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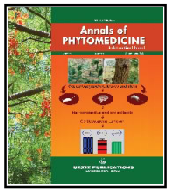

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Complete assessment of methanolic extract of *Euphorbia tirucalli* L. in wistar rat model by subacute oral toxicity

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Abstract

Euphorbia tirucalli L. (Euphorbiaceae), often known as Aveloz, is a native plant of Africa and America that is widely utilized in traditional medicine in both developed and developing countries for a variety of ailments. The goal of this current study was to determine the subacute oral toxicity of *E. tirucalli* methanol extract in wistar rats. In this investigation, sixteen albino rats of both sexes were employed. The Group I rats were received Normal saline for 28 days and served as control, while the Groups II, III, and IV rats were received a single dose of 250, 500, and 1000 mg/kg of E. *tirucalli* extract orally for 28 days, respectively. Cage-side observations like behavior and morphology were monitored daily. Weekly measurements of food consumption, water intake and body weight were taken. Biochemical and hematological parameters were examined. Gross findings were obtained from histopathological examinations of key organs like the liver, kidney, spleen and lungs. There was no mortality at the end of the study, and there were no significant alteration in relative organ and body weights, hematological and biochemical data, or physical abnormalities when compared to the control group. However, the level of malonaldehyde, was significantly reduced compared to control (p<0.05 and p <0.01). Our oral subacute toxicity study results suggested the methanol extract of *E. tirucalli* was safe and it did not produce any significant change in albino rats.

1. Introduction

Herbal medicine, whether as an extract, a pure component, or a derivative, provides limitless possibilities for medication discovery (Hsu *et al*., 2021).The use of a medicinal plant for illness treatment without sufficient scientific evidence to support findings of safety and efficacy can be both harmful and ineffective. Misuse of these medicinal herbs can also result in serious toxicity in humans (Bushra *et al*., 2020). This raise worries about the potential harmful effects of using medicinal plants as medications on a long-term basis. As a result, assessing the toxicological effects of any medicinal plant extract intended for clinical or preclinical use is an crucial aspect of the risk evaluation process.

E. tirucalli (ET) belongs to the Euphorbiaceae family and is a huge unarmed shrub that exudes a milky sap with phylloclade (Brunetti *et al*., 2019). It is an African and American native plant that can be found throughout India. It is a significant medicinal plant with a wide range of pharmacological applications. Whooping cough, dyspepsia, toothache, dropsy, enlargement of spleen, gonorrhea, asthma, leprosy colic, jaundice, and bladder stones, sexual impotence, hemorrhoids, epilepsy, snakebites, and bone fracture are all treated using areal components of this plant (Kgosiemang *et al*., 2020; Kritikar and Basu, 2006). Furthermore, ET latex has pharmacological properties such as analgesic, anti-inflammatory antiarthritic and larvicidal, molluscicidal, antihelminthic, antimicrobial and antifungal, antiviral, immunomodulatory, hepatoprotective and antioxidant, anticancer, anti-HIV actvity. Several compounds have been identified in the stem of *E. tirucalli*, including euphol, -sitosterol, euphorbolhexacosonate,12-deoxy-4-hydroxyphorbol-13-phenylacetate-20-acetate,12,20-dideoxyphorbol-13-isobutyrate, glut-5-en-3-taraxerol,3,3'-di-omethylellagicacid, euphorone, cycloeuphordenol, cyclotiru-canenol, tirucalicine, tri-methylellagicacid, euphorcinol, gallicacids, euphorbins, terpenicalcohol, isoeuphorol, taraxasterol, tirucallol, euphorbin-A (polyphenol), tirucallin-A (7) (tannin), tirucallin-B (11), euphorbin-F (14) (dimers), cycloartenol, ingenol triacetate, 12-deoxy-4β-hydroxyphorbol-13-phenyl acetate-20-acetate, campesterol, stigmasterol, methylene-cycloartenol, taraxerone, euphorginol, palmitic acid, linoleic acid, β-amyrin. The decoction branches are used to treat gastralgia and colic, while the cooked root liquid is used as an emetic in snake bites (Prashanty and Shitals, 2017). An acute toxicity research with ET was previously published

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in experimental animals, in which rats tolerated oral ingestion of ET at a dose of 2500 mg/kg/bw. (Jyothi *et al.*, 2008). However, there has been no report of a subacute oral toxicity investigation with ET in an experimental setting. As a result, the current study examines the subacute oral toxicity of *E.tirucalli* methanol extract (MEET) in albino rats.

