

Toxicity Effects of Oral Administration of *Clinacanthus nutans* Ethanolic Leaf Extract on Blood, Liver and Kidneys of Mice

A. ALIYU^{1*}, M. R. SHAARI², NURUL SYAHIRAH AHMAD SAYUTI, M. F. H. REDUAN³, SAJJARATTUL NURUL NADIA ASYURA², S. SITHAMBARAM², M. M. NOORDIN, KHOZIRAH SHAARI^{4,5} AND HAZILAWATI HAMZAH

Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia, ¹Department of Veterinary Pathology, Faculty of Veterinary Medicine, Usmanu Danfodiyo University, Sokoto 840212, Sokoto State, Nigeria, ²Animal Science Research Centre, Malaysian Agricultural Research and Development Institute Headquarter, Serdang 43400, Selangor, ³Department of Paraclinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Kota Bharu 16100, Kelantan, ⁴Department of Chemistry, Faculty of Science and Environmental Studies, ⁵Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia

Aliyu et al.: Sub-Chronic Toxicity Effects of *Clinacanthus nutans* Ethanolic Leaf Extract in Mouse Model

Clinacanthus nutans has been used traditionally in the treatment of herpes simplex viral infection. This research evaluated the toxicity effects of sub-chronic oral administration of *Clinacanthus nutans* ethanolic leaf extract in Institute of Cancer Research mice. A total 50, 8 w old female mice were divided into five groups of 10 mice each; Group A (control), Group B (125 mg/kg), Group C (250 mg/kg), Group D (500 mg/kg) and Group E (1000 mg/kg). The extract was administered orally for 90 d. The mice were monitored and sacrificed on d 91. Blood, liver and kidney samples were collected for analyses. There was significant ($p < 0.05$) alterations in the haematological parameters of the mice in Group E and a significant increase in creatinine levels in groups B, C, D and E compared to A. The plasma level of alanine aminotransferase was significantly ($p < 0.05$) higher in Groups D and E, compared to A. Histopathological evaluation of liver and kidneys revealed a moderate cytoplasmic vacuolation, eosinophilic cytoplasm and pyknosis of hepatocytes, as well as mild to moderate activated Kupffer cells in Group E. Similarly, the renal tubular cells showed mild to moderate renal cytoplasmic vacuolation, eosinophilic cytoplasm, pyknotic and karyolytic cells in Group E. It is concluded that repeated oral doses of *Clinacanthus nutans* ethanolic leaf extract for 90 d induced hepato-renal toxicities in female Institute of Cancer Research mice.

Key words: *Clinacanthus nutans*, Institute of Cancer Research mice, sub-chronic toxicity, haematological parameters, liver, kidneys

Clinacanthus nutans (*C. nutans*), which is locally referred to as “Belalai Gajah” (in Malaysia) belongs to the family Acanthaceae; which is considered as one of the leading families of dicotyledonous flowering plants. The family consists of 250 genera and about 2500 species^[1]. The genus *Clinacanthus* consists of two species, *C. nutans* (Burm. F.) Lindau and *C. siamensis* Bremek which are found throughout Southeast Asia. The two species have been reported to have different pharmacological activities^[2,3]. The plant has been used traditionally for the treatment of snake bites, skin rashes, diabetes mellitus, diarrhoea and cancers^[4]. Moreover, the plant has been used as an anti-viral agent against Herpes Simplex Virus (HSV) and Varicella-Zoster Virus (VZV)^[3]. Interestingly, the leaves of

C. nutans have gained its popularity as supplement and medicine to treat various types of illnesses including cancer^[5]. The mode of action of this plant is ascribed to its anti-cell lysis property rather than an anti-neuromuscular transmission blocker^[4]. Phytochemical investigation of *C. nutans* extracts revealed the presence of various bioactive compounds including C-glycosyl flavones^[6], phytosterols, triterpenoid^[7], stigmasterol, glycolglycerolipids^[8], lupeol, b-sitosterol,

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*Address for correspondence

E-mail: abdullahi.aliyu@udusok.edu.ng

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belutin, sulfur containing glycosides and some compounds related to chlorophyll A and chlorophyll B. The extraction method has a significant role on the phytochemical yield of *C. nutans* extract^[9]. Alam *et al.*^[4] reported that vitexin, isovitexin, schaftoside, isomollupentin 7-O- β -glucopyranoside, orientin and isoorientin were isolated from the n-butanol and water-soluble fractions of methanolic extract of this plant in Thailand. Furthermore, a previous study in our group by Aliyu *et al.*^[10] reported that *C. nutans* ethanolic leaf extract cultivated in Pahang, Malaysia contained phytochemical compounds including myricetin, isookanin and ferulic acid^[10]. These compounds were in addition to those reported earlier by previous researchers^[11-13].

