EVALUATION OF SUB-ACUTE TOXICITY OF THE ETHANOLIC ROOT EXTRACT OF COLEUS VETTIVEROIDES IN ALBINO WISTAR RATS.

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Abstract: Background: In this study, we evaluated the oral sub-acute toxicity study of ethanolic roots extract of *Coleus vettiveroides* on animal models. **Methods:**The sub-acute oral toxicity study was carried out as per OECD 423 guidelines. The study was approved by the Institutional Animal Ethics Committee (IAEC). In sub-acute toxicity study, the oral dose (250, 500 and 1000 mg/kg) of *coleus vettiveroides* root extract was administered for 28 days to the three animals groups and their body weight, hematological, serum hepatic biochemical parameters were evaluated and compared to normal group by sacrificing all group animals. **Results:** *Coleus vettiveroides*treated groups revealed neither mortality nor any significant changes were observed. The result indicates that the oral administration of ethanolic root extract of *Coleus vettiveroides* plant did not produce any significant toxic effect in albino wistar rats. **Conclusions:** The extract can be utilized safely for therapeutic use in pharmaceutical formulations.

Keywords: Sub acute toxicity, *Coleus vettiveroides*, hematology, liver**.**

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INTRODUCTION

Ethnomedicinal plants have been widely used in humansand animals as therapeutic remedies for the treatment, mitigation and prevention of diseases in traditional medicine for centuries in both developed and developing countries [1]. The general perception is that medicinal plants are natural products devoid of synthetic preservatives and therefore, safe for discretional uses. Over 85%of disease conditions of humans and animals, ranging from bacterial infections to cancer and immunological disorders are treated with either natural products or compounds derived from natural products [2]. Drug-induced liver injury and nephrotoxicity are the leading causes of pharmaceutical withdrawals of promising drug candidates in clinical trials [3].Whereas, aminotransferases and to a lesser extent, alkaline phosphatase, sorbitol dehydrogenase, glutamate dehydrogenase,gamma-glutamyltransferase, total bilirubin, total bile acids, and 5′-nucleotidase are the commonly evaluated biomarkers in drug safety assessment of hepatotoxicity[4]; blood urea nitrogen, serum creatinine, sodium and phosphorous are the common endpoint indicators used to evaluate renal function[5]. Therefore, assessment of at least four serum parameters, involving a minimum of two for each of the hepatocellular and hepatobiliary serum biomarkers has been recommended for a safety study of xenobiotics[6].

MATERIALS AND METHODS

Chemicals and reagents

GSH, MDA,NBT and SDS were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Folin's - Ciocalteau reagent, H& E stain and TBA were obtained from Sisco Research Laboratories, Mumbai - India. Acids, bases, solvents and salts used for the investigation were of analytical grade (AR) and were obtained from, SRL, Mumbai, India and The diagnostic kits purchased from Enzo life Sciences.

Animals

Albino wistar rats of either sex (150 - 200 g) were used for the present study. They were housed in clean polypropylene cages and maintained under standard laboratory condition at an temperature 22±2°C and 12 hr alternating light-dark cycle. They were allowed free access to standard pellet diet (Hindustan Lever, Kolkata, India) and water ad libitum. The experimental protocol the animal studies were approved by the Institutional Animal Ethics Committee (IAEC), Saveetha Institute of Medical and Technical Sciences, Chennai, Tamilnadu, India with BRULAC/SDCH/SIMATS/IAEC/12-2019/036.

Figure 1: Albino wistar rats

Plant materials: The roots of *Coleus vettiveroides* werecollected from forest area of Coimbatore, India and the sample was identified and authenticated by botanist Dr.V Vasubabu and the material was kept under shade drying, pulverized by a mechanical grinder and passed through a 30 mesh sieve.