2. Materials and Methods

2.1 Chemicals

Tween-80 (National chemicals, Baroda, India), methanol, formaldehyde, ethylenediaminetetra acetic acid (EDTA) (SD Finechem Pvt. Ltd., Boisor, India), and diethyl ether (SD Finechem Pvt. Ltd., Boisor, India) (Standard reagents Pvt. Ltd. Hyderabad, India). The remaining chemicals were all purchased locally and were of analytical grade.

2.2 Animals

Male and female albino rats (*Rattus norvegicus)* were used in this current study. The rats were purchased from Sainath Agencies, an animal house in Hyderabad, Telangana, India. Prior to the studies, the animals were acclimatized to laboratory settings for one week. The rats were kept in a room with a temperature range of 22–240°C, a 12-h light/dark cycle, and humidity levels of 50-5%. The rats were divided into experimental and control groups during acclimation and housed separately in sterilized polypropylene cages with sterile rice husk as bedding. The animals had full access to water and a standard pellet diet. The Institutional Animal Ethical Committee (IAEC), 2018/IAEC/Ph.D./01, approved the experimental procedure, which followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in India.

2.3 Plant material collection and identification

During the month of June 2017, ET was collected in the rural Vizianagram area. Dr. D.Apparao, Botanist, Dr.V.S. Krishna Government Degree College, Visakhapatnam, Andhra Pradesh, India, identified and authenticated the plant, and the voucher specimen was deposited (D.O.B/BH/ET-001/V.S.K.D.C) for future reference. Preparation of the sample freshly obtained aerial parts of the plant were shade dried for two weeks. With a mortar and pestle, it was pounded to a coarse powder. An electric blender was used to grind the powder into a fine powder. One kilogram of fine ET powder was combined with two liters of 90% methanol (Jyothi *et al.*, 2008) for an 80-h extraction at 55^{o}C. After that, a rotary evaporator (Kadavil electromechanical Industries, Kerala, India) was used to evaporate the extract under reduced pressure, and the resultant extract was dissolved in 10% tween.

2.4 Designing experiments

Subacute oral toxicity study was carried out according to the Organization for Economic Cooperation and Development (OECD) guideline 407 for chemical testing and the World Health Organization's guideline (OECD, 2008). Wistar rats of both sexes (160-180 g) were placed into four groups, each with four animals. The vehicle (normal saline) was given to the control group, while the other rats were given logarithmic oral dosages of the plant extract (250, 500, and 1000 mg/kg weight) by gavage. The dose ranges listed above were chosen based on (Jyothi *et al.*, 2008) previous oral acute toxicity research. Several toxic symtoms and observations were examined over the course of 28 days, including body weight, mortality, and food and drink intake. On the 29th day, blood was taken from overnight fasting rats to examine biochemical and hematological markers. After that, the rats were euthanized, and their internal organs, such as the liver, kidney, lungs, and spleen, were removed and weighed to assess relative organ weights, as well as examined for any physical lesions.. For histological investigation, the internal organs were maintained in a 10% buffered formaldehyde solution.

2.5 Toxic symptoms and mortality

After MEET ingestion, various changes in physical appearance, behaviour (sleepy, salivation, lethargy), visual observations of mortality, and any accident or sickness were documented daily, during twenty eight days.

2.6 Parameters of hematology

Cardiac puncture was used to draw blood, which was then placed in EDTA-coated tubes. Hematological markers including haemoglobin (Hb), red blood cells (RBC), total white blood cells (WBCs), differential WBCs (neutrophil, lymphocyte, and monocyte), PCV (packed cell volume), and platelet count were assessed using standard techniques at MIMS, Vizianagaram, Andhra Pradesh, India.

2.7 Analytical biochemistry

The blood was drawn into non-heparinized tubes and centrifuged at 3000 rpm for 10 min to separate the serum. Creatinine, urea, uric acid, total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin and malonaldehyde (MDA), total bilirubin and malonaldehyde (Erba Diagnostic Manheim, Germany).