Despite these numerous pharmacological potentials of *C. nutans*, the sub-chronic toxicity effects of ethanolic leaf extract of this plant on the vital organs especially liver and kidney have not been thoroughly investigated. Previous studies have reported the acute and sub-acute (28 d) toxicity effects of *C. nutans* administration in laboratory rodents^[10,14-17]. However, treatment of various chronic diseases including cancers required daily administration of the treatment for longer than 28 d duration. Moreover, Aliyu *et al.*^[10] reported that daily administration of *C. nutans* ethanolic leaf extract for 28 d induced hepatic and renal toxicity in mice^[10]. Therefore, it is necessary to evaluate the safety levels of medicinal herbs (including *C. nutans*) when administered for longer duration of 90 d or more, in order to avoid administering higher doses of the plant that could exert toxic effects on the vital organs in the course of treating chronic illnesses in both humans and animals.

MATERIALS AND METHODS

Collection of plant materials:

Leaves of *C. nutans* were collected from the Malaysian Agriculture Research and Development Institute (MARDI) research station, Muadzam Shah, Pahang between the months of July to October 2017. It was processed as described by Aliyu *et al.*^[10]. The leaves were cleaned with water and were dried under the sun for 48 h. The leaves were later ground into powdered form and kept at 4° for subsequent analyses^[10,18].

Identification of the plant material:

The botanical identification of the plant (to confirm its kingdom, family and species) was performed at the Faculty of Science and Technology (FST), University

Kebangsaan Malaysia (UKM). The voucher specimen has been deposited at the herbarium of FST, UKM with reference number: UKMB40367.

Extraction of the plant material:

The method described by Aliyu *et al.*^[10] was employed. Briefly, about 100 g of the ground leaf samples was dissolved in about 4000 ml 95 % ethanol (Merck, German) in a glass flask for the extraction process^[10,19]. The mixture was placed at 200 rpm at about 25° (room temperature) in an orbital shaker (Heidoph Unimax 1010, German) for about 2 h^[5,10,20,21]. Whatman No. 1 filter paper was used to filter the extract and later concentrated to a semi solid form using a rotary evaporator (BUCHI Rotavapor R-200, Switzerland). The processed extract was stored at 4° for future analysis.

Preparation of extracts:

The weekly doses of the extracts were prepared freshly by dissolving the dried extracts of the plants leaf in 5 % Dimethyl Sulfoxide (DMSO) as previously described by Aliyu *et al.*^[10]. The doses were prepared based on the average body weight of the mice in each group^[10]. Five percent (5 %) DMSO was reported earlier by Nurul *et al.*^[22] not to be associated with any toxicity in rats, therefore, it was chosen as a solvent in this study to dissolve the extract properly prior to the administration.

Animal husbandry and experimental design:

The animals used in this study were purchased from a commercial vendor at Selangor, Malaysia. The experiment was conducted according to Organisation for Economic Co-operation and Development (OECD) guidelines 408 and was conducted at the Animal Metabolism, Toxicology and Reproductive Centre (AMTREC), Malaysian Agricultural Research and Development Institute (MARDI), Serdang. The experimental animals were treated according to the guide for the care and use of laboratory animals as approved by the Animal Ethics Committee (AEC) of MARDI (20170717/R/MAEC00023). The mice were placed in polycarbonate plastic cages individually and allowed to acclimatize to the housing conditions for 7 d. The housing was conditioned with temperature of 22-25°, humidity at 40 %-70 % and equal 12 h light/dark cycle^[10].

A total of 50, 8 w old female mice were randomly allocated into 5 groups of 10 mice each for the study.

For 90 d, once daily, repeated doses of the extracts were administered orally via oral gavage using stainless steel needle, the details of the doses are shown in Table 1. The extract was administered at a volume of 1 ml/100 g body weight to Groups B to E accordingly, while Group A received equal volume of distilled water only.

Determination of weekly body weight gain:

The method of Aliyu *et al.*^[10] was employed for the determination of weekly bodyweight gain of the individual mouse in each of the experimental groups. The weights were measured and recorded accordingly.

