (6.6% w/w) component of the plant *Carex baccans*,[13] crude root peel extract of which is consumed by indigenous people of Jaintia Hills (Area : 1,693 km^2; Population : 270,352) in Northeast India to get rid of intestinal helminth infection.[21] In the traditional use, different tribes consume 3-4 mg of crude aqueous extract having 200-250 μM (+)-α-viniferin per kg body weight per day continuously for 10 to 15 days. Therefore, considering the public health importance of the compound and lack of information on its toxic effect, the present study was conducted to evaluate *in vivo* sub-acute cytotoxic potential of the compound (+)-α-viniferin in Swiss albino mice taking ultrastructural, biochemical and immunohistochemical criteria as parameters.

MATERIALS AND METHODS

Chemicals

Enzymes and co-enzymes were procured from Sisco Research Laboratories and/or Himedia (Mumbai, India). (+)-α-viniferin was purchased from BioBioPha Co. Ltd., China. ELISA Kit for detection of Proinflamatory cytokines (IL-6, IL-1β and TNF-α) were obtained from Thermo Fisher Scientific, Mumbai, India. All other chemicals were purchased from Himedia, India.

Experimental animals and sub-acute toxicity test

The assessment of sub-acute oral toxicity was carried out following the guidelines of Organization for Economic Cooperation and Development (OECD) 407[22] and with approval of Institutional Animal Ethics Committee, North-Eastern Hill University, Shillong, India. Healthy Swiss albino mice (5 weeks age) weighted about 25 -35 g was procured from Pasture Institute, Shillong. The mice were kept in metal cages in the animal house with uniform temperature of 23±2 °C with 12 h light/dark cycle and were fed with standard rat chows (Pranav Agro Feed Ltd., New Delhi, India), and water ad libitum. Animals were acclimatized for one week before starting the experiment. Twenty four mice (both male and female) were divided into four groups, having 6 animals (3 males and 3 females) in each. Different doses like 1.0, 2.0 and 5.0 mg (+)-α-viniferin/Kg body weight (b.w.) of mice were prepared by dissolving the compound in phosphate buffer saline (PBS) having 0.1% Dimethyl sulfoxide (DMSO), and were administered orally to different groups of mice at a single dose per day for 14 days. The control group received only the vehicle (PBS having 0.1% DMSO) and were kept at the same

experimental conditions. All animals were kept under observation for 4 h after administration of the compound to see behavioral changes and mortality, if any. Body weight, food intake and water consumption of each animal was measured every day during the experiment. Oral administration of AVF was terminated on the day 14, after which the mice were fasted for 24 h. On the day 15, the mice in each group were weighed and sacrificed through CO_2 asphyxiation to collect different organs and blood samples.

Haematological assay

Blood samples were collected by retro-orbital bleeding in EDTA vials for assessment of total white blood corpuscles (WBC), red blood corpuscles (RBC), platelets count, and hemoglobin (Hb) content. Analysis of the selected blood parameters were done using AVANTOR BC-2800Vet Auto Haematology Analyzer.

Spleenic Lymphocyte Count was carried out through isolation of spleens from the mice followed by mincing using two glass slides. Fragments of minced spleen were passed through 100 µm-nylon mesh and suspended in balanced salt solution (BSS) to make cell suspension. Lymphocytes were then isolated by gradient centrifugation in lymphocyte separating medium, Hisep LSM 1077 (Himedia) followed by two washes in BSS. The lymphocyte pellets were then dissolved in 1 ml of BSS and counted using a haemocytometer.

Liver and kidney bio-markers assay

For analysis of liver and kidney functions, serum was isolated from blood. In brief, blood was collected in non-EDTA vials and allowed to clot for ~45 minutes at room temperature, then centrifuge for 15 minutes at 3000 rpm to isolate the serum. Serum activities of aspartate aminotransferase(AST) and alanine aminotransferase (ALT) were carried out by calorimetric method of Reitman & Frankel (1957).[23] In brief, 0.1 ml of serum was added to a tube containing 0.5 ml of substrate (ALT/AST substrate). The reaction mixture was mixed thoroughly (30 minutes for ALT and 60 minutes for AST). 0.5 ml of 2,4-dinitrophenyl hydrazine and the mixture was re-