2.8 Histopathology study

The formalin-fixed liver, lung, kidney, and spleen tissues were processed and embedded in paraffin blocks using standard histological techniques. A rotary microtome was used to cut the slices, which were then stained with Ehlrich'shematoxylin and eosin (H and E). To analyze histopathological alterations generated by MEET, histological sections were inspected and photos were acquired using an Olympus light microscope (Olympus, Japan, magnification 40x) (Vipul *et al.*, 2019).

2.9 Statistical analysis

The results were analyzed using software GraphPad Prism (Graph Pad Software, Inc., La Jolla, CA, USA) version 5.0. One-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests was used for statisticalanalysis. $p<0.05$ was considered significant.

3. Results

3.1 MEET induced changes in the behavior, body weight and relative organ weight

MEET did not cause death in the animals, and their skin, fur, eyes, sleep, salivation, diarrhoea, and behavior showed no signs of poisoning. At any stage during the study, the treated rats' food and water consumption were not statistically different from the controls. At the end of days 7, 14, 21, and 28, the effects of MEET on body weight were measured. Twenty-eight days of daily MEET adminisrtation did not show in a significant change in body weight in rats, when compared to the control group, (Figure 1). The relative organ weights were not significantly different from the control group at necropsy (Table 1).

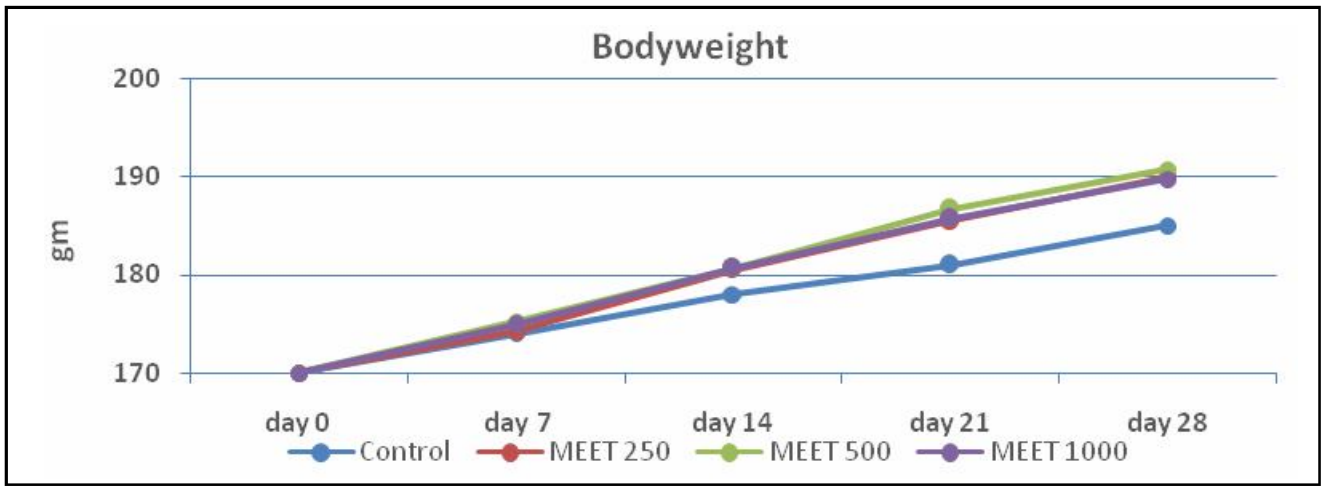


Figure 1: Methanol extract of *E. tirucalli* (MEET)-induced changes in body weight.

Table 1: Methanol extract of *E. tirucalli* (MEET)-induced changes relative organ weight

Parameters (gm)	Control	MEET250	MEET500	MEET1000
Liver	3.74 ± 0.83	3.89 ± 0.71	3.89 ± 0.55	4.0 ± 0.31
Kidney	0.92 ± 0.15	0.88 ± 0.09	0.97 ± 0.17	0.89 ± 0.08
Lung	1.22 ± 0.33	1.29 ± 0.14	1.33 ± 0.41	1.27 ± 0.42
Spleen	0.36 ± 0.01	0.33 ± 0.15	0.33 ± 0.08	0.36 ± 0.03

Values indicate the mean ± S.D. (n = 4). Relative organ weight was calculated as (organ weight (g)/ body weight of animal on sacriûce day (g)) × 100.

