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Acute and Subacute Toxicity Assessment of Ethyl Acetate Extracts from Aerial Parts of *Clerodendrum thomsoniae* Balf.f in Rodents

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Abstract: *Clerodendrum* is a genus of about 500 species belongs to the family Lamiaceae. Several species of this genus have been proved for the treatment of various diseases. *Clerodendrum thomsoniae* Balf.f were selected in this study; according to the literature available, there is no systematic toxicity studies for this plant were described. The current study was therefore carried out to evaluate the acute and sub-acute toxicity in mice and rats. The acute oral toxicity study was performed in mice following OECD guidelines 425, and the sub-acute toxicity was performed in male and female rats following OECD guidelines 407. The results showed that mice given a single dose of up to 2000 mg/kg orally did not show any toxicity signs or mortality. In the sub-acute toxicity analysis in rats, 3 specific daily doses of 150, 300, and 600 mg/kg for 28 days did not induce any major changes to the hematological and biochemical parameters. Histopathological studies revealed normal architecture that did not indicate any morphological disturbances. In our study, no deaths or any signs of toxicity were found in acute and subacute toxicity studies after oral administration according to OECD guidelines, which concluded that ethyl acetate extract of *Clerodendrum thomsoniae* Balf (EACT) could use for in vivo biological activity studies in laboratory animals to explore its various medicinal activity before study in human subjects.

Keywords: *Clerodendrum thomsoniae* Balf; mice; acute toxicity; sub-acute toxicity; rats.

1. Introduction

In the most recent decade, medicinal plants characterize a significant and rich source of novel drugs [1]. A massive number of the world's residents depend on medicinal plants as a substitute and complementary remedy for various illnesses. Lacks of experimental reports on their safety make it essential to perform toxicological analysis [2]. Herbal preparations' beneficial uses have been limited because of a shortage of healthy-clear chemical classification, dose, and toxicity data to assess plant safety concerns [3]. The plant-based products consist of secondary metabolites that undergo preclinical studies, including toxicological investigation followed by experimental investigation in laboratory animals, and the successful molecules will become novel molecules with a definite therapeutic activity [4]. The medicinal plants contain bioactive components that act as protective mechanisms against numerous ailments, but the plant itself might be poisonous [5]. Obviously, all plant products should not be incorrectly measured as safe or harmless as they are naturally obtained. Some secondary metabolites in plants may be harmless to the plant, but they might be poisonous or even can be

lethal when used in humans. The information from toxicity studies on secondary metabolites or other plant derivatives must be obtained to document their safety for humans' usage, which is important in drug discovery courses [6]. In addition, certain chemical ingredients existing as naturally harmless may show poisonous properties at a definite dose or sustained contact [7]. The chief principle of toxicity investigation is to study and document adverse effects of the test substance that are proposed to be used or consumed by humans. The progress of toxicity from herbal medicine could be extrinsic, intrinsic, or other additional causative aspects [8]. Herb-herb and herb-drug interactions play vital responsibility in activating adverse reactions [9].

Clerodendrum thomsoniae Balf .f. is a twined, rambling, wine-like shrub native to the tropical part of West Africa, and the collective name is Bleeding-heart or Bag-flower [10, 11]. They have beautiful white flowers and are available in a different part of the world because of ornamental demand. These species are not extensively studied for pharmacological studies, even though many plants listed under genus *Clerodendrum* are well studied for various pharmacological studies. No clinical evidence or studies for the oral acute and sub-acute toxicity analysis for this plant are available in the literature. Therefore, acute and sub-acute toxicity studies were carried out by the OCED guidelines to determine and establish the safety for its use in clinical practice.

2. Materials and Methods

2.1. Plant material and preparation of extracts.

The plant materials were identified and authenticated by Dr. A.K. Pradeep., Assistant Professor -Department of Botany, Calicut University (Calicut, India). Voucher specimens were deposited in the same department herbarium as specimen No. 148249. The aerial parts of *C. thomsoniae* were selected for the study. The dried plant sample was extracted with the help of the Soxhlet apparatus using ethyl acetate as solvent.

