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Evaluation of acute and subacute toxicity induced by methanol extract of *Terminalia citrina* leaves in Sprague Dawley rats

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ABSTRACT

Objective: To evaluate acute and subacute toxicity of methanol extract of *Terminalia citrina* leaves (family: Combretaceae) in Sprague Dawley rats.
Methods: The acute toxicity studies were conducted where the limit test dose of 3 200 mg/kg body weight used. Observations were made and recorded systemically on 1, 2, 4, 24 and 48 h after dose administration for behavior, breathing, cutaneous effects, sensory nervous system response or gastrointestinal effects. For the subacute toxicity, four groups of 10 female rats were received; distilled water (control), 250, 500 and 1 000 mg/kg of extracts respectively every 24 h orally for 28 days.
Results: No significant variation in the body and organ weights between the control and the treated group was observed after 28 days of treatment. Haematological analysis and biochemical parameters revealed no toxic effects of the extract. Pathologically, neither gross abnormalities nor histopathological changes were observed. No mortality was recorded in 28 days.
Conclusions: It was safer and non toxic to rats even at higher doses and therefore could be well considered for further investigation for its medicinal and therapeutic efficacy.

1. Introduction

The usage of medicinal plants has a great importance from ancient times[1], because plants produce a wide range of drugs to widen the therapeutic arsenal[2,3]. However, during the past few decades, traditional system of medicine has drawn tremendous attention for *in vivo* studies[4] and for this reason, more researches are carried out in order to determine the toxicity of medicinal plants and their products. Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This mechanism of action may vary depending on the cell membrane and chemical properties of the toxicants. It may occur within the cell membrane or on the cell surface or tissue beneath as well as at the extracellular matrix. In most of the cases vital organs such as liver and kidney are affected by the toxicants[5].

Terminalia citrina [(*T. citrina*) (Bengali name: Haritaki; family: Combretaceae)] is a deciduous tree wide spread throughout the forest of Gazipur, Tangail, Sylhet, Chittagong, Rangamati and Chittagong hill tracts of Bangladesh. It is an important medicinal plant having various ethno-pharmacological uses. Different parts of the plant are used for various ailments. The fruit is used in long-term fever, loss of appetite and as sexual stimulant in Bangladesh [6]; diarrhea, helminthes and other digestive disorders in Iran [7]. Its bark is diuretic and cardio tonic[8]. Seed is used in stomach aches and intestinal disease[9]. The plant is also used in asthma, diarrhea, boils, burns, constipation, migraine, dental disease, haemoptysis, dizziness, bleeding hemorrhoids, eye disease, gastric hyperacidity, anemia, arthritis, hoarse voice, dysentery, pyrexia, infections, traumatic cuts, cardiac diseases, cough, hepatomegaly, urolithiasis and for life longevity in Myanmar [10]. A detailed literature survey revealed that seed of plant was reported to possess antioxidant properties[11] and five tannins identified as corilagin (1) (3), punicalagin (2) (4), 1,3,6-tri-O-galloyl-β-D-glucopyranose (3) (5), chebulagic acid (4) (6), and 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose (5) (7) was isolated from methanol extract of fruit[12].

However, no detailed pharmacological study has been reported in the literature. Therefore in the present investigation, we aimed to investigate toxicity activities of *T. citrina* leaves for

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ensuring safety and exploring the beneficial role of this plant to treat various ailments.

2. Materials and methods

2.1. Collection of plant material

T. citrina leaves were collected from Rangamati District, Bangladesh during the month of May 2013. The plant was identified and authenticated by Sardar Nasir Uddin, Senior Scientific Officer, Bangladesh National Herbarium Mirpur, Dhaka and a voucher specimen (Accession No.: DACB 38094) was deposited there for future reference.

2.2. Preparation of methanolic extract

The leaves of the plant were collected in fresh condition. The dried and coarse powder (1 000 g) was extracted with methanol (4.0 L) in an air tight flat bottomed container for 15 days at room temperature with occasional stirring. The extract was then filtered through a cotton plug followed by a Whatman No. 1 filter paper. The filtrate was concentrated using a rotary evaporator at low temperature and pressure to afford crude methanol extract (50 g).