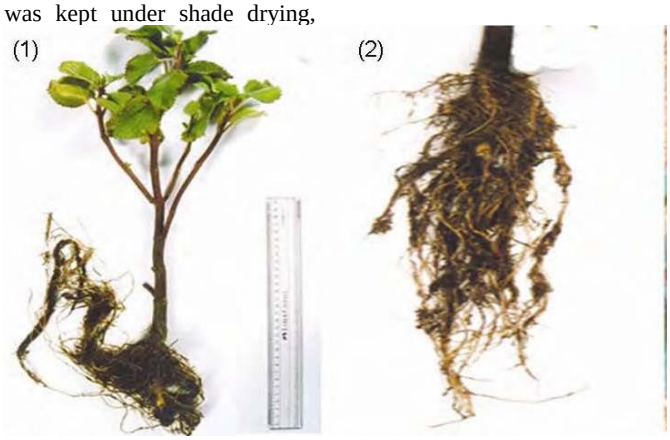

Figure 2: 1 Coleus vettiveroides Fresh plant; 2 Freshly cut roots of Coleus vettiveroides

Figure 3: Coleus vettiveroides dried roots

Extract preparation

The 500g powder of *Coleus vettiveroides*root was taken, were soaked in 2L of absolute alcohol in a 5 L round flask for about 24 h and reflux condensation was done at 60-80° C for 3 h. It was cooled and alcohol was drained. Reflux condensation was repeated twice with 2L of alcohol each time. The filtrate was

concentrated by distillation till a syrupy consistency of extract was obtained. The extract was evaporated to dryness in a china dish on a water bath. The extract was filtered and concentrated to dryness in vacuum and stored in an air tight container for further use. Ethanolic root extract of *Coleus vettiveroides*was suspended in distilled water and administered.

Qualitative estimation of Phytoconstituents: The collected root extracts were analyzed to explore chemical profile using standard phytochemical tests qualitatively

Experimental design

The acute oral toxicity study was carried according to OECD 423 guidelines (OECD, 2001).Animals were given normal diet and had normal accessibility to water.The animals were divided into four groups each group comprising of three animals. Group I rats served as control, Group II, III and IV rats were orally administered with 250, 500 and 1000mg/kg, b.w. respectively. The rats were monitored for behavioural and morphological changes daily for 28 days and sacrificed on 29th day and various biochemical and histopathological changes were observed.

Mortality and Toxic Signs

The visual observations of mortality, various changes in physical appearance, behaviour (sleepy, salivation, lethargy), and any injury or illness were conducted once daily for 28 days, especially after dosing.

Relative Organ Weight

On the 29th day, all the animals were anesthetized by an intraperitoneal injection of ketamine. Blood samples were collected by cardiac puncture into EDTA containing tubes and non-heparinized tubes for haematological and biochemical analysis, respectively. Rats were then euthanized after blood collection and the internal organs (liver, spleen, kidney, heart and lungs) were removed and weighed and observed for any gross lesions.

Blood analysis

Biochemical analysis included liver function markers (AST, ALT), bilirubin (total), nephrotic markers (urea, creatinine and uric acid) were analysed using Enzo Life Sciences diagnostic kits and measured using Robonik semi auto analyser. Hematologic parameters included red and white blood cells, hemoglobin, hematocrit, platelets using the unit XN1000 (Sysmex).

Antioxidant parameters

Estimation lipid peroxidation

This method depends on the formation of MDA as an end product of lipid peroxidation which reacts with thiobarbituric acid producing thiobarbituric acid reactive substance (TBARS), a pink chromogen, which can be measured spectrophotometrically at 532 nm, an MDA standard was used to construct a standard curve against which readings of the samples were plotted (Ohkawa et al.,1979). Briefly, 0.2 mL of tissue homogenate, 0.2 mL of SDS, 1.5 mL of acetic acid, and 1.5 mL of TBA were added. The mixture was made up to 4 mL with water and then heated in a water bath at 95°C for 60 min. After cooling, 1 mL of water and 5 mL of n-butanol/pyridine mixture were added and shaken vigorously. After that it was centrifuged at 4000 rpm for 10 min; then, the organic layer was taken and measured at 532 nm absorbance. The level of MDA was expressed as nmoles /mg protein.