2.2. Experimental design.

Adult healthy young female Swiss albino mice, nulliparous, non-pregnant and weighing 25-30, and Wistar rats of either sex (10-12 weeks old, 125-175 g) were used for the study. The study was conducted according to CPCSEA and IAEA guidelines. The protocol was approved by the ethical committee (Reference number IAEC/MUHAMMED ASHRAF VK/Ph.D/AU/ 1661130022/KMCP/91/2020**).**

2.3. Acute toxicity.

The oral acute toxicity study of the ethyl acetate extract of Clerodendrum thomsoniae Balf (EACT) was conducted following the Organization for Economic Development (OECD) guideline 425 using the ‘‘Up-and-Down” method of testing in mice and rats at single doses of 175, 500, and 2000 mg/kg [12]. For each dose level in the study, five female mice were used. An animal with the equivalent volume of extract dissolved in distilled water was picked, weighed, and dosed at a time. The extract was orally administered through a gastric feeding tube. After dosing, each animal was observed for regurgitation signs for the first 5 min and kept in a metallic cage. Each animal was then monitored every 15 minutes in the first 4 hours after dosing, every 30 minutes for 6 hours, and every day for 48 h for behavioral symptoms of toxicity (skin, hair, lips, mucous membranes, and gastrointestinal, circulatory, autonomic and

central nervous systems, muscle function, seizures, tremors, salivation, vomiting, lethargy or sleep) as per the OECD requirements (2001). The animals were monitored for a total of 14 days for the possible long-term lethal result. The animals' body weights were measured on Days 1, 7, and 14.

2.4. Sub-acute toxicity study.

Wistar rats of both sexes were randomly allocated to four classes (n=12 / group: six males and six females). The experiment was carried out in compliance with OECD recommendations 407 [13]. Group I-III provided 150, 300, and 600 mg/kg of the extract, while category IV obtained only distilled water (5 ml/kg). The rats were fed by oral gavage, utilizing a 28-day flat, ball-tipped stainless steel feeding stick. The female rats received daily feed and water intake. The animals' weights were assessed regularly. The rats were weighed and sacrificed on day 29. Blood samples were separated for biochemical and hematological analysis using a cardiac puncture. The heart, liver, lungs, kidneys, brain, and spleens have been extracted, weighed, and histological analysis was performed.

2.5. Body weight, food, and water consumption.

The rats' body weight was recorded in all groups before the doses were given, further body weight was taken weekly during the treatment, and finally on the day of sacrifice. Food and water intakes were reported daily. Before they supplied each group, the amount of food and water consumed was measured, their residue was calculated the next day to obtain the difference, which was recorded as daily food (g/rat/day) and water use (ml/rat/day).

Blood (1.5 ml) was obtained after 14 days and 28 days from the retro-orbital area of the rats to calculate hematological (EDTA-coated tubes) and biochemical (dry tubes) parameters.

2.6. Hematological analysis.

For hematological tests, the blood samples obtained in heparinized tubes were used. The following parameters were tested by an automatic analyzer (KX-21-Hematology-analyzer, Sysmex Company, USA): red blood cell count (RBC), white blood cell count (WBC), neutrophils (NP), lymphocytes (LC), monocytes (MC), eosinophils (EP), hemoglobin (Hb), platelets (PL), and packed cell volume (PCV).

2.7. Biochemical analysis.

Dry tubes carrying blood collected for investigation were centrifuged at 3000 rpm at 25°C for 15 min to get the serum, which was stored at -20 °C for the analysis of biochemical parameters.