2.3. Experimental animal

Eight-week old female albino rats (*Rattus norvegicus*: Sprague-Dawley strain) weighing 110–140 g were used for the present study. They were purchased and maintained in the animal house of Jahangirnagar University, Bangladesh for experimental purpose. The animals were maintained under controlled conditions of temperature (23 ± 2) °C, humidity (50 ± 5)% and 12 h light–dark cycles. All the animals were acclimatized for seven days before the study. The animals were randomized into experimental and control groups and housed individually in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellets as basal diet and water ad libitum. Animals were habituated to laboratory conditions for 48 h prior to experimental protocol to minimize if any of non-specific stress. All experimental protocols were in compliance with Dhaka University Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

2.4. Phytochemical screening

The preliminary phytochemical group test was carried out by following standard procedure[13].

2.5. Acute toxicity test

The acute toxicity of *T. citrina* methanolic extract was determined in rats according to the method[14]. Rats fasted for 16 h were randomly divided into groups of ten rats per group. Graded doses of the extract (200, 400, 800, 1 600 and 3 200 mg/kg *p.o.*) were separately administered to the rats in each of the group by means of bulbed steel needle. All rats were then allowed free access to food and water and observed over a period of 48 h for signs of acute toxicity. The number of deaths within this period was recorded.

2.6. Subacute toxicity test

Sub-acute toxicity test was performed according to the World Health Organization guideline[15] and the Organization of Economic Co-operation and Development guideline 407 for testing of chemicals[16].

The plant extract at the doses of 250, 500 and 1 000 mg/kg body weight were administered orally to 4 groups of ten female rats respectively at every 24 h for 28 days and control received vehicle at the same volume. The toxic manifestation such as body weight, mortality, food and water intake was monitored. After 28 days all surviving animals were fasted overnight and anaesthetized with ether. The heparinized blood samples were collected for determining hematological parameters and the serum from non-heparinized blood was carefully collected for determining clinical blood chemistry. Animals were sacrificed after blood collection and the internal organs were removed and weighed to determine the relative organ weights and observed for gross lesions. The internal organs were preserved in 10% buffered formaldehyde solution for histological examination.

2.6.1. Weekly body weight

The body weight of each rat was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing, once weekly during the dosing period and once on the day of sacrifice.

2.6.2. Mortality and clinical signs

During the four-week dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 4 h after dosing[17]. On 29th day, the animals were anaesthetized with ketamine, blood was collected by sino-orbital puncture and centrifuged for 30 min at 2 000 r/min to separate serum for biochemical analysis. The liver and kidney were excised immediately and thoroughly washed in normal saline and weights were recorded.

2.6.3. Relative organ weight

On 29th day, all the animals were anaesthetized with ketamine. Organs namely, liver and kidneys were carefully dissected out and weighed in grams. The relative organ weight of each animal was then calculated as follows:

Relative organ weight = absolute organ weight (g) × 100/ body weight of rat on sacrifice day (g).

2.6.4. Hematological parameters

Estimation of hemoglobin (Hb) content (Sahli's hemoglobinometer), total white blood count (WBC), red blood cell (RBC) and platelet count (Neubauer hemocytometer; Feinoptik, Germany) was done using standard technique and differential WBC count (neutrophil, eosinophil, basophil, lymphocyte and monocyte) was done by Leishman's staining method. Mean cell volume (MCV) indicated the volume of the average red cell in a sample expressed in femtoliters (fL) and calculated by using the formula: MCV = packed-cell volume/RBC × 10. Mean cell hemoglobin (MCH) represents the absolute amount of Hb in the average red cell in a sample in units of picogram per cell. The MCH is calculated from the Hb and the RBC using the following formula: MCH = (Hb × 10)/RBC. Mean corpuscular hemoglobin concentration (MCHC) is the average Hb concentration in the RBCs. The MCHC expressed as the amount of Hb