Collection of blood and organ samples:

The mice were humanely sacrificed on d 91 of the experiment using Carbon dioxide (CO₂) chamber^[10,23,24]. Blood samples were collected through cardiac puncture^[10,24] into clean test tubes containing Ethylenediamine Tetraacetic Acid (EDTA) anticoagulant for the analysis of haematology and blood chemistry parameters^[10,24-26]. Post-mortem was performed on each mouse and tissues from liver, kidney, heart, brain, spleen, lungs and uterus were collected and weighed accordingly^[10]. The relative organ weight of each organs collected was also determined using the formula below^[10,24,27].

Relative organ weight=Organ weight/ Body weight×100

Haematological analyses:

Automated haematology analyser (ABC Vet®, ABX Diagnostics, France) was used for the complete blood count analyses^[5,10,24,28].

Clinical biochemistry analysis:

Plasma sample was obtained by centrifuging the anti-coagulated blood samples collected at 3000 rpm for 15 min for blood biochemical analyses^[24-26,29-32]. The plasma was subsequently analysed using fully automated clinical chemistry analyser (BioLis 24i Chemistry Analyzer, Japan) for the concentration of urea, creatinine, Alanine Aminotransferase (ALT),

Aspartate Aminotransferase (AST), Creatinine Kinase (CK), Total Protein (TP), Albumin (ALB)^[10,24].

Histopathological analyses:

Liver and kidneys were collected from each mouse as described by Aliyu *et al.*^[10] at the end of the experiment and cleaned using cold normal saline to remove excess blood from the tissues. The method described earlier by Sajjaratul *et al.*^[5], Aliyu *et al.*^[10] and Aliyu *et al.*^[24] were used to process the tissues for Haematoxylin and Eosin (H&E) stain at the histopathology laboratory, Faculty of Veterinary medicine, Universiti Putra Malaysia. Lesions were evaluated at 4x, 10x, 20x and 40x objective lenses of light microscope^[10,24] from each of the processed sample.

Lesion scoring:

Lesion scoring was conducted according to the method of Aliyu *et al.*^[10]. Lesions including eosinophilic cytoplasm, pyknosis, karyolysis, karyorrhexis, activated Kupffer cells, sinusoidal dilatation, cytoplasmic vacuolation, regeneration and inflammation were analysed and scored in each stained section of liver from all groups of mice while hydropic degeneration, pyknosis, inflammation, protein casts, cellular casts and granular casts, were equally scored in the kidneys of each mouse^[10]. The detailed scores of each lesion and its interpretation are presented in Table 2.

Statistical analysis:

The results of the study were evaluated using International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) statistical software version 23 and were expressed as mean±Standard Error of Mean (SEM). One-way Analysis of Variance (ANOVA) with Tukey post hoc test and non-parametric Kruskal-Wallis H statistical tool were used accordingly to test for significant differences between groups at p<0.05^[10].

RESULTS AND DISCUSSION

The extract administered daily for 90 d showed significant (p<0.05) differences in the average body weight gain of the mice between groups across the 13 w experimental period as determined by repeated measures ANOVA with a Greenhouse-Geisser correction and Bonferroni post hoc (fig. 1). There were 106.51 %, 112.04 %, 53.13 %, 272.73 %, 37.56 %, 28.32 %, 35.84 %, 747.73 %, 149.67 % and 70.09 % reductions in the average body weight gains of

TABLE 1: SUB-CHRONIC TOXICITY STUDY OF CELE

| Group (n=10) | Treatments |
|--------------|-----------------|
| A | Control |
| B | 125 mg/kg CELE |
| C | 250 mg/kg CELE |
| D | 500 mg/kg CELE |
| E | 1000 mg/kg CELE |