2.8. Histological analysis.

The fixed tissues were dehydrated in an ascending alcohol series, cleared in xylene, and embedded at 60 ^{0}C in paraffin wax melting. Serial sections (5 mm thick) obtained by microtome cutting of the embedded tissue were fixed on 3- aminopropyl triethsilane-coated slides and dried at 37 ^{0}C for 24h [14]. The sections on the slides were deparaffinized with xylene and were hydrated in a descending alcohol series. They were then stained with Mayer’s

hematoxylin and eosin dyes, dried, and mounted for histopathological examination on a light microscope (20X).

2.9. Statistical analysis.

The study results were expressed as mean ±SEM (the mean, standard error). The results were interpreted using version 6 of the Graph Pad Prism software. The comparison was made in all groups using a one-way variance analysis (ANOVA) followed by Dunnett's post hoc test. The p values < 0.05 were noted as significant.

3. Results and Discussion

3.1. Acute toxicological evaluation.

The acute toxicity test using the Up and Down method at an oral dose limit of 175, 500, and 2000 mg/kg of the *Clerodendrum thomsoniae* Balf (EACT) ethyl acetate extract did not trigger death in mice. The whole course of the short and long-term observation period was not observed with any lethal effects. During the 14-day study period, no signs of poisonousness were detected in the animals. Therefore, the extract may be harmless at these doses, and the oral LD_{50} is considered to be more than 2000 mg/kg.

3.2. Sub-acute toxicity.

During 28 days of treatment, all of the treatment rats of both sexes at the 150, 300, and 600 mg/kg doses survived. Compared with the control, no observable toxicity signs were noticed in the rats treated with the extract.

3.3. Effect of EACT on body weight, food intake, and water consumption in rats.

During the study, no major changes in the animal's body weight were observed (Fig . 1). The food and water intake were also not affected after 28 days of oral administering EACT. The extract indicated that there were no significant appetite changes and no adverse impact on animal growth. In comparison with control, no significant variations were found in rats' physiological and metabolic activity. The effect of EACT on food and water intake were given in table 1.

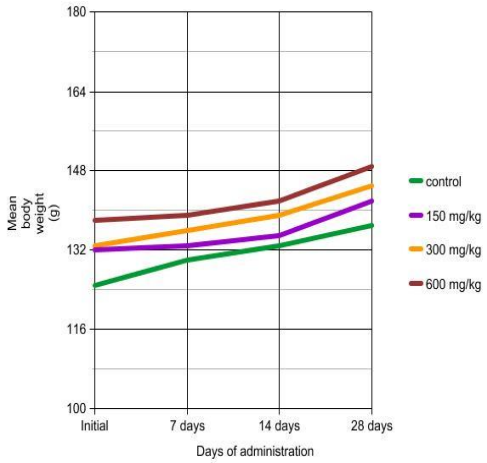

Figure 1. Bodyweight assessment in sub-acute toxicity study of treated rats.

Table 1. EACT effect on rat food intake and water consumption during 28 days of treatment

Treatment	Average food intake (g/day/rat)	Average water intake (ml/day/rat)
Control	16.27±1.23	19.34±1.34
150 mg/kg	16.74±1.73	18.37±1.77
300 mg/kg	16.74±1.94	17.74±1.82
600 mg/kg	15.73±1.98	19.36±1.79

Values are given in Mean ± SEM, n=6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

3.4. Impact of EACT on blood parameters.

The 14th and 28th-day hematological analysis results are given in Tables 2 and 3. No significant variation in hematological parameters were observed.

Table 2. Blood parameters of rat treated in sub-acute toxicity with different dose levels of EACT after 14th day.