per deciliter of red cells (g/dL) and calculated as follows: MCHC = Hb/packed-cell volume. The hematocrit is the ratio of RBCs to plasma and is expressed as a percentage of the whole blood volume. The hematocrit is calculated from the RBC count and the MCV as follows: hematocrit = (RBC × MCV)/10. Red cell distribution width (RDW) is a measure of the heterogeneity of the RBC population. The CELL-DYN 3700 system reports a relative RDW equivalent to a CV in percent. The RDW is derived from the RBC histogram using the width of the RBC distribution at 50% of the peak height. The mean platelet volume (MPV) is derived from the platelets histogram after the platelets count has been determined. The MPV is expressed in fL. The platelet distribution width (PDW) is a measure of the heterogeneity of the platelets population. It is expressed as the geometric standard deviation. Erythrocyte sedimentation rate (ESR) was determined by Westergren method. For the determination of the bleeding time, modified procedure of Mohamed *et al.*[18] was used. Clotting time test was performed using capillary tubes.

2.6.5. Biochemical estimations

Blood collected in non-heparinized tubes were than centrifuged at 3000 r/min for 10 min. The serum separated was analyzed for various parameters such as creatinine, urea, was done in Star 21 Plus, autoanalyzer by using the commercially available standard kit (ERBA Diagnostics Mannheim GmbH, Mannheim, Germany). Total protein content of serum was estimated following modified biuret method using a standard kit of Span Diagnostics Ltd., Dhaka, Bangladesh. Uric acid and albumin were estimated using kit obtained from Lab-Care Diagnostics Pvt. Ltd., Dhaka, Bangladesh[19].

2.6.6. Histology of liver and kidney

Paraffin sections of liver and kidney were prepared, stained with hematoxylin and eosin and processed for light microscope following the technique[20].

2.7. Statistical analysis

All values were expressed as the mean ± SEM and the results were analyzed statistically by One-way ANOVA followed by Dunnett's *t*-test by using SPSS version 16. $P < 0.05$ compared to standard was considered to be statistically significant.

3. Results

3.1. Phytochemical screening

The phytochemical screening test showed the presence of alkaloids, flavonoids, tannins, reducing sugar and carbohydrates in the leaves of *T. citrina*.

3.2. Acute toxicity test

In acute toxicity study, oral administration of graded doses (200, 400, 800, 1600 and 3200 mg/kg *p.o.*) of the methanol extract of *T. citrina* to rats showed no significant changes in behavior, breathing, cutaneous effects, sensory nervous system responses or gastrointestinal effects during the observation period. No mortality or any toxic reaction was recorded in any group at 48 h after administration. *T. citrina* was safe up to a dose level of 3200 mg/kg body weight.

3.3. Subacute toxicity test

3.3.1. Effect on body weight

Body weight was determined on day 0, 9th, 18th and 28th days. The body weight of rat in control group showed gradual increase during the 28th day's treatment period up to [(140.20 ± 5.31) g] of weight gain. In contrast administration of extract at 250, 500 and 1000 mg/kg per day doses showed respectively, [(143.40 ± 6.15), (137.50 ± 6.11) and (130.90 ± 4.86) g] increase in body weight (Table 1).

3.3.2. Effect on relative weight of liver and kidney

The intact weight of liver and kidney was converted to relative weight of 100 g body weight as shown in Table 2. The result showed that extract in different doses (250, 500 and 1000 mg/kg per day) administered for 28 days has non-significant effect on liver and kidney weight compared to vehicle control.

3.3.3. Effect on hematological parameters

The effect of extracts on hematological indices was examined at the end of treatment (Table 3). Treatment for 28 days has non-significant effect on WBC, RBC, platelet count, Hb, neutrophil, lymphocyte, monocyte, eosinophil, hematocrit, MPV, PDW, ESR, BT and CT. At 1000 mg/kg per day dose MCH show significant ($P < 0.05$) while MCV and MCHC at same concentration showed ($P < 0.001$) compared to control (Table 3).