Estimation of reduced glutathione

Reduced glutathione was determined by the method of Sedlak and Lindsay (1968). 0.5 mL tissue homogenate was mixed with 0.2 M Tris buffer with pH of 8.2, and then contents were mixed with 0.1 mL of 0.01 M Ellman's reagent, (5,5′-dithiobis-(2-nitro-benzoic acid)) (DTNB), then centrifuged at 3000 g for 15 min. The absorbance was read at 412 nm. A series of standards treated in a similar way also run to determine the glutathione content. The amount of glutathione is expressed as µM of GSH/mg protein.

Assay of superoxide dismutase

Superoxide dismutase was assayed following the method of Misra and Fridovich (1972). The tissue homogenate (0.1 mL) was mixed by reaction mixtures that contained sodium carbonate (1 mL, 50 mM), nitrobluetetrazolium (0.4 mL, 25 µm), and hydroxylamine hydrochloride (0.2 mL, 0.1 mM). The mixture were observed at 560 nm using UV-spectrophotometer (MINDRAY 91).

Assay of catalase

The activity of catalase was assayed according to the method of Takahara et al. (1978). Phosphate buffer (1.2 mL) and 0.2 mL of tissue homogenate were mixed, and the reaction was started by the addition of 1.0 mL of H_2O_2 solution. Decrease in the absorbance was measured at 240 nm at 30 sec intervals for 3 min. For the enzyme blank, distilled water was used instead of hydrogen peroxide. The activity of enzyme was expressed as µM of H_2O_2 decomposed/min/mg of protein.

Histopathological examinations

The vital organs isolated from sacrificed rats were fixed in 10% formalin, then after processing embedded in paraffin wax. Paraffin sections were made at 5 µm and stained with haematoxylin and eosin. The slides were studied under a light microscope (Olympus model) and captured the magnified images of tissues structure for further study.

Statistical analysis

Values were expressed as Mean ± SD. To determine the significance of inter group differences, one way ANOVA followed by Post Hoc t tests were used. $P < 0.05$ was considered statistically significant**.**

RESULTS

Preliminary and qualitative analysis of Phytoconstituents:

The qualitative phytochemical analysis of Ethanolic Root Extract of *Coleus vettiveroides* (ERECV) was shown in the (table1).

Table 1: Qualitative phytochemical analysis of Ethanolic Root Extract of Coleus vettiveroides (ERECV)

S.No	Phytochemicals	ERECV
1	Alkaloids	+
2	Tannins	+
3	Proteins	+
4	Saponins	-

5	Aminoacids	+
6	Flavanoids	+
7	Phenols	+
8	Carbohydrates	+
9	Glycosides	-
10	Sterols	+
11	Terpinoids	+

+ indicates Present - indicates Absent

ERECV - Ethanolic Root Extract of *Coleus vettiveroides*

Table 2: Effect of oral administration of ethanolic root extract of Coleus vettiveroides on average body weight (g) of rat.

Body weight(g)	Control	ERECV(250mg)	ERECV(500mg)	ERECV(1000mg)
Day 1	172.6 ± 2.51	181.0 ± 1.0	179.3 ± 1.15	178 ± 2.64
Day 28	213 ± 2.64	207.3 ± 6.42	194.3 ± 4.04	183.3 ± 3.05

ERECV- Ethanolic Root Extract of *Coleus vettiveroides*

Values are expressed as mean ± STD. P < 0.05 when compared to control group.

Table 3: Effect of oral administration of ethanolic root extract of Coleus vettiveroides on average organ weight (g) of rat.