Parameters	Normal ranges	Control	150 mg/kg	300 mg/kg	600 mg/kg
Hemoglobin (%)	10.2-16.6	14.43±0.34	15.13±1.34	12.14±1.22	13.43±1.36
Total RBC (10^6/µL)	5-10	5.12±1.64	5.35±0.77	8.68±1.76	8.77±1.67
WBC (10^3/µL)	6-15	9.34±0.36	14.67±0.78	13.87±1.74	12.77±1.37
Platelets(10^3/L)	782-985	839±1.45	859±0.78	753±1.86	799±0.79
PCV (%)	39-49	42.34±1.32	46.78±0.47	39.88±1.68	46.78±1.89
LC (%)	55-95	63.12±0.94	76.35±1.53	78.23±1.46	92.45±1.47
NP (%)	10-40	32.32±1.96	30.23±0.35	33.44±0.46	37.25±1.66
MC (%)	1-4	3.23±1.38	3.55±0.45	2.56±0.55	3.47±1.24
EP (%)	0-4	2.11±0.72	2.56±1.58	3.56±1.76	2.56±0.58

All values are expressed in Mean ± SEM, n=6 animals/group, $p< 0.05$ (ANOVA/ Dunnett's test).

Table 3. Blood parameters of rat treated with different dose levels of EACT in sub-acute toxicity after 28th day.

Parameters	Normal ranges	Control	150 mg/kg	300 mg/kg	600 mg/kg
Hemoglobin (%)	10.2-16.6	15.67±1.24	13.34±0.45	15.46±0.35	11.54±0.46
Total RBC (10^6/µL)	5-10	7.62±0.26	9.46±1.56	7.56±1.57	8.64±0.56
WBC (10^3/µL)	6-15	10.34±1.76	13.53±1.64	11.53±0.35	13.56±1.48
Platelets(10^3/L)	782-985	919±0.26	848±0.78	799±0.78	919±0.57
PCV (%)	39-49	47.14±0.29	47.77±1.57	42.75±0.37	48.35±0.67
LC (%)	55-95	79.76±1.98	75.56±1.66	89.56±1.73	86.72±1.77
NP (%)	10-40	39.32±0.26	19.55±1.74	30.56±1.36	23.56±0.63
MC (%)	1-4	3.23±1.22	2.46±1.66	3.63±1.67	2.67±1.67
EP (%)	0-4	2.81±0.29	3.47±0.67	2.77±0.67	3.56±1.68

All values are expressed in Mean ± SEM, n=6 animals/group, $p< 0.05$ (ANOVA/ Dunnett's test).

3.5. Effect of EACT on biochemical parameters.

The state of the kidney and liver function can be assessed using a biochemical assessment without sacrificing the animals, so it is very important to measure any new substance's toxicity. The parameters such as SGOT, SGPT, and ALP are very important for a liver function test. Serum urea and creatinine are the most important parameters for the assessment of kidney function. Any variations in the above parameter after the intake of any test compound from the normal ranges indicate toxicity of that compound in animals. The results showed no significant variations in SGPT, SGPT, ALP, urea, and créatinine at each trial dose, in contrast, to control in animals' biochemical parameters after 14th & 28th days (Tables 4 and 5).

Table 4. Biochemical estimation of rats' blood serum at different dose levels after 14 days of treatment in the sub-acute toxicity study.

Parameters	Normal ranges	Control	150 mg/kg	300 mg/kg	600 mg/kg
SGOT (U/L)	54-298	176.12±19.45	144.65±14.45	236.54±17.73	211.45±19.56
SGPT (U/L)	17-77	56.23±1.86	53.63±3.55	42.42±3.61	59.44±1.57
ALP (U/L)	64-128	102.35±2.76	129.45±1.66	73.46±1.56	118.55±2.45

Parameters	Normal ranges	Control	150 mg/kg	300 mg/kg	600 mg/kg
Creatinine (mg/dL)	0.2-0.9	0.25±0.12	0.54±0.35	0.64±0.19	0.72±0.35
Urea (U/L)	35-96	44.39±1.39	55.43±1.35	67.25±4.45	65.34±3.14
BUN (mg/dl)	8-33	22.95±1.94	31.26±0.23	25.14±1.34	25.24±1.55

All values are expressed in Mean ± SEM, n=6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

Table 5. Biochemical estimation of rats' blood serum at different dose levels after 28 days of treatment in the sub-acute toxicity study.