incubated at 37°C for 20 minutes. 5 ml of 0.4N NaOH was added to stop the reaction and the absorbance of the mixture was read at 505 nm. The concentration of ALT/AST was calculated in U/L using a set of standard with known concentration of sodium pyruvate as reference. Alkaline phosphatase activity was determined following the method of Plummer (1988).[24] Briefly, a 10% (w/v) of the liver tissue was homogenized in glycine buffer. The homogenate was centrifuged at 5,000 rpm at 4°C for 20 minutes. The supernatant obtained was used as the enzyme source for estimation of ALP activity. The absorbance of both the blank and incubated solutions were measured at 405 nm in UV-VIS Spectrophotometer (Systronics model 119, India). The enzyme activity was calculated from a linear standard graph of p-nitrophenol. One unit of ALP activity was defined as that amount which catalyzed the formation of 1 mM of p-nitrophenol/h at 37°C. Serum level of creatinine was measured using Jaffe' method. Briefly, to 1ml of working reagent (containing picric acid and 0.4 M NaOH/12.5 mM EDTA Disodium salt in 1:1 ratio), 100 µL of serum was added while 100 µL of 2mg/dL creatinine was added to the standard. Absorbance A1 was taken at 420 nm after 30 seconds of mixing. The second absorbance A2 was taken 90 seconds later. The concentration of serum creatinine in the sample was measured in mg/dL using the following formula

Creatinine (mg/dL) = Δsample absorbance/Δstandard absorbance x standard concentration

Pro-inflammatory cytokines assay

Pro-inflammatory cytokine viz., Interleukin-1β, Interleukin-6 and Tumour Necrosis Factor-α were assayed using solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) as per kit instructions. To assess Interleukin-1β, 50 µL of incubation buffer, 50 µL Standards/Controls/Samples and 50 µL of Biotin conjugate were added into appropriate wells of the supplied microtiter plate and the plate was incubated for 90 minutes at 37°C. The content of the wells were aspirated and washed 4 times. 100 µL of Streptavidin-HRP working solution was added and incubated for 30 minutes at room temperature. The content of the wells were aspirated