Organ (g)	Control	ERECV (250mg)	ERECV(500mg)	ERECV(1000mg)
LIVER	7.84 ± 0.05	7.99±0.04	7.80 ± 0.02	7.50 ± 0.05
LUNG	1.40 ±0.02	1.33 ± 0.03	1.34 ± 0.05	1.22 ± 0.04
KIDNEY	0.71±0.02	0.80 ± 0.04	0.80 ± 0.04	0.63 ± 0.005
SPLEEN	0.51±0.02	0.5 ± 0.01	0.55 ± 0.02	0.44 ± 0.02

ERECV- Ethanolic Root Extract of *Coleus vettiveroides*

Values are expressed as mean ± STD. P < 0.05 when compared to control group.

Table 4: Effect of oral administration of ethanolic root extract of Coleus vettiveroides on haematological parameters of rat.

BLOOD PARAMETERS	Control	ERECV (250mg)	ERECV (500mg)	ERECV (1000mg)
RBC (10^6 / µl)	6.91 ± 0.24	7.46 ± 0.42	6.61 ± 0.45	6.80 ± 0.34
WBC (10^3 / µl)	8.71 ± 0.15	8.32 ± 0.22	7.66 ± 0.32	7.35 ± 0.23
PLATELETS (10^3/µl))	1193 ± 4.0	1174 ± 4.04	1128 ± 7.57	1090 ± 39.9
Hb (g/dl)	14.8 ± 0.56	15.3 ± 0.55	14.7 ± 0.45	14.3 ± 0.41
PCV (%)	44.4 ± 1.04	44.0 ± 0.76	43.4 ± 0.72	42.0 ± 1.13

PCV = Packed cell volume; Hb= haemoglobin concentration; RBC = red blood cell count; WBC = white blood cell count

ERECV- Ethanolic Root Extract of *Coleus vettiveroides*

Values are expressed as mean ± STD. P < 0.05 when compared to control group.

Table 5: Effect of oral administration of ethanolic root extract of Coleus vettiveroides on biochemical parameters of rat.

PARAMETERS	Control	ERECV(250mg)	ERECV(500mg)	ERECV(1000mg)
AST (IU/L)	144.1 ± 4.45	137.1 ± 1.25	142.8 ± 2.93	139.9 ± 1.19
ALP(IU/L)	150.0 ± 2.0	153.3 ± 3.51	160 ± 1.0	162 ± 1.73
ALT(IU/L)	57.2 ± 1.12	57.3 ± 0.91	60.4 ± 1.27	57.9 ± 3.65
GGT(IU/L)	0.38 ± 0.01	0.43 ± 0.02	0.49 ± 0.01	0.52 ± 0.01
LDH(IU/L)	7.56 ± 0.20	6.8 ± 0.1	8.46 ± 1.02	9.43 ± 1.30
BILIRUBIN (g/dl)	0.56 ± 0.05	0.66 ± 0.15	0.633 ± 0.05	0.766 ± 0.15
PROTEIN (g/dl)	6.93 ±0.24	6.95 ± 0.18	6.92 ± 0.16	6.51 ± 0.36
GLUCOSE (mg/dl)	83.23 ± 2.80	84.4 ± 0.76	91.4 ± 0.77	83.1 ± 1.72
UREA(mg/dl)	25.5 ± 0.78	26.9 ± 1.61	27.0 ± 2.72	22.5 ± 1.26
URIC ACID (mg/dl)	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
CREATININE(mg/dl)	0.76 ± 0.11	1.1 ± 0.1	1.43 ± 0.15	0.76 ± 0.15

AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; GGT=gamma-glutamyltranspeptidase

ERECV- Ethanolic Root Extract of *Coleus vettiveroides*

Values are expressed as mean ± STD. P < 0.05 when compared to control group.

Table 6: Effect of oral administration of ethanolic root extract of Coleus vettiveroides on Oxidative stress in liver tissue of rat.