Parameters	Normal ranges	Control	150 mg/kg	300 mg/kg	600 mg/kg
SGOT (U/L)	54-298	176.12±19.45	99.34±14.25	163.34±13.34	211.34±13.35
SGPT (U/L)	17-77	56.23±1.86	77.35±1.35	55.35±2.25	70.42±1.19
ALP (U/L)	64-128	102.35±2.76	99.32±1.45	119.34±3.44	125.84±2.46
Creatinine (mg/dL)	0.2-0.9	0.25±0.12	0.53±0.24	0.73±0.35	0.45±0.26
Urea (U/L)	35-96	44.39±1.39	80.35±1.24	89.43±2.31	76.34±2.35
BUN (mg/dl)	8-33	22.95±1.94	21.34±0.56	19.44±1.44	23.44±1.45

All values are expressed in Mean ± SEM, n=6 animals/group, $p < 0.05$ (ANOVA/ Dunnett's test).

3.6. Histopathology.

In the histopathological study, there are no changes at the cellular level compared to the control were observed after 14 and 28 days (Fig 1 and 2, respectively).

The main focus of any remedial plant's safety assessment is to recognize the nature and depth of side effects and ensure the level of exposure at which any toxic effects are detected [15]. Prior to conducting a pharmacological activity, secondary metabolites from any plant's acute and sub-acute toxicity studies are compulsory according to the standard protocol like OECD guidelines [16]. Toxicity studies will help the researcher determine the dose conformation of the test sample, which is considered one of the important steps in the drug discovery journey [17]. The results of the acute toxicity study in our study specify that the aerial parts of EACT administered by oral route to mice at 175, 500, and 2000 mg/kg using the up and down technique of acute toxicity testing did not give rise to any indication of toxicity and mortality in the animals used. According to the OECD criteria under its Globally Harmonized Classification System (GHS), any substances with LD50 greater than 2000 mg/kg are considered safe. Final and regular clinical monitoring in repeated dose analysis has foremost significance [18]. Due to the increasing demand for herbal drugs or plant-based medicines, several researchers focus on toxicity studies as an initial step to figure out its safety and confirm the safe, effective dose. According to the literature review, many herbal formulations, herbal extracts, isolated compounds from plants and, synthetic compounds derived from plants have been tested for toxicity evaluation. Some of the examples are *Cocos Nucifera* [19], *Haloxylon Scoparium* Pomel [20], *Syzygium Guineense* [21], Oxyclozanide [22], *Aegialitis Rotundifolia* Roxb [23], *Fagaropsis Hildebrandtii* [24], *Withania Frutescens* [25], Polyherbal Preparation containing six plant extracts *Camellia sinesis, Ocimum sanctum, Withania somnifera, Centella asiatica, Bacopa monnieri and Hypericum perforatum* [26], *Geophila Obvallata* [27], Ayurvedic medicine Dhatryadi Ghrita [28], Triplotaxis Stellulifera [29], *Hibiscus Sabdariffa* Calyces [30]. A polyherbal formulation contains the roots of *Withania somnifera* (Ashwagandha), *Hemidesmus indicus*, the fruit of *Aegle marmelos* (Bael), the pericarp of *Emblica officinalis* (Amla) and fresh juice of aerial parts of *Ocimum sanctum* (Tulsi) [31], *Solanum Torvum* [32], and *Achillea Wilhelmsii* [33].