Note: CELE-C. *nutans* ethanolic leaf extract

the mice in Group B at 1st w (-0.07 ± 0.42 g; $p < 0.05$), w 3 (-0.14 ± 0.34 g; $p < 0.05$), w 5 (0.42 ± 0.17 g; $p < 0.05$), w 7 (0.40 ± 0.19 g; $p > 0.05$), w 8 (0.40 ± 0.16 g; $p > 0.05$), w 9 (0.41 ± 0.23 g; $p > 0.05$), 10 (0.29 ± 0.19 g; $p < 0.05$), 11 (-0.08 ± 0.41 g; $p > 0.05$) and 12 (0.14 ± 0.26 g; $p > 0.05$) of the experiment respectively, compared to Group A at w 1 (1.12 ± 0.43 g), w 3 (1.20 ± 0.17 g), w 5 (0.89 ± 0.34 g), w 7 (0.64 ± 0.27 g), w 8 (0.56 ± 0.25 g), w 9 (0.64 ± 0.28 g), w 10 (-0.04 ± 0.18 g), w 11 (0.15 ± 0.18 g) and w 12 (0.46 ± 0.27 g) (fig. 1). Furthermore, the mice in Group C showed 61.11 %, 6.78 %, 107.61 %, 13.18 %, 79.31 %, 241.67 %, 95.93 %, 81.72 %, 129.55 %, 103.06 % and 25.95 % reductions in the average body weight gains at w 1 (0.44 ± 0.36 g; $p < 0.05$), w 2 (0.65 ± 0.20 g; $p > 0.05$), w 3 (-0.09 ± 0.20 g; $p < 0.05$), w 4 (0.61 ± 0.25 g; $p > 0.05$), w 5 (0.19 ± 0.25 g; $p < 0.05$), w 7 (0.03 ± 0.17 g; $p > 0.05$), w 8 (0.10 ± 0.13 g; $p < 0.05$), w 10 (0.01 ± 0.26 g; $p > 0.05$), w 12 (-0.01 ± 0.18 g; $p < 0.05$) and w 13 (0.21 ± 0.18 g; $p > 0.05$) of the experiment respectively, compared to Group A gains at w 1 (1.12 ± 0.43 g), w 2 (0.69 ± 0.16 g), w 3

(1.20 ± 0.17 g), w 4 (0.70 ± 0.36 g), w 5 (0.89 ± 0.34 g), w 7 (0.64 ± 0.27 g), w 8 (0.56 ± 0.25 g), w 10 (-0.04 ± 0.18 g), w 12 (0.46 ± 0.27 g) and w 13 (0.29 ± 0.52 g) (fig. 1). Similarly, there were 140.05 %, 87.12 %, 89.59 %, 456.06 %, 69.48 %, 97.13 % and 71.28 % significant ($p < 0.05$) decrease in the average body weight gains of the mice in Group D at w 1 (-0.45 ± 0.40 g; $p < 0.05$), w 3 (0.15 ± 0.28 g; $p < 0.05$), w 5 (0.09 ± 0.17 g; $p < 0.05$), w 7 (0.20 ± 0.19 g; $p < 0.05$), w 8 (0.02 ± 0.26 g; $p < 0.05$) and w 13 (0.08 ± 0.19 g; $p > 0.05$) of the experiment respectively, compared to those in Group A at w 1 (1.12 ± 0.43 g), w 3 (1.20 ± 0.17 g), w 5 (0.89 ± 0.34 g), w 7 (0.64 ± 0.27 g), w 8 (0.56 ± 0.25 g) and w 13 (0.29 ± 0.52 g) (fig. 1). In addition, the treated mice in Group D also showed 78.07 %, 9.17 %, 17.99 %, 327.27 %, 71.24 % and 13.32 % increases in the average body weight gains at w 2 (1.23 ± 0.41 g; $p > 0.05$), w 4 (0.76 ± 0.32 g; $p > 0.05$), w 9 (0.75 ± 0.18 g; $p > 0.05$), w 10 (-0.19 ± 0.12 g; $p > 0.05$), w 11 (0.26 ± 0.31 g; $p > 0.05$) and w 12 (0.52 ± 0.22 g; $p > 0.05$) of the experiment respectively, compared to the mice in Group A at w 2 (0.69 ± 0.16 g), w 4 (0.70 ± 0.36 g), w 9 (0.64 ± 0.28 g), w 10 (-0.04 ± 0.18 g), w 11 (0.15 ± 0.18 g) and w 12 (0.46 ± 0.27 g) (fig. 1). Moreover, there were 106.16 %, 83.36 %, 48.21 %, 85.92 %, 131.00 %, 44.91 %, 1415.91 %, 496.73 %, 59.39 % and 96.65 % reductions in the average body weight gains of the mice in Group E, at w 1 (-0.07 ± 0.40 g; $p < 0.05$), w 3 (0.20 ± 0.23 g; $p < 0.05$), w 5 (0.46 ± 0.20 g; $p < 0.05$), w 7 (0.09 ± 0.31 g; $p < 0.05$), w 8 (-0.17 ± 0.58 g; $p < 0.05$), w 9 (0.35 ± 0.12 g; $p > 0.05$), w 11 (-0.61 ± 0.28 g; $p < 0.05$), w 12 (0.19 ± 0.73 g; $p < 0.05$) and w 13 (0.00 ± 0.35 g; $p > 0.05$) of the experiment respectively,