Table 2: Methanol extract of *E. tirucalli* (MEET)-induced changes in hematological parameter

Parameters (n=4)	Control	MEET250	MEET500	MEET1000
HB (g/dl)	12.12 ± 0.62	12.32 ± 0.39	12.50 ± 1.49	12.73 ± 0.85
PCV (%)	34.75 ± 1.89	35.5 ± 0.58	36.50 ± 5.00	35.50 ± 2.52
RBC ($10^{6/cumm}$)	8.30 ± 0.47	8.22 ± 0.20	8.65 ± 0.61	8.7 ± 0.52
Platelets ($10^{5/cumm}$)	7.75 ± 0.64	7.4 ± 0.16	8.27 ± 0.57	7.72 ± 0.35
WBC ($10^{3/ cumm}$)	9.15 ± 0.5	10.1 ± 0.84	9.25 ± 0.59	9.55 ± 0.55
Neutrophils (%)	67 ± 5.72	57 ± 6.78	56.25 ± 6.65	65.5 ± 5.51
Lymphocyte (%)	29 ± 4.55	40.25 ± 5.85	40.50 ± 11.73	31.5 ± 4.43
Monocytes (%)	2 ± 0.82	2.25 ± 0.95	1.75 ± 0.95	1.5 ± 0.57
Eosinophils (%)	2 ± 0.81	1 ± 0.81	1.0 ± 0.81	1.5 ± 0.57

Values indicate the mean ± S.D. HG-hemoglobin, RBC-red blood cell, WBC-white blood cell, PCV-packed cell volume.