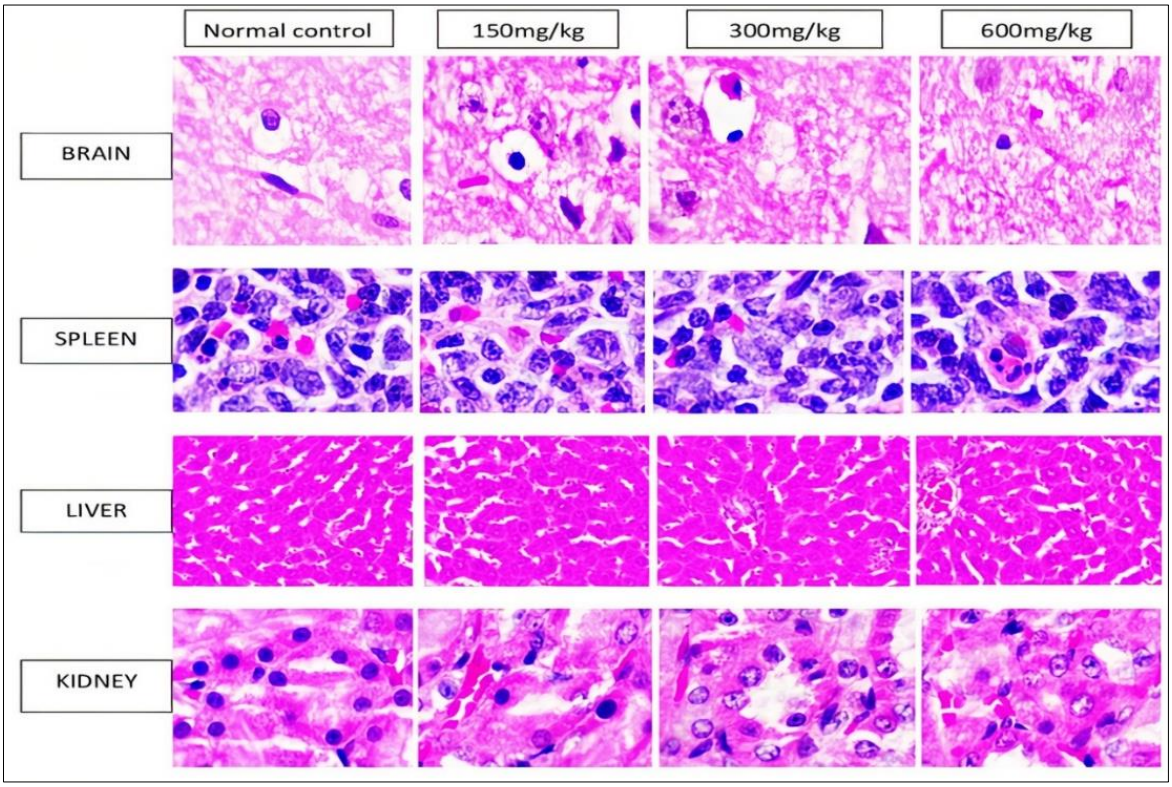

Figure 2. Histopathology (20X) of rat tissues of treated and control groups after 14th days.

The histopathological slides also confirmed that, in 28 days, the EACT treated group showed no toxicity up to a dose of 600 mg/kg.