TABLE 2: INTERPRETATION OF SCORES IN LIVER AND KIDNEY LESION SCORING FOR THE TOXICITY STUDIES OF CELE IN ICR MICE

| *Score | Percentage | Severity |
|--------|----------------|-----------------|
| 0 | None | None |
| 1 | Less than 10 % | Mild |
| 1.5 | 10-30 % | Mild-moderate |
| 2 | 30-50 % | Moderate |
| 2.5 | 50-70 % | Moderate-severe |
| 3 | More than 70 % | Severe |

Note: CELE-C. *nutans* ethanolic leaf extract; *-adopted from Aliyu et al.^[10]

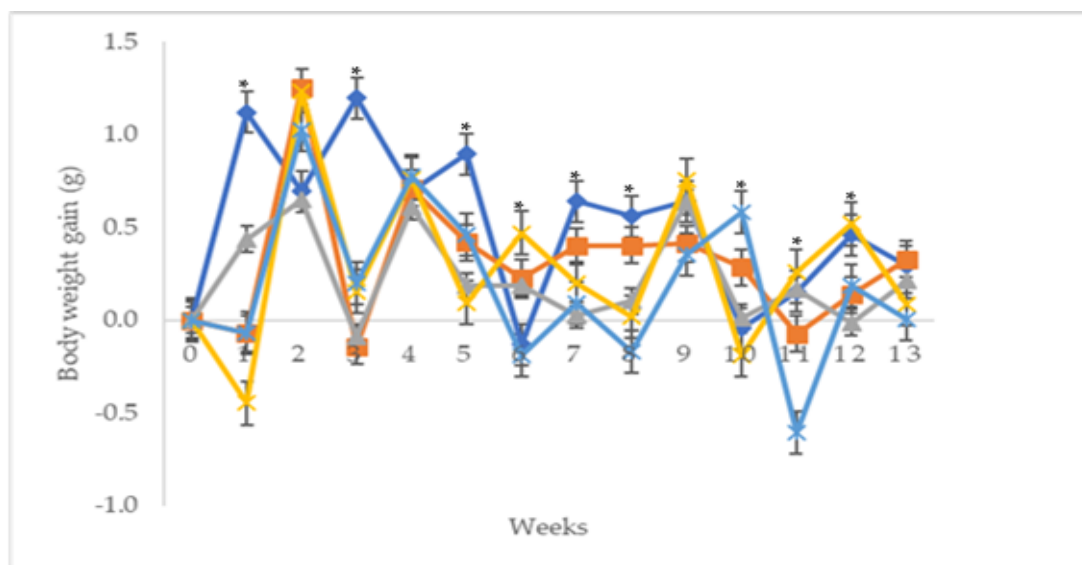


Fig. 1: Average (mean±SEM) weekly body weight gain (g) of female ICR mice in sub-chronic toxicity study of CELE. A-control; F-125 mg/kg CELE; G-250 mg/kg CELE; I-1000 mg/kg CELE; *differ significantly at $p < 0.05$; SEM-standard error of mean; CELE-C. *nutans* ethanolic leaf extract, (—●—) A; (—■—) B; (—▲—) C; (—◆—) D; (—×—) E

compared to Group A at w 1 (1.12 ± 0.43 g), w 3 (1.20 ± 0.17 g), w 5 (0.89 ± 0.34 g), w 7 (0.64 ± 0.27 g), w 8 (0.56 ± 0.25 g), w 9 (0.64 ± 0.28 g), w 11 (0.15 ± 0.18 g), w 12 (0.46 ± 0.27 g) and w 13 (0.29 ± 0.52 g) (fig. 1).