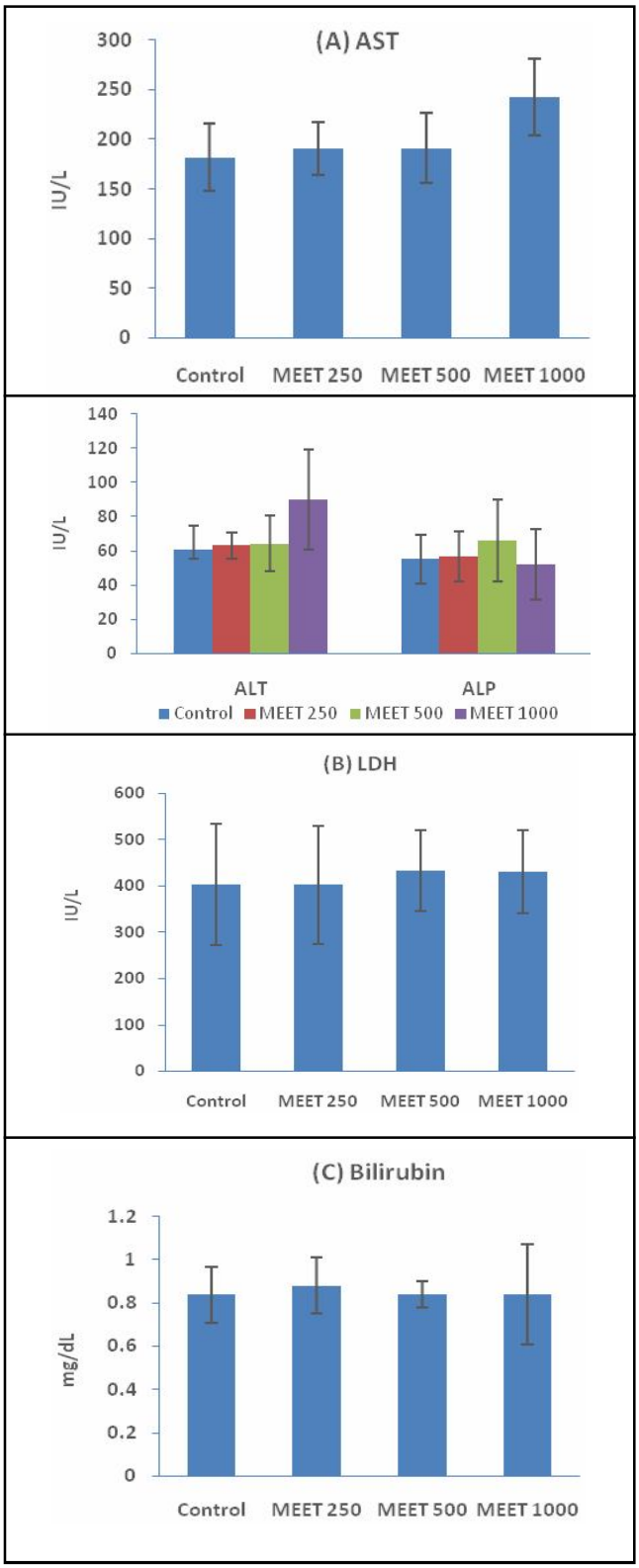
3.2 Hematological parameters

The effect of repeated oral ingestion of MEET for twenty-eight days on hematological markers is shown in Table 2. The rats administered MEET for 28 days , there was no significant difference in hemoglobin, total RBCs, WBCs, neutrophils, lymphocytes, monocytes, or platelet count, compared to control rats.

3.3 Biochemical analysis

The effects of repeated oral MEET delivery on liver toxicity indicators are discussed in this paper (Figure 2).There was no significant alteration in serum bilirubin or hepatotoxicity marker enzymes like AST, ALT, ALP, and LDH (Figures 2 A-E). MEET treatment had no effect on serum protein levels (Figure 2F). When compared to control

rats, MEET treatment for 28 days significantly (p<0.01) reduced serum MDA levels (Figure 2G). Kidney function measures including as creatinine, urea, uric acid, and glucose exhibit no significant alterations between control and MEET groups (Table 3).



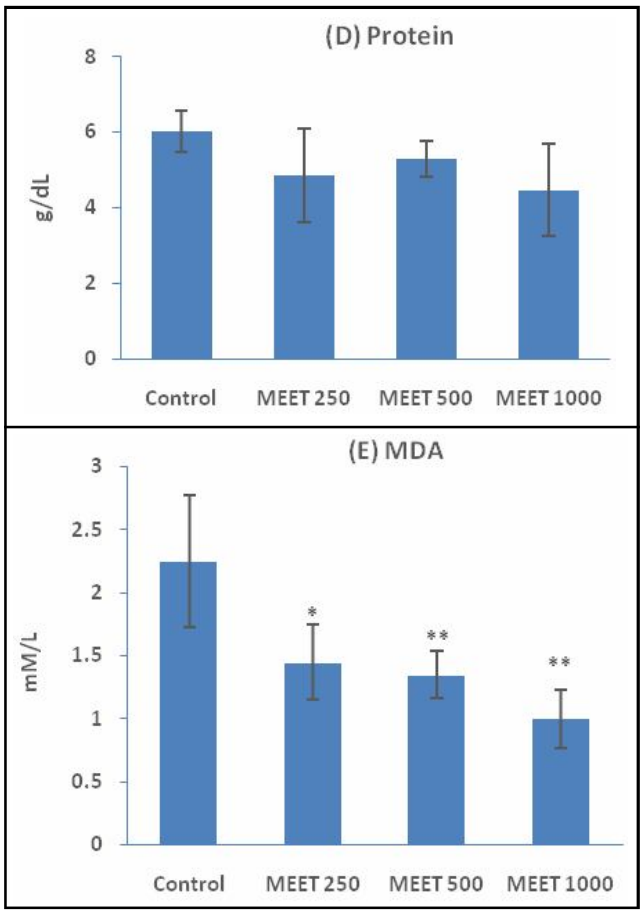


Figure 2: Methanol extract of *E. tirucalli* (MEET)-induced changes biochemical markers of toxicity. Values are expressed as the mean ± S.D. (n = 4). AST- aspartate aminotransferase, ALT- alanine amino transferase; ALP - alkaline phosphatase, LDH - lactate dehydrogenase, MDA- malondialdehyde. *p<0.05; **p<0.01 vs control.

Table 3: Methanol extract of *E. tirucalli* (MEET)-induced changes in nephrotoxic marker

Parameters (mg/dl)	Control	MEET 250	MEET 500	MEET 1000
Urea	27.18 ± 5.14	28.12 ± 8.2	30 ± 7.56	34.06 ± 5.62
Uric acid	3.26 ± 0.52	4.66 ± 2.17	4.50 ± 1.68	3.41 ± 0.76
Creatinine	0.91 ± 0.16	0.87 ± 0.08	0.74 ± 0.05	0.91 ± 0.16
Glucose	79. 96 ± 14.46	87.23 ± 36.08	71.92 ± 13.30	70.90 ± 5.98

values indicate mean ± S.D.