Table 2

Effect of *T. citrina* leaves on relative weight of liver and kidney (g/100 g body weight).

Group	Liver weight	Kidney weight
Control	3.95 ± 0.83	0.89 ± 0.07
I	3.46 ± 0.45^{ns}	0.84 ± 0.03^{ns}
II	3.24 ± 0.73^{ns}	0.86 ± 0.04^{ns}
III	2.94 ± 0.36^{ns}	0.76 ± 0.02^{ns}

Groups I–III methanolic extracts with doses (I: 250 mg/kg; II: 500 mg/kg; III: 1000 mg/kg); ^{ns}: Not significant.

Table 1

Effect of methanol extract of *T. citrina* leaves on body weight of rat at different days (g).

Group	Weight			
	Initial day	9th day	18th day	28th day
Control	114.10 ± 5.60	121.40 ± 5.78	131.10 ± 5.75	140.20 ± 5.31
I	117.40 ± 5.22^{ns}	127.40 ± 6.24^{ns}	134.70 ± 6.28^{ns}	143.40 ± 6.15^{ns}
II	117.00 ± 5.97^{ns}	124.60 ± 6.88^{ns}	127.40 ± 6.59^{ns}	137.50 ± 6.11^{ns}
III	111.00 ± 4.24^{ns}	116.80 ± 4.34^{ns}	122.90 ± 4.54^{ns}	130.90 ± 4.86^{ns}

Groups I–III methanolic extracts with doses (I: 250 mg/kg; II: 500 mg/kg; III: 1000 mg/kg); ^{ns}: Not significant.

Table 3
Effect of methanol extract of *T. citrina* leaves for 28-day treatment on hematological parameter of rats.

Group	WBC (10^9/L)	RBC (10^{12}/L)	Platelets (10^9/L)	Hb (g/dL)	Neutrophil (10^9/L)	Lymphocyte (10^9/L)	Monocyte (10^9/L)	Eosinophil (10^9/L)	Hematocrit (%)
Control	5.9 ± 0.4	6.6 ± 0.1	540.5 ± 17.5	11.7 ± 0.1	1.2 ± 0.1	4.6 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	37.7 ± 0.5
I	5.3 ± 0.4$^{n.s.}$	6.9 ± 0.1$^{n.s.}$	541.0 ± 22.1$^{n.s.}$	11.9 ± 0.1$^{n.s.}$	0.9 ± 0.1$^{n.s.}$	4.2 ± 0.3$^{n.s.}$	0.1 ± 0.1$^{n.s.}$	0.1 ± 0.1$^{n.s.}$	38.2 ± 0.4$^{n.s.}$
II	5.2 ± 0.4$^{n.s.}$	6.6 ± 0.1$^{n.s.}$	490.5 ± 13.8$^{n.s.}$	11.5 ± 0.3$^{n.s.}$	0.9 ± 0.1$^{n.s.}$	4.2 ± 0.3$^{n.s.}$	0.1 ± 0.1$^{n.s.}$	0.1 ± 0.1$^{n.s.}$	36.3 ± 0.4$^{n.s.}$
III	5.9 ± 0.4$^{n.s.}$	6.8 ± 0.1$^{n.s.}$	535.0 ± 20.1$^{n.s.}$	11.7 ± 0.1$^{n.s.}$	1.1 ± 0.1$^{n.s.}$	4.7 ± 0.3$^{n.s.}$	0.1 ± 0.1$^{n.s.}$	0.1 ± 0.1$^{n.s.}$	36.8 ± 0.3$^{n.s.}$

MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	MPV (fL)	PDW (%)	ESR (Mm/h)	Bleeding time (sec)	Clotting time (min)
56.9 ± 0.2	17.5 ± 0.1	31.1 ± 0.2	13.9 ± 0.3	4.1 ± 0.1	16.1 ± 0.1	2.1 ± 0.1	34.5 ± 2.2	4.1 ± 0.1
55.3 ± 0.2^{a}	17.2 ± 0.1$^{n.s.}$	31.6 ± 0.1^{a}	12.7 ± 0.2^{b}	4.1 ± 0.1$^{n.s.}$	16.5 ± 0.2$^{n.s.}$	2.2 ± 0.1$^{n.s.}$	37.5 ± 2.5$^{n.s.}$	4.1 ± 0.1$^{n.s.}$
54.1 ± 0.3^{c}	17.1 ± 0.1^{a}	31.7 ± 0.1^{b}	12.5 ± 0.1^{c}	4.1 ± 0.1$^{n.s.}$	16.4 ± 0.2$^{n.s.}$	2.2 ± 0.1$^{n.s.}$	37.5 ± 2.5$^{n.s.}$	4.1 ± 0.1$^{n.s.}$
54.1 ± 0.5^{c}	17.1 ± 0.1^{a}	31.7 ± 0.1^{b}	12.5 ± 0.1^{c}	4.1 ± 0.1$^{n.s.}$	16.1 ± 0.1$^{n.s.}$	2.2 ± 0.1$^{n.s.}$	37.5 ± 2.5$^{n.s.}$	4.1 ± 0.1$^{n.s.}$

Groups I–III methanolic extracts with doses (I: 250 mg/kg; II: 500 mg/kg; III: 1 000 mg/kg); BT: Bleeding time; CT: Clotting time; $^{n.s.}$: Not significant; ^{a}:$P < 0.05$; ^{b}:$P < 0.01$; ^{c}:$P < 0.001$.

3.3.4. Effect on serum biochemical parameters

The effect of extracts for 28 days doesn't showed significant creatinine, urea, uric acid, total protein and albumin at doses 250, 500 and 1 000 mg/kg per day compare to control (Table 4).

3.3.5. Histology of liver and kidney

Light microscopic examination of liver and kidney sections of the control group showed a normal histology of liver and kidney. Similarly, extracts with all three doses 250, 500 and

Table 4
Effect of methanol extract of *T. citrina* leaves for 28-day treatment on biochemical parameter of rats.

Group	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)	Total protein (g/dL)	Albumin (g/dL)
Control	0.87 ± 0.03	6.08 ± 0.12	3.06 ± 0.11	6.16 ± 0.12	3.83 ± 0.13
I	0.86 ± 0.03^{ns}	5.97 ± 0.08^{ns}	3.08 ± 0.14^{ns}	6.19 ± 0.15^{ns}	3.77 ± 0.18^{ns}
II	0.84 ± 0.03^{ns}	5.91 ± 0.12^{ns}	2.85 ± 0.14^{ns}	5.95 ± 0.15^{ns}	3.69 ± 0.15^{ns}
III	0.86 ± 0.03^{ns}	5.89 ± 0.11^{ns}	3.01 ± 0.16^{ns}	6.24 ± 0.16^{ns}	3.83 ± 0.17^{ns}

Groups I–III methanolic extracts with doses (I: 250 mg/kg; II: 500 mg/kg; III: 1 000 mg/kg); ^{ns}: Not significant.