and washed again 4 times. Now, 100 µL of stabilized chromogen was added to the wells and the plate was incubated for 30 minutes at room temperature in dark. After incubation, 100 µL of stop solution was added to each well and the absorbance was taken at 450 nm within 30 minutes. For assessment of Interleukin-6, 100 µL of standards and sample (50 µL sera+50 µL standard diluents) were added to the appropriate wells of a microtiter plate. The plate was covered and incubated for 2 hours at room temperature (RT). The content of the wells were aspirated and wash 4 times. Then, 100 µL Ms IL-6 Biotin Conjugate solution was added into each well except chromogen blanks and the plate was incubate for 30 minutes at room temperature. The content of the wells were aspirated and washed 4 times. 100 µL Streptavidin-HRP was added into each well except the chromogen blanks and the plate was incubated for 30 minutes at room temperature. The contents of the well were aspirated and washed 4 times. Then, 100 µL Stabilized Chromogen was added to each well and incubated for 30 minutes at RT in the dark. Then 100 µL stop solution was added to each well to stop the reaction and the absorbance was taken at 450 nm within 30 minutes. For Tumour Necrosis Factor α, 100 µL of standards, controls and sample (50 µL sera+50 µL standard diluents) were put in the appropriate microtiter wells, 50 µL Ms IL-6 Biotin Conjugate was added and incubated for 90 minutes at RT. The content of the wells were aspirated and washed 4 times. 100 µL Streptavidin-HRP Working Solution was added to each well and incubated for 30 minutes at RT. The content of the wells were aspirated and washed again 4 times. Then, 100 µL Stabilized Chromogen was added to each well and incubated for 30 minutes at RT in dark. 100 µL of Stop Solution was added to each well and the absorbance was read at 450 nm within 30 minutes.
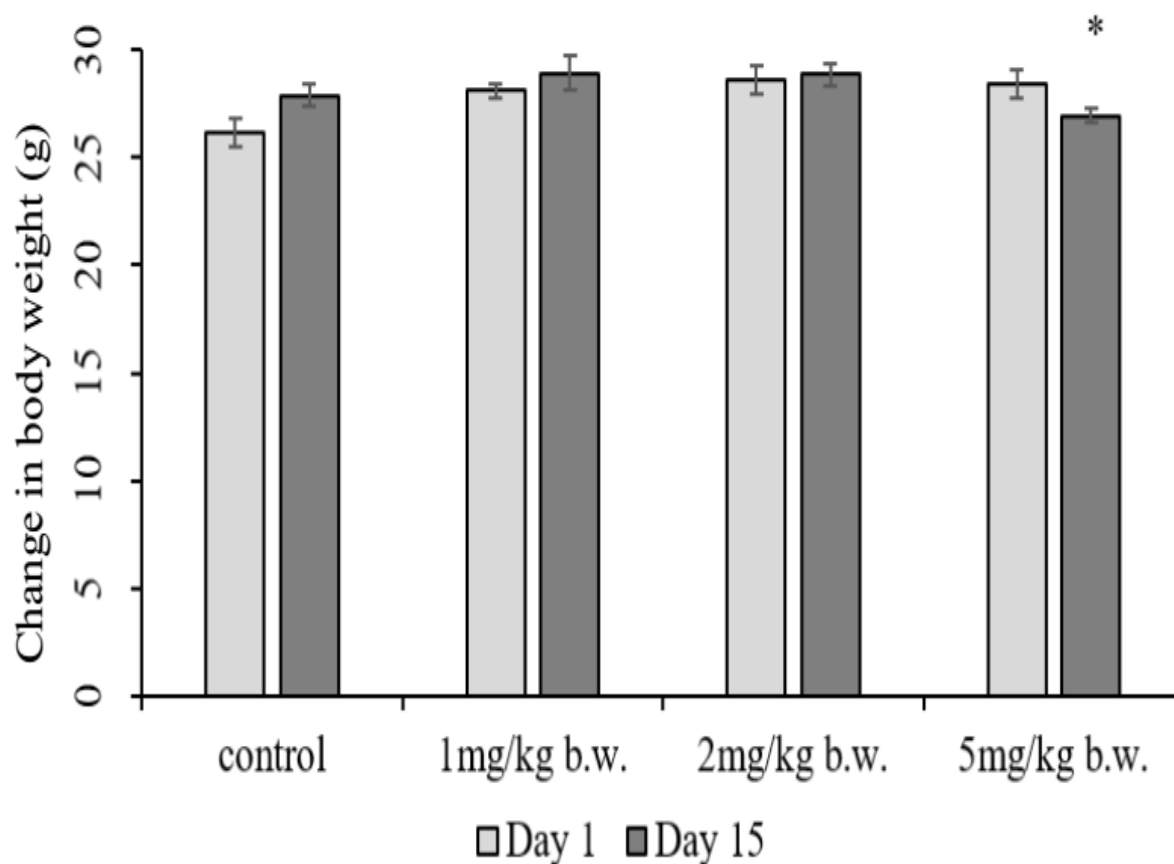
Ultrastructural preparations

Intestine and liver tissue from control and (+)-α-viniferin exposed mice were fixed in modified Karnovsky's fixative, washed and post fixed in 1% OsO_4 buffered with 0.2 M sodium cacodylate for 4 h. For scanning electron microscopy, tissue pieces were dehydrated in graded series of acetone, followed by treatment with Tretramethylsilane for 5 minutes and drying in air.[25,26] Surface architecture of gold coated liver was viewed in JEOL JSM 6360 scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at 15 kV. For transmission electron microscopy, fixed pieces of tissues were dehydrated with ascending grades of acetone series and embedded in araldite. Ultrathin sections were stained with uranyl acetate followed by lead citrate and viewed in a JEM 2100 (JEOL) transmission electron microscope operated at 200 kV.