3.4 Histopathology

The architecture of the control liver, kidney, lung, and spleen is normal. MEET treatment for 28 days had no effect on the liver architecture, and the liver tissue showed intact sinusoids and central veins with no inflammatory alterations. The architecture of the kidney tissue was normal, with complete glomeruli and tubules. The epithelium, smooth muscles, and bronchioles in the lung tissue are all intact. The red pulp in the spleen tissue is normal, with no negative alterations (Figure 3).

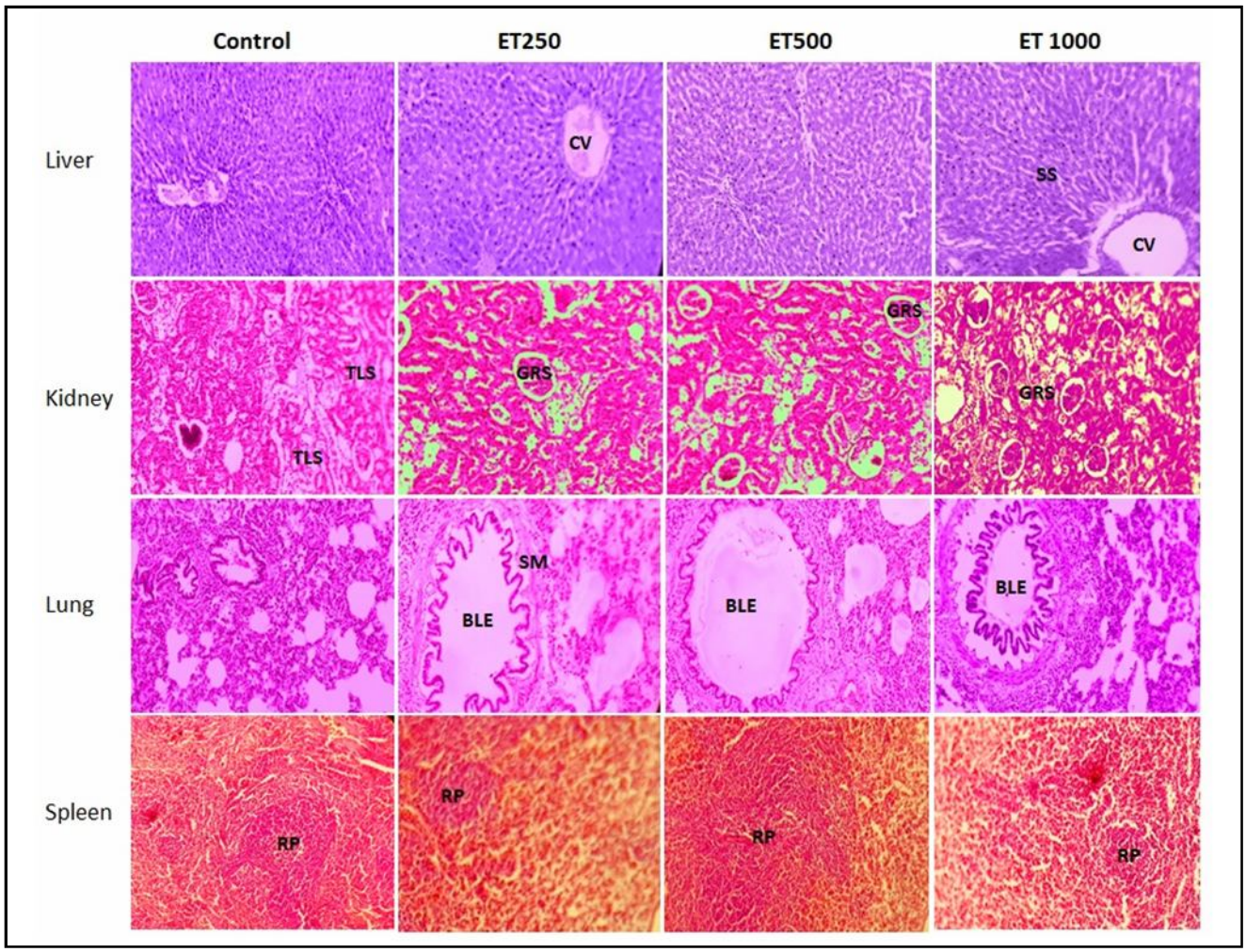


Figure 3: Methanol extract of *E. tirucalli* (MEET)-induced changes in the histology of liver, kidney, lung and spleen. CV-central vein, SS-sinusoidal space, TLS-renal tubules, GRS-glomerulus, SM-smooth muscle, BLE-bronchiole, RP-red pulp.