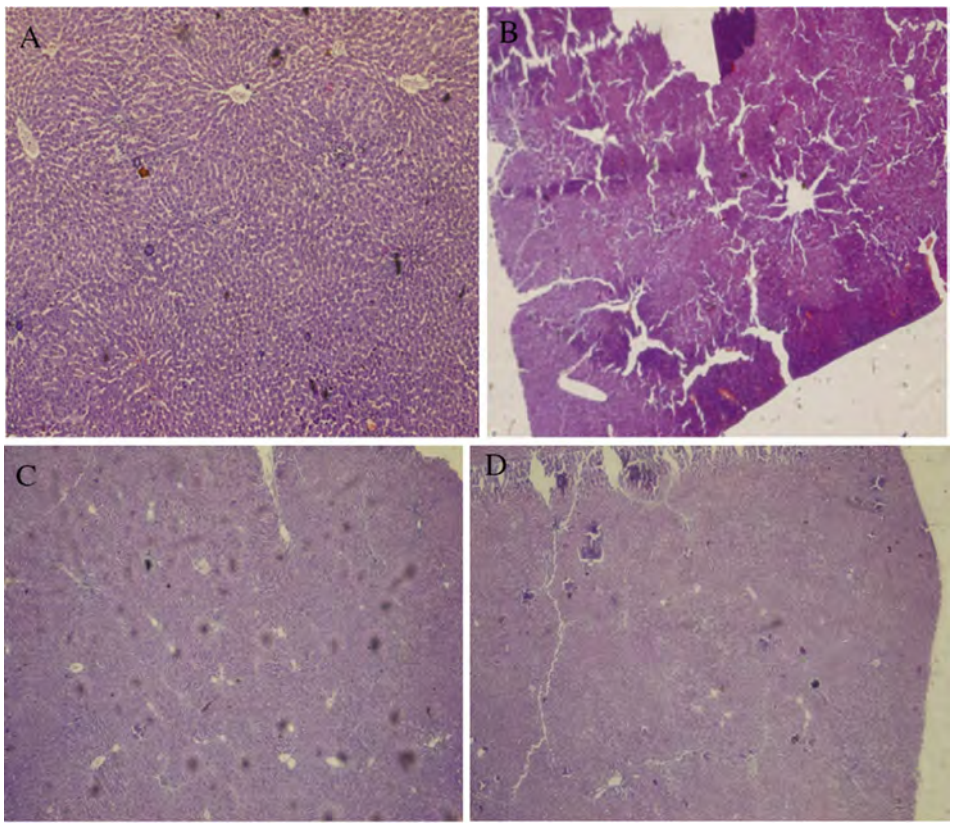

Figure 1. Photomicrograph of rat liver treated with different doses of extracts for 28 days.
A: Control group; B: *T. citrina* (250 mg/kg per day) group; C: *T. citrina* (500 mg/kg per day) group; D: *T. citrina* (1 000 mg/kg per day) group showing normal liver histology.