OXIDATIVE STRESS	Control	ERECV(250mg)	ERECV(500mg)	ERECV(1000mg)
MDA (µmol/mg protein)	1.81 ± 0.03	1.72 ± 0.06	1.89 ± 0.05	2.02 ± 0.10

SOD (µ /ml protein)	2.74 ± 0.06	1.94 ± 0.04	2.33 ± 0.48	3.05 ± 0.13
CAT (µ mole / L protein)	19.0 ± 0.25	14.9 ± 0.69	13.3 ± 0.65	12.8 ± 0.268
GSH (Units / mg protein)	5.67 ± 0.04	5.86 ± 0.04	5.14 ± 0.07	5.01 ± 0.02

ERECV- Ethanolic Root Extract of *Coleus vettiveroides*
Values are expressed as mean ± STD. P < 0.05 when compared to control group.

Sub-Acute toxicity study

The Ethanolic Root Extract of *Coleus vettiveroides* (**ERECV**) was determined as per the OECD guideline 423, where the limit test dose of 1000 mg/kg was used. Treatment related toxic symptom or mortality were not observed after oral administration of ethanolic root extract of *Coleus vettiveroides* at a dose of 200, 500 and 1000 mg/kg. The general behavioural of animals which was treated with extract and control group was observed first for short period of 4 hours followed by long period of 72 hours. The animals which was treated with extract did not show any changes in behaviour, breathing, skin effects, water consumption, impairment in food intake and temperature when compared with control group. Therefore, the extract seems to be safe at a dose level of 1000 mg/kg.

Effect of Plant Extract on Relative Organ Weight and Body Weight

There was no significant difference in average organs and relative organs weight between control and extract treated group at a dose of 250, 500 and 1000 mg/kg. The effect of plant tested extract on principal organ weights relative to body weight are shown in Table 2 and 3. There were no significant differences in the changes of each weight. The results revealed that, the vital organs such as liver, kidney, spleen and lung were not adversely affected throughout the treatment by extract. The average and relative organ weight of tested plant extract and control treated groups shown statistically non-significant differences (P < 0.05).

DISCUSSION

Toxicology tests are used to observe products such as individual compounds, mixture of compounds, crude extract, pesticides, medications, food additives, packing materials or their chemical ingredients. World health organization (WHO) recommends that medicinal herbs would be the dominant source to obtain a range of drugs. Therefore, such medicinal plants must be investigated for better understanding of their medicinal properties, safety and effectiveness. Safety of plant extract is evaluated by sub-acute oral toxicity analysis.In the present study, even a higher dose of plant extract i.e.1000 mg/kg did not show any signs of toxicity or mortality for animals. Thus, plant extract even at 1000 mg/kg may be considered as safe. There were no behavioral changes and no mortality was observed in animals at 250 mg/kg, 500 mg/kg and 1000 mg/kg concentrations.

The ethanolic extract of the plant contains alkaloids, tannins, proteins, amino acids, flavonoids, phenols, carbohydrates, sterols, and terpenoids as phytochemical constituents.

There is a significant increase (P<0.05) in the average body wt of rat in Group II (ERECV-250mg) than Group III (ERECV-500mg), Group IV (ERECV-1000mg) and control on 28th day compared to 1St day (Table 2)

There is significant increase (P<0.05) in average weight of liver in ERECV 250mg compared to control, Group III (ERECV-500mg), Group IV (ERECV-1000mg) but there is an significant decrease (P<0.05) in average weight of lung in ERECV 250mg (Group II), ERECV 500mg (Group III), ERECV 1000mg (Group IV) compared with control group. There is an significant increase in average weight of kidney in ERECV 250mg (Group II), ERECV 500mg (Group III) compared with control and Group IV (ERECV-1000mg).There is an significant increase (P<0.05) in average weight of spleen in ERECV 500mg (Group III) compared with control group, ERECV 250mg (Group II) and Group IV (ERECV-1000mg) (Table 3).