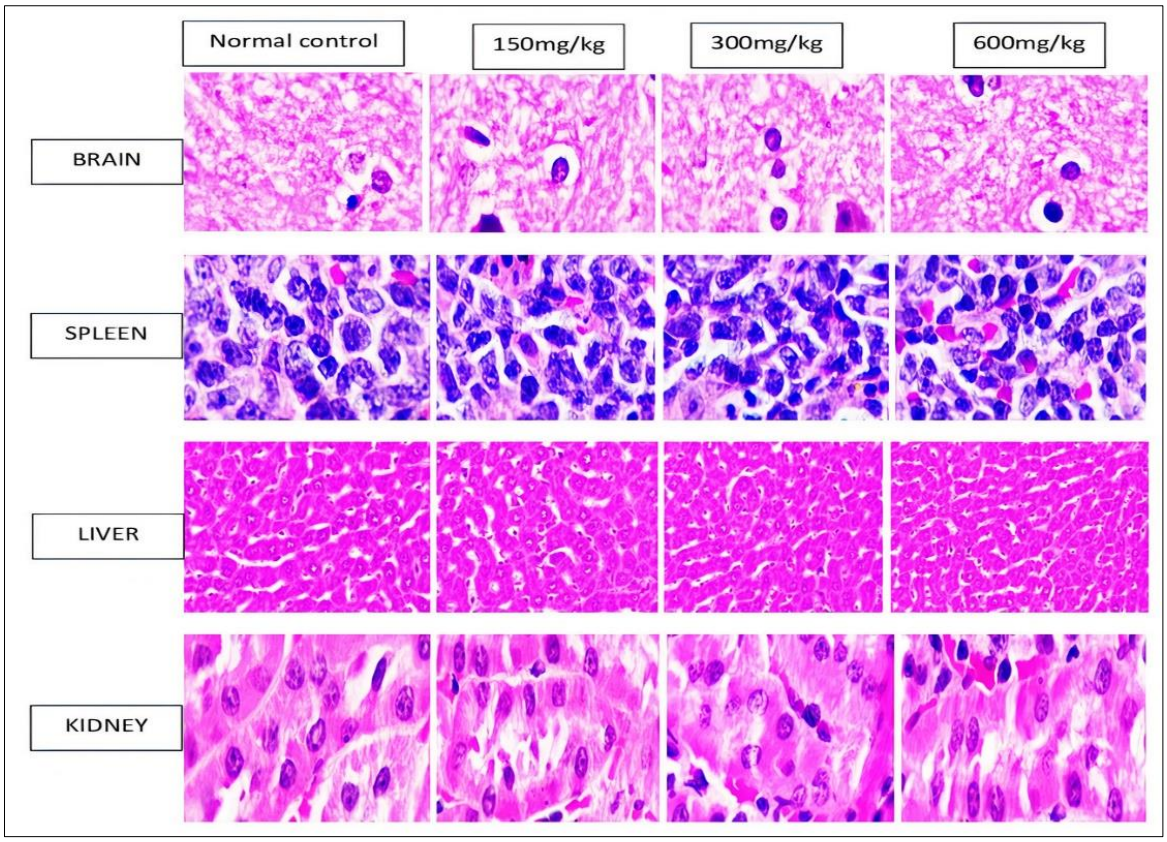

Figure 3. Histopathology (20X) of rat tissues of treated and control groups after 28th days.

The 28 days long sub-acute toxicity study found no harm or any mortality in any treated animals. During the study period, food and water intake were not affected, which indicates that

the EACT did not interfere with the appetite and poisonous events on animal growth. In contrast, to control, no significant variations were detected in rats' physiological and metabolic activity.

Changes in hematological parameters are considered either at the pathological or physiological level as an indication of the test compound's toxic properties in rat blood. Suppose the test sample shows toxicity in the body. In that case, blood components such as hemoglobin, white blood cells, red blood cells, and platelets will be disturbed. Any changes in blood components will affect the body's immune system and the organs' functions directly [34]. Our test results revealed that EACT produced no significant changes in hematological components when compared with control in sub-acute studies (Tables 2 & 3). The liver and kidney are the greatest vital organs responsible for metabolism and excretion, respectively [35]. To assess any novel compound's toxicity, knowing the status of these two vital organs, which can be tested by biochemical evaluation without killing rats, is crucial.

In the histopathological examination, we observed that the organs showed no major variations at the cellular level, similar to control in all treated groups after 14 and 28days (Figures 2 and 3, respectively).

4. Conclusions

The oral LD_{50} of the ethyl acetate extract of aerial parts of *Clerodendrum thomsoniae* Balf (EACT) is greater than 2000 mg/kg and is generally supposed to be safe. This study intimates that the EACT can further evaluate pharmacological activities on the above-mentioned doses of the extract.

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Conflicts of Interest

The authors declare no conflict of interest.

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