4. Discussion

People in underdeveloped nations have increased their usage of herbal medications because they believe these products are natural and reasonably safe (Alam, 2019).There is also a misconception that natural products and herbal medicines harm the human body less than manufactured pharmaceuticals. Even though, herbal remedies are thought to be harmless, they could contain impurities, a mix of hazardous substances, pollutants, microorganisms, and heavy metals (Ezhilarasan, 2019). As a result, toxicological studies are now required to assess the safety of herbal medications in development. Furthermore, published data on adverse reactions and toxicity is the most important source for determining the regulatory safety of natural herbal products. Examining the hazardous qualities of medical goods (extract, isolated compounds, and formulation) is a common first step in pharmacological activity screening of natural products. As a result, the current study was carried out to determine the subacute oral toxicity. Changes in body and organ weight are crucial indicators of an animal's physiopathological condition. The metabolic process triggered by toxicants affects important organs including the liver, kidney, spleen,heart and lungs (Harshad *et al.*, 2020 ; Abid and Mahmood, 2019). The tolerance of rats for MEET may explain why subacute oral treatment of MEET for twenty-eight days did not reveal in any significant alteration in total body weight gain or relative organ weight in rats. It is also crucial to track food and water consumption during a study of a product's safety for medicinal purposes, because correct supplementation is essential for the animal's physiological well-being and a better response to the test chemical under investigation. The oral administration of MEET, even at a high dose of 1000 mg, had no effect on food intake or water consumption.

Plant extract's blood-related functions can be determined using hematological measures. The haemopoietic system is one of the most precise targets for hazardous compounds in both human and animals, as well as an crucial indicator of physiological and pathological condition. After 28 days, there was no significant changes in hematological indicators like hemoglobin, red blood cells, white blood cells, platelets, and total leucocyte count, between MEET treated as well as control group rats (Jaimin *et al.*, 2019).

The evaluation of serum biochemical analysis is critical in identifying the possible effects of extract on renal and hepatic functions.

Hepatotoxicity indicators such as AST, ALT, ALP, LDH, protein, and bilirubin are extremely sensitive (Jyothilekshmi *et al*., 2020) . Serum urea, uric acid, and creatinine are all good indices of renal function, and increases in the renal index are frequently associated with visible damage to functional nephrons (Susmita *et al.*, 2020). Hepatotoxicity marker enzymes and renal parameters such as urea, uric acid, creatinine and glucose did not exhibit significant alterations between control and MEET-treated rats. Malondialdehyde is produced when polyunsaturated lipids are degraded by reactive oxygen species (ROS) (Roopam and Jessy, 2021) . It is one of the most widely used lipid peroxidation indicators (Mohamed and Said Ahmed, 2019; Smita *et al*., 2020). It is worth noting that MEET treatment resulted with a dose-dependent reduction in MDA concentrations in the blood.

In a recent acute oral toxicity investigation, a single dosage of ET aqueous extract generated a considerable drop in MDA levels in experimental animals (Jyothi *et al.*, 2008) and our current findings are consistent with the above data. MEET's antioxidant potential may also be responsible for its MDA-decreasing properties. The histopathological evaluation corroborated our serum biochemical data.

5. Conclusion

The current findings reveal that oral ingestion of MEET for twenty-eight days did not cause any substantial toxicity in wistar rats. In MEET-treated rats, no death or other symptoms of toxicity were seen. Histopathology examinations of critical organs including the liver, kidney, spleen and lung, found no changes in histology. MEET was found to be safer and non-toxic, and it can be utilized in known levels for pharmacological and therapeutic objectives, according to our findings. However, more long-term toxicity research is needed before it can be considered a viable therapeutic option.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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