Table 3 showed that there were no statistically significant ($p > 0.05$) differences between groups in the relative organs weight of the mice treated with *C. nutans* Ethanolic Leaf Extract (CELE) daily for 90 d, as determined by one-way ANOVA (Table 3). However, there was 26.5 % ($p > 0.05$) decrease in the relative weight of brain in Group E (1.67 ± 0.05 %) compared to A (2.27 ± 0.10 %). Similarly, the relative weights of uterus were 33.1 %, 31.5 %, 30.6 % and 37.1 % lower respectively in Groups B (0.83 ± 0.03 %), C (0.85 ± 0.05 %), D (0.86 ± 0.06 %) and E (0.78 ± 0.03 %) compared to A (1.24 ± 0.17 %) (Table 3). Furthermore, the relative organs weights of right kidney were 14.6 % and 16.9 % lower ($p > 0.05$) in Groups C (0.76 ± 0.02) and

E (0.74 ± 0.03) respectively, compared to A (0.89 ± 0.08) (Table 3).

There were statistically significant ($p < 0.05$) differences between groups in the haematological parameters of the mice treated with different doses of CELE daily for 90 d as determined by one-way ANOVA (Table 4). Tukey post hoc test demonstrated sub-chronic administration of CELE showed 21.2 %, 9.1 % and 9.1 % significant ($p < 0.05$) increases in the values of Packed Cell Volume (PCV) respectively in Groups C (0.40 ± 0.02 l/l), D (0.36 ± 0.02 l/l) and E (0.36 ± 0.03 l/l) of the mice, compared to A (0.33 ± 0.02 l/l) (Table 4). Furthermore, there was 61.7 % significant ($p < 0.05$) increase in total leucocyte counts in Group E ($11.85 \pm 0.69 \times 10^9$ /l) compared to A ($7.33 \pm 0.45 \times 10^9$ /l). The increase in total leucocyte count was accompanied by significant ($p < 0.05$) increase in neutrophils (67.3 %) in Group E ($3.38 \pm 0.289 \times 10^9$ /l) compared to A ($2.02 \pm 0.16 \times 10^9$ /l),

TABLE 3: RELATIVE ORGAN WEIGHTS IN PERCENTAGE (%) (MEAN \pm SEM) OF FEMALE ICR MICE IN SUB-CHRONIC TOXICITY STUDY OF CELE

| Organs | A | B | C | D | E |
|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Liver | 6.49 ± 0.36 | 7.40 ± 0.39 | 6.52 ± 0.26 | 6.30 ± 0.24 | 6.25 ± 0.22 |
| Right Kidney | 0.89 ± 0.08 | 0.84 ± 0.04 | 0.76 ± 0.02 | 0.83 ± 0.05 | 0.74 ± 0.03 |
| Left kidney | 0.82 ± 0.05 | 0.85 ± 0.03 | 0.76 ± 0.03 | 0.80 ± 0.03 | 0.74 ± 0.03 |
| Spleen | 0.62 ± 0.06 | 0.63 ± 0.05 | 0.58 ± 0.08 | 0.76 ± 0.06 | 0.62 ± 0.10 |
| Heart | 0.67 ± 0.05 | 0.76 ± 0.05 | 0.69 ± 0.04 | 0.67 ± 0.05 | 0.65 ± 0.04 |
| Lungs | 1.43 ± 0.07 | 1.43 ± 0.13 | 1.46 ± 0.10 | 1.29 ± 0.12 | 1.58 ± 0.23 |
| Brain | 2.27 ± 0.10 | 2.21 ± 0.09 | 2.08 ± 0.08 | 1.97 ± 0.06 | 1.67 ± 0.05 |
| Uterus | 1.24 ± 0.17 | 0.83 ± 0.03 | 0.85 ± 0.05 | 0.86 ± 0.06 | 0.78 ± 0.03 |

Note: CELE-*C. nutans* ethanolic leaf extract; A-Control; B-125 mg/kg CELE; C-250 mg/kg CELE; D-500 mg/kg CELE; E-1000 mg/kg CELE; Values in the same row without asterisk are comparable ($p > 0.05$)