There is a significant increase (P < 0.05) in RBC count and Hb in ERECV -250 mg (Group II) compared with control group, Group IV (ERECV-1000mg), ERECV 500mg (Group III). There is a significant decrease (P < 0.05) in WBC, Platelets count in ERECV 250mg (Group II) compared with control. There is a significant increase in PCV in control group than ERECV -250 mg (Group II), ERECV 500mg (Group III), Group IV (ERECV-1000mg) (Table -4)

There is a significant increase (P < 0.05)in AST level in control group compared with other extract groups, significant increase in ALP, GGT, LDH and Bilirubin levels in ERECV -1000mg (Group IV) compared with other extract groups and control groups, significant increase in ALT, Glucose, Urea, Creatinine level in ERECV 500mg (Group III) compared with other extract groups and control group, significant increase in Protein level in ERECV 250mg (Group II) compared with other extract groups and control group. But there is significant decrease in uric acid levels ERECV 250 mg (Group II) compared with other extract groups and control group. (Table-5).

The role of liver and kidney functions are important for survival of animals. Theirfunctionality can be measured by serum biochemical analysis, which are crucial in the toxicological evaluation of xenobiotics[7]. Serum liver functiontests provide information about the status of the liver. The liver enzymes (aminotransferases; ALT and AST) describe its cellular integrity, while albumin and total protein levels describe its functionality [8]. AST and ALT are principally produced by the liver cells and any assault to the liver may lead to an increase in the serum level of these enzymes [9]. High levels of liver enzymes are signs of hepatocellular toxicity [10], whereas a decrease may indicate enzyme inhibition [11]. However, ALT is the most sensitive marker of liver damage or toxicity since AST is also found in abundance in kidneys, testes, cardiac and skeletal muscles [7,13].The functionality of the liver was assessed by the serum total protein, bilirubin and albumin. A reduction in serum levels of total proteins, bilirubin and albumin depicts reduced synthetic function, which is evident in liver damage or diseases. An increase in these parameters is usually seen in cancerous conditions, or following high protein diet [12, 14].

There is a significant decrease (P < 0.05)in MDA and SOD levels in ERECV 250mg (Group II) compared with ERECV 500mg (Group III), control group, ERECV 1000mg (Group IV). There is a significant decrease (P < 0.05) in CAT and GSH levels in ERECV 1000mg compared with ERECV

500mg (Group III), ERECV 250mg (Group II), control group (Table 6).

Histopathological study

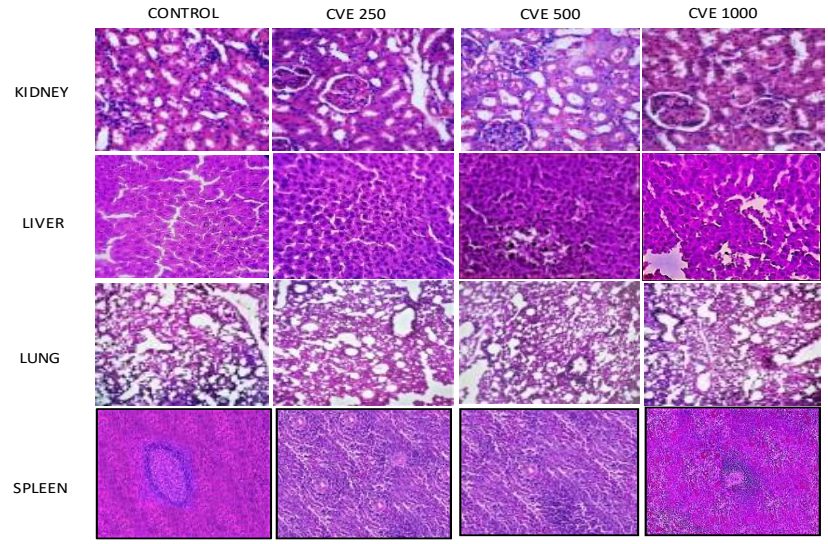

Fig Histopathological Study Slides

Liver

Control (Group I): Normal liver architecturewith no pathological damage was observed. Normal portal triad with bile duct, hepatic artery, portal vein in centri lobular region .No damage and no degeneration and no inflammation in hepatocytes.