RESULTS
Body weight and relative organ weight
Observations on body weight of mice exposed to AVF for 14 days revealed decrease in body weight-gain significantly ($P<0.001$) at a dose of 5 mg/kg b.w. as compared to the control (Fig. 1). However, weight of different organs relative to body weight of mice treated with 0 mg, 1 mg, 2 mg and 5 mg extract per kg b.w. caused an increase in liver weight/body weight ratio to an extent of 0.051 ± 0.044, 0.044 ± 0.047, 0.05 ± 0.121 and 0.045 ± 0.068; kidney weight/body weight ratio to 0.013 ± 0.038, 0.013 ± 0.046, 0.015 ± 0.058 and 0.014 ± 0.021; heart weight/body weight ratio to 0.005 ± 0.007, 0.005 ± 0.009, 0.005 ± 0.005 and 0.005 ± 0.009, and spleen weight/body weight ratio to an extent of 0.005 ± 0.024, 0.003 ± 0.043, 0.007 ± 0.065 and 0.004 ± 0.013, respectively.
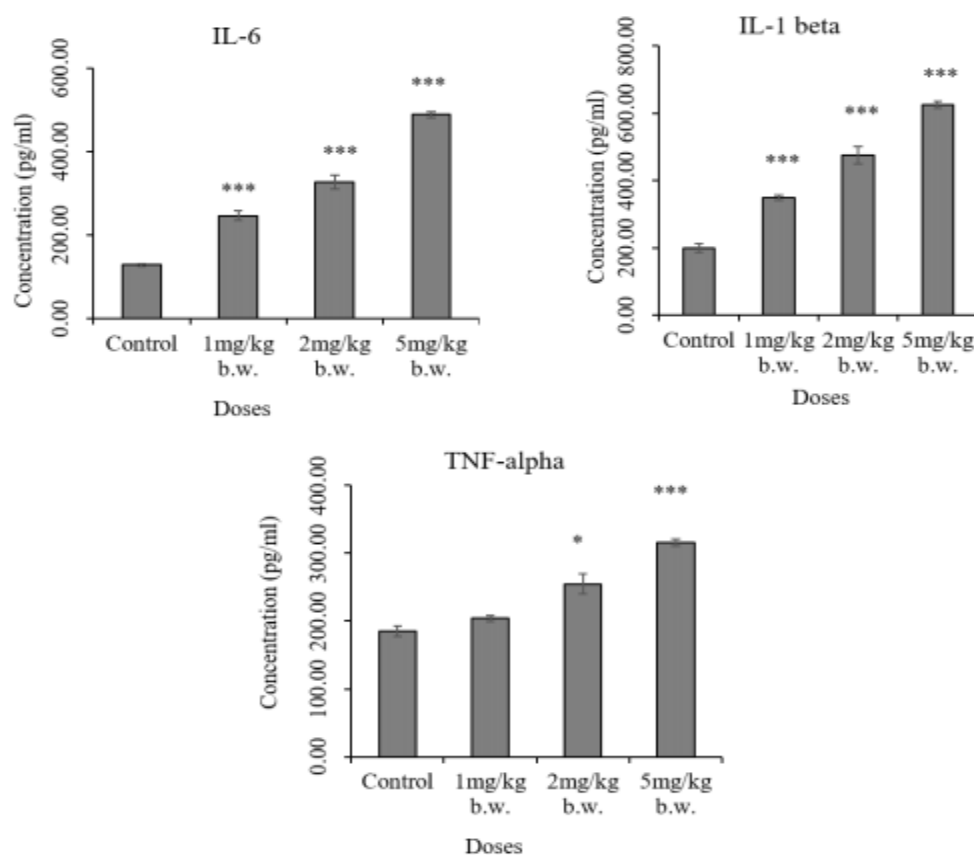
*Figure 1. Changes in the body weight of mice following 14 days oral administration of (+)-α-viniferin at doses 1mg, 2mg, and 5mg/kg body weight compared with control. *Significant (p< 0.001) reduction in body weight.*

Observations on haematological parameters

Table 1 shows WBCs, RBCs, platelet counts and haemoglobin level in the blood of control and AVF exposed mice. Though WBC count showed a dose dependent increase compared to the control, a significant (P<0.05) increase was observed in the mice when the dose of AVF increased to 5 mg/kg b.w. A dose dependent significant (P<0.001) increase in platelet count was observed in mice exposed to all the three concentration of AVF i.e. 1, 2 and 5mg/kg b.w.

compared to the control. However, a significant (P<0.001) decrease in Hb level was recorded at the lowest dose of 1mg/kg as compared to the control. A dose-dependent significant (P<0.01) increase in lymphocyte number was observed with increasing dosages (1 to 5 mg/kg b.w.) of the active compound (Table 1). The mice exposed to the highest dose (5 mg/kg) shows the highest increase (P<0.001) in lymphocyte number as compared to the others.