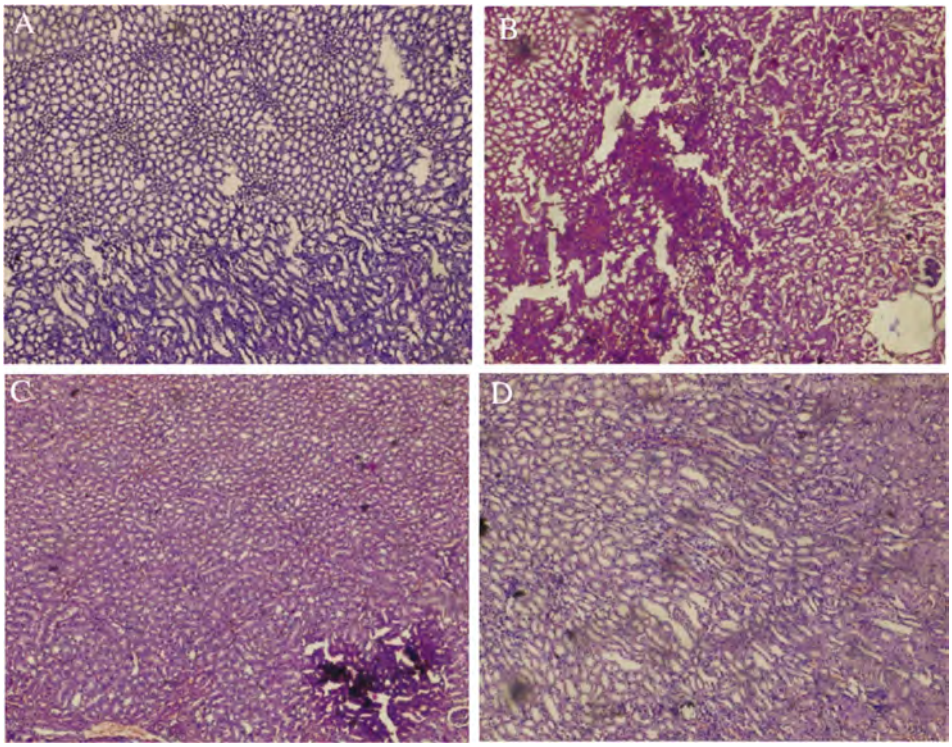

Figure 2. Photomicrograph of rat kidney treated with different doses of *T. citrina* for 28 days.
A: Control group; B: *T. citrina* (250 mg/kg per day) group; C: *T. citrina* (500 mg/kg per day) group; D: *T. citrina* (1 000 mg/kg per day) group showing normal kidney histology.

1 000 mg/kg per day did not have any deleterious effect on histological features of rat liver and kidney (Figures 1 and 2).

4. Discussion

Due to negligible adverse effects, natural compounds take an important role in therapeutic applications. However, there is a lack of scientific validation on the toxicity and adverse effects of these natural compounds. Therefore, scientific knowledge towards acute oral toxicity study is much needed, which will not only help identify the range and concentration of dose that could be used subsequently, but also to reveal the possible clinical signs elicited by the substances under investigation. In addition, it is also a useful parameter to investigate the therapeutic index of drugs and xenobiotics[21]. In our work, the phytochemical analysis of methanol extracts of *T. citrina* was carried out. The methanolic extract contained alkaloids, flavonoids, tannins, reducing sugar and carbohydrates.

After 28 days treatment of methanolic extract of *T. citrina* leaves, there were no significant changes in weight of body and organs. All the animals exhibited a normal increment in body weight without drastic difference between both control and treated groups.

The haematological parameters between control and treated groups, showed that the extract was not toxic to circulating red cells, nor interfered with the production and that of platelets. The haematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in man and animals[22]. The RBC indices, MCV, MCH and MCHC showed marginal decrease in the extract treated compared to the control. The importance of calculated blood indices in anemia diagnosis has been reported[23]. The non-significant difference on the RBC indices suggested that the extract did not affect a change in the average size of RBCs. By extension, it did not induce anemia. There was also, notably no change in WBC count which is known to rise as body defense in response to toxic environment[24]. On the other hand, lymphocyte, the main effector's cell of the immune system[25] recorded fluctuation suggesting that the extract might not have exerted challenge on the immune system of the animals.

In addition, most of the biochemical parameters were not also altered by the methanol extract of *T. citrina*. The lack of significant alterations in the levels of alanine aminotransferase, aspartate amino transferase, alkaline phosphatase, glucose and creatinine, which are good indicators of liver and kidney functions, suggests that sub-chronic administration of extract neither altered hepatocytes and kidneys of rats nor the normal metabolism of the animals[26]. Because these clinical blood chemical parameters are the index of kidney and liver function, it suggests that the extract does not induce toxicity to the kidneys and liver. These observations were further confirmed by the histological assessment of the organs showed in Figures 1 and 2. Some of the species of *Terminalia genus* such as *Terminalia chebula*, *Terminalia belerica*, *Terminalia arjuna*, *Terminalia mollis*, *Terminalia avicennioides* and *Terminalia paniculata* have been previously reported to exhibit similar activities and have been used as good therapeutic agents[27–32]. Based on the results found in our study, we concluded that *T. citrina* methanol extract was safer and non toxic and could be well used for pharmacological and therapeutic purposes.

In conclusion, the present results show that methanol extract of leaves of *T. citrina* does not cause any apparent *in vivo* toxicity in an animal model. No death or signs of toxicity were observed in rat treated with extract at doses 250, 500 and

1 000 mg/kg body weight, thus its safety in use. The histology examination revealed no changes in the architecture of the internal organs kidney and liver of rat, in both control and treated groups. Hence, leaves of *T. citrina* can be used as a medicinal agent in known dosages, especially in rural communities where conventional drugs are unaffordable because of their cost. A detailed experimental analysis of its chronic toxicity is essential for further support of this plant.

Conflict of interest statement

The authors report no conflict of interest.

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