ERECV 250 mg (Group II): Normal peribillary lining along with hepatocytes was observed, No fatty degeneration of hepatocytes were observed in lobular and radiating pattern.

ERECV 500 mg (Group III): The liver tissue exhibited preserved structure, cords of normal hepatocytes with no features of acute or chronic damage and portal tracts with no significant inflammation

ERECV 1000 mg (Group IV): No peri portal inflammation with infiltration of inflammatory cells was observed. Hepatocytes are normal with no infiltration of inflammatory cells in peri vascular region.

Kidney

Control (Group I): Normal kidney structure with bowman's capsule, distal and proximal tubules was observed. Cytoplasmic vacuolation in renal tubules with no inflammatory signs

ERECV 250 mg (Group II): The glomerulus showed squamous epithelium, glomerular tuft of capillaries with normal thickness of endothelium in the Bowman's capsule.

ERECV 500 mg (Group III): No decrease in the thickening in the cellular structure of the Bowman's capsule. No abnormalities were noted in the histo architecture of the kidneys.

ERECV 1000 mg (Group IV): There is no degeneration of glomerular capillaries, no thickness in renal tubules, no congestion of the afferent and efferent arterioles with migrating foam cells in the glomerulus tuft of capillaries.

Lung

Control (Group I): Normal lung architecture within septa with normal alveolar walls and vascular bed and no pathological changes in alveoli and thin septal formation

ERECV 250 mg (Group II): Alveolar wall is lined by pseudo stratified ciliated columnar epithelium with goblet cells.

ERECV 500 mg (Group III): No changes in the pulmonary morphological characteristics and no infiltrates inside the alveoli, showed very less macrophages and small cellular infiltrates.

ERECV 1000 mg (Group IV): Alveoli are thin walled spaces lined by simple squamous epithelium. Smaller bronchioles are lined by simple columnar epithelium, no cartilage is present in the wall.

Spleen

Control (Group I): Normal spleen architecture was observed with red pulp and white pulp in parenchyma with normal thickness of spleen capsule.

ERECV 250 mg (Group II): In extract treated group no abnormalities were observed in the architecture of spleen parenchyma.

ERECV 500 mg (Group III): There is no change in the architecture of spleen parenchyma

ERECV 1000 mg (Group IV): Normal thickness of spleen capsule with red and white pulp was observed like in control group.

CONCLUSION

The non toxic nature of ethanolic root extract prepared from ***Coleus vettiveroides*** was confirmed by sub-acute oral toxicity test conducted as per the OECD guidelines. The normal behavior of animals during the observation of twenty eight days suggests the safety and harmless nature of ethanolic root extract even up to 1000 mg/kg body weight of animals.The LD_{50} of the samples for rat greater than 1000 mg/kg in the subacute test, suggesting a potential for safe use.

The obvious lack of morbidity and mortality does not rule out pathology but because rats are not immediately sacrificed (eg, keeping animals for 28 days) after last administration (for

continuous dosing in the case of long-term studies) of plant extract(s) or pharmaceutical agents, the transient rise in the traditional biomarkers of liver (transaminases and other enzymes) are reversible and might have resolved before the animals were sacrificed. Single large dose administration in short-term use produces greater reversible toxic effects on important organs of metabolism and excretion than one-quarter of the same dose used over a prolonged period of medium (4 weeks) and long-term (12–14 weeks) uses. The ability of these plant extracts to maintain adequate hematological parameters, bodyweight and absence of mortality may be responsible for free usage of preparations made from these plants in folkloric medicine.

The obvious lack of morbidity and mortality does not rule out pathology but because rats are not immediately sacrificed (eg, keeping animals for 14 days) after administration (single dose in the case of acute toxicity study) or last administration (for continuous dosing in the case of long-term studies) of plant extract(s) or pharmaceutical agents, the transient rise in the traditional bio-markers of liver (transaminases and other enzymes) and kidney function tests are reversible and might have resolved before the animals were sacrificed. Single large dose administration in short-term use produces greater reversible toxic effects on important organs.

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