*Figure 2. Changes of pro-inflammatory cytokines IL-6, IL-1β and TNF-α in different doses of α-viniferin exposed mice compared to control. Results are expressed as Mean ± SEM, n=3. *Significant at (p<0.05); ***Significant at (p<0.001)*

Analysis of liver and kidney markers

Activities of AST, ALT, sCr and ALP in control and AVF exposed mice were presented in Table 1. All the parameters have been observed to be increased in a dose dependent manner in the AVF treated mice. Serum level of AST and ALT showed a significant (p<0.01) increase at doses 2 mg/kg b.w. and above, while serum level of creatinine showed a significant (p<0.001) increase at a dose 1 mg/kg and above. Concentration of ALP also showed a significant (p<0.05) increase at doses 1 mg/kg b.w. and above.

Analysis of pro-inflammatory cytokines

Figure 2. shows the activities of pro-inflammatory cytokines in the control mice and mice exposed to different dosages of AVF. A dose dependent increase in pro-inflammatory cytokines namely IL- 1β and IL-6 was observed in the serum of mice exposed to 1 to 5 mg AVF. However, TNF-α show a significant increase when the concentration of AVF was raised to 2 mg and above/kg body weight.

Ultrastructural observations

SEM observations on the fine surface topography of control liver of mice revealed normal intra-hepatic cells, intact endothelial lining having endothelia with flattened processes and small pores (Fig. 3a). Complete distortion and deformation of surface topography of liver, retraction of endothelium with very few intact pores were evident in the AVF exposed mice (Fig. 3b). Regular arrangement of microvilli having smooth surface was observed in the intestinal lumen of the control mice (Fig. 3c). However, in the AVF treated mice collapsed lumen surface of intestine

with deformed microvilli was evident (Fig. 3d). TEM of control liver showed normal cells with intact nuclear membrane, nucleus and chromatin granules (Fig. 4a). AVF exposed mice on the other hand showed disruption of nuclear membrane, nucleolus and chromatin granules (Fig. 4b). Epithelial layer of control intestinal lumen revealed regular arrangement of microvilli with compact cytoplasm having normal mitochondria, ribosome, endoplasmic reticulum and other cell organelle (Fig. 4c). In contrast, mice administered with AVF showed distorted microvilli, vacuolated cytoplasm and deformed nucleus (Fig. 4d).
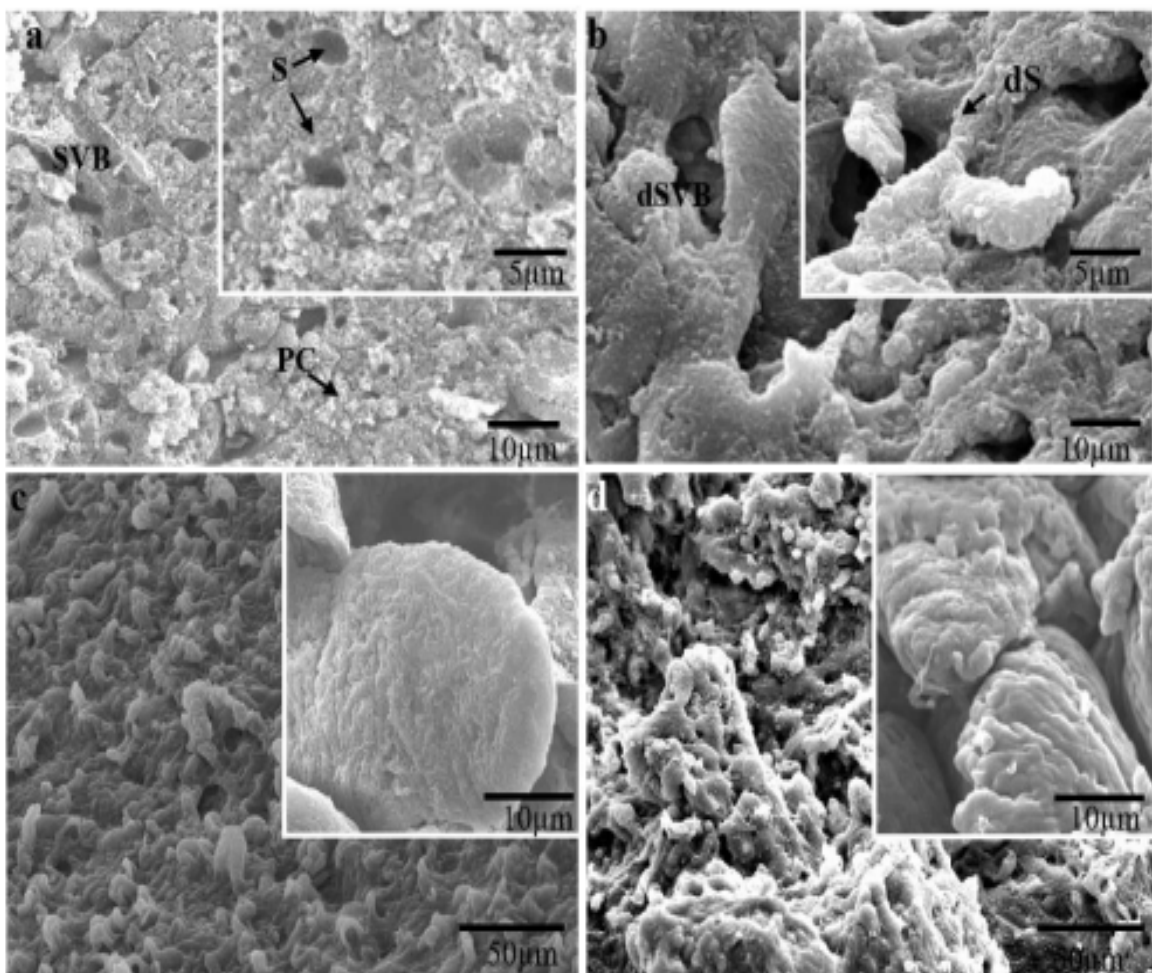

Figure 3. Scanning electron microscopic images of liver (a, b) and intestinal surface (c, d) of mice – (a) liver surface showing normal sinusoidal vascular bed (SVB) with normal sinusoids (S) and parenchyma cells (PC) of control; (b) liver surface showing disruption of sinusoidal vascular bed (dSVB) and destruction of liver sinusoids (dS) of mice treated with 5mg α-viniferin/kg body weight; (c) control microvilli and (d) α-viniferin exposed mice showing deformation and disruption of microvilli. Inset showing enlarge view of the respective liver and microvilli.
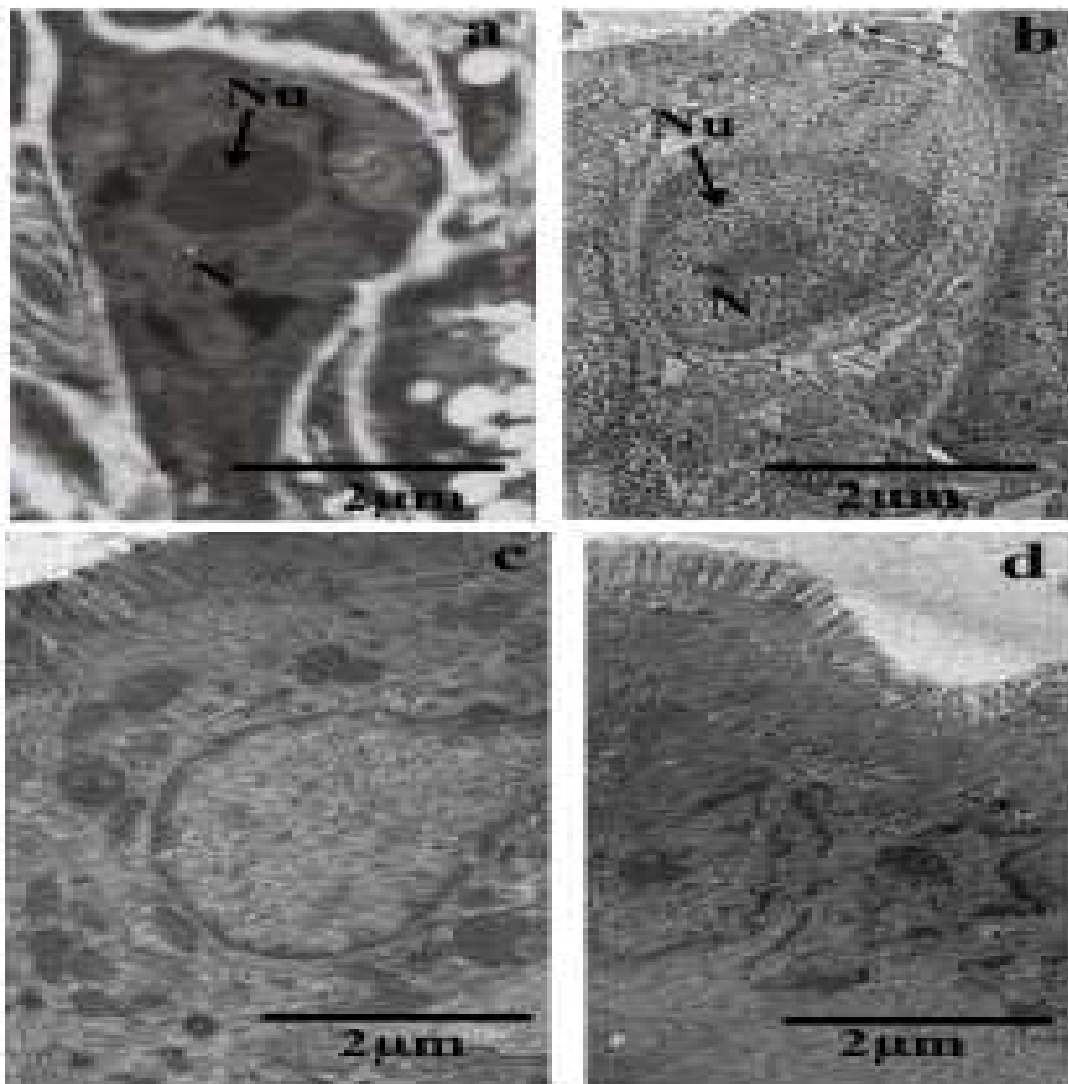
Figure 4. Transmission electron microscopic images of liver (a, b) and intestine (c, d) of mice: (a) control cell of liver with intact plasma membrane and distinct nucleus (N) and nucleolus (Nu); (b) liver cells of α-viniferin (5mg/kg body weight) exposed mice showing disrupted plasma membrane, nucleus (N) and nucleolus (Nu); (c) control mice showing normal arrangement of microvilli, intact cell organelle, nucleus, nuclear membrane, mitochondria and (b) α-viniferin (5mg/kg body weight) exposed mice showing distorted microvillus, vacuolization of cytoplasm and distorted nucleus.

Parameters	Control	1mg/kg body weight	2mg/kg body weight	5mg/kg body weight
WBC (cells x 10^3/mm³)	6.84 ± 0.36	7.54 ± 0.46	8.39 ±0.64	9.68 ± 0.91*
RBC (cells x10^6/mm³)	3.16 ± 0.17	3.92 ± 0.13**	4.025 ± 0.15**	4.43 ± 0.35**
Platelet (cells x 10^6/mm³)	3.43 ± 0.27	5.46 ± 0.35***	6.66 ± 0.31***	7.55 ± 0.28***
Hb g/dl	17.76 ± 0.27	15.98 ± 0.12***	15.46 ± 0.18***	15.1 ± 0.34***
AST (U/L)	31.10046 ± 0.50	31.92 ± 1.54	38.88 ± 0.6***	52.06 ± 1.94***
ALT (U/L)	48.32 ± 2.94	59.07 ± 5.04	60.92 ± 2.0 **	110.00 ± 2.46**
Lymphocyte (cells x 10^4/g spleen)	2.3 ± 0.03	2.62 ± 0.03**	3.78 ± 0.02**	4.19 ± 0.04***
sCr (mg/dl)	0.16 ± 0.01	0.38 ± 0.02 ***	0.48 ± 0.01***	0.79 ± 0.04***
ALP (U/mg wet tissue)	104.433 ± 0.33	106.54 ± 0.41**	137.86 ± 0.68***	138.89 ± 3.11***

*Table 1. Effects of α-viniferin on different hematological and biochemical parameters of mice compared to control. Results are expressed as Mean ± SEM, n = 6. *Significant (at $p<0.05$); **Significant (at $p<0.001$); ***Significant (at $p<0.0001$)*

DISCUSSION

One of the vital functions of liver and kidney is to eliminate waste products and toxic substances, and to perform this function integrity of the liver and kidney cells has to be maintained. Whenever damage of liver and kidney cells occur due to toxic substances or parasitic infections, leakage of biomarkers enzymes like AST and ALT from liver/kidney to serum takes place.[27] Thus an increased value of these substances in the blood is an indication of abnormal liver and kidney function.[28] A significant increase in quantity of AST and ALT in the blood of AVF assaulted mice along with deformation and destruction of liver morphology and ultrastructure of hepatocytes as observed in our present study indicates the extent of liver damage in the experimental animals which is generally caused by toxic chemicals.[29,30,31] A similar type of liver damage was also observed in rats exposed to crude extract of *Potentilla fulgens* and *Carex baccans*, two anthelmintic medicinal plants of northeast India.[32] Insecticidal plant *Stemona aphylla*

has also been reported to alter structure and function of liver in albino rat.[33]Alexis *et al*. (2003) observed an increased level of AST and ALT in the serum of rat exposed to fruit extracts of *Punica granatum* and are on the view that the elevation of the enzymes could be an adaptation by liver to the stress imposed by the phytochemicals or due to de novo synthesis of the protein molecules.[34]An increased level of ALP in the blood of mice exposed to increased amount of (+)-α-viniferin as observed in the present study indicate possible inflammation and scarring of liver.[35] Likewise an elevated level of AST, ALT and ALP together with dilation of sinusoids, reduction in size of hepatocytes, whole liver and nucleus of liver cells were also observed in animals treated with ciprofloxacin, a broad spectrum antibiotic.[36,37,38] Creatinine level in serum indicates the creatinine clearance capacity of kidney through glomerular infiltration. Therefore, malfunction of kidney leads to increase in the level of serum creatinine (sCr). A significant increase in the quantity of creatinine as

observed in the blood of (+)-α-viniferin exposed mice at a dose of 1mg/kg body weight and above as compared to the control, indicates the extent of nephrotoxic potential of the phytochemical.[39] A similar type of malfunction and damage of kidney in rat exposed to crude extract of *Carex baccans* was observed by Giri and Roy .[6] The spleen houses and aids in the maturation of lymphocytes which in turn plays an important role in the immune system. In the present study a significant increase in lymphocyte count in the spleen due to long-time exposure to (+)-α-viniferin indicates the possible long term association between the spleen tissue and the phytochemical that resulted in activation and hyperplasia of lymphocytes to maximize elimination of the toxic chemicals.[40,41]

A dose dependent significant increase in cytokines like IL-1β, IL-6 and TNF-α in the AVF exposed mice indicates the possible cytokine-induced cytotoxic affect in the animal.[42] IL-1β, IL-6 and TNF-α are potent pro-inflammatory cytokines known to exert a synergistic cellular cytotoxic effect through promoting inflammatory cell infiltration and cell proliferation. IL-6 is the major inducer of acute phase reactions in response to inflammation or tissue injury, and together with IL-1β and TNF-α, it induces synthesis of acute phase proteins by hepatocytes. Toxins/chemicals are responsible for production of different cytokines in the activated cells of experimental animals and modulate the functions of cells under different circumstances.[43,44] Similar to our present observations, an increase in the level of cytokines was also observed by Simpson *et al*. in mice where a cytotoxic dose of paracetamol caused an increased in TNF-α, coinciding with liver necrosis.[45] Liver cells, in particular, are easily susceptible to toxic chemicals leading to injury and subsequent release of different cytokines, reactive oxygen species and hydrolytic enzymes, thus exaggerating the injury process.[46] Thus, the phytochemical AVF seems to be responsible for several fold increase in inflammatory process (through releasing IL-1β, IL-6 and TNF-α) resulting an extensive tissue damage as also revealed through biochemical and ultrastructural observations.[47,48]

CONCLUSIONS

Based on the present observations, it may be concluded that (+)-α-viniferin is certainly toxic when consumed at a dose 2 mg and above per kg b.w. per day for 14 days, since at these dosages it can cause weight loss, allergic reaction, damage to liver, kidney, intestine and alter blood parameters. Toxicity of (+)-α-viniferin revealed to be dose dependent; therefore, the undesired effects can be reduced through use of lower concentrations for therapeutic purposes, as practiced in traditional system of healing.

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