TABLE 4: HAEMATOLOGICAL PARAMETERS (MEAN \pm SEM) OF FEMALE ICR MICE IN SUB-CHRONIC TOXICITY STUDY OF CELE

| Parameters | A | B | C | D | E |
|--|------------------|---------------------|------------------|--------------------|--------------------|
| Red blood cells ($\times 10^{12}$ /l) | 9.50 ± 0.25 | 9.90 ± 0.31 | 9.79 ± 0.29 | 9.11 ± 0.23 | 9.56 ± 0.30 |
| Haemoglobin (g/l) | 158.40 ± 3.3 | 170.10 ± 3.9 | 163.40 ± 4.3 | 153.10 ± 5.1 | 159.89 ± 6.0 |
| PCV (l/l) | 0.33 ± 0.02 | $0.40 \pm 0.02^*$ | 0.37 ± 0.01 | $0.36 \pm 0.02^*$ | $0.36 \pm 0.03^*$ |
| Platelets ($\times 10^9$ /l) | 871.6 ± 124 | 1126.0 ± 154 | 821.2 ± 130 | 840.1 ± 137^a | 1175.1 ± 132 |
| MCV (fl) | 65.80 ± 0.79 | 67.20 ± 0.76 | 66.20 ± 1.05 | 64.60 ± 0.98^a | 65.33 ± 1.09 |
| MCH (pg) | 16.71 ± 0.33 | 17.33 ± 0.25 | 16.75 ± 0.30 | 16.80 ± 0.39^a | 16.70 ± 0.32 |
| MCHC (g/l) | 253.50 ± 3.5 | 257.60 ± 3.5 | 253.10 ± 2.3 | 259.00 ± 3.9^a | 255.67 ± 3.7 |
| Plasma proteins (g/l) | 77.60 ± 3.34 | 82.00 ± 2.02 | 80.50 ± 1.71 | 80.60 ± 1.55^a | 84.89 ± 3.74 |
| WBCs ($\times 10^9$ /l) | 7.33 ± 0.45 | $10.07 \pm 0.589^*$ | 6.81 ± 0.44 | 8.72 ± 0.67 | $11.85 \pm 0.69^*$ |
| Neutrophils ($\times 10^9$ /l) | 2.02 ± 0.16 | 2.73 ± 0.20 | 1.81 ± 0.17 | 2.52 ± 0.20 | $3.38 \pm 0.289^*$ |
| Lymphocytes ($\times 10^9$ /l) | 4.59 ± 0.31 | $6.68 \pm 0.449^*$ | 4.57 ± 0.34 | 5.62 ± 0.49 | $7.55 \pm 0.379^*$ |
| Monocytes ($\times 10^9$ /l) | 0.47 ± 0.03 | 0.46 ± 0.03 | 0.34 ± 0.02 | 0.43 ± 0.04 | $0.63 \pm 0.049^*$ |
| Eosinophils ($\times 10^9$ /l) | 0.24 ± 0.04 | 0.20 ± 0.02 | 0.09 ± 0.01 | 0.16 ± 0.02 | $0.29 \pm 0.049^*$ |
| Basophils ($\times 10^9$ /l) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

Note: CELE-*C. nutans* ethanolic leaf extract; A-Control; B-125 mg/kg CELE, C-250 mg/kg CELE; D-500 mg/kg CELE; E-1000 mg/kg CELE; PCV-Packed cell volume; MCV-Mean corpuscular volume; MCH-Mean corpuscular haemoglobin; MCHC-Mean corpuscular haemoglobin concentration; Values in the same row with asterisk are not comparable ($p < 0.05$)

significant ($p<0.05$) increase in lymphocyte counts (64.5 %) in Group E ($7.55\pm0.379\times10^9/l$) compared to A ($4.59\pm0.31\times10^9/l$), significant ($p<0.05$) increase in monocyte count (34 %) in Group E ($0.63\pm0.049\times10^9/l$) compared to A ($0.47\pm0.03\times10^9/l$), as well as 20 % significant ($p<0.05$) increase in eosinophil counts in Group E ($0.29\pm0.049\times10^9/l$) compared to A (0.24 ± 0.04) (Table 4).

The levels of the plasma biochemical parameters of the mice treated with varying doses of CELE sub-chronically (90 d) are shown on Table 5. There were statistically significant ($p<0.05$) differences between groups in the plasma biochemical parameters of the mice as demonstrated by one-way ANOVA (Table 5). Tukey post hoc test revealed that the mice treated with CELE had 45.4 % significant ($p<0.05$) increase in the urea in Group E (10.22 ± 0.74 mmol/l) compared to A (7.03 ± 0.25 mmol/l) as well as 37 %, 45.6 %, 40.3 % and 41.6 % significant ($p<0.05$) increases in creatinine levels respectively in Groups B (41.80 ± 1.17 μ mol/l), C (44.40 ± 0.99 μ mol/l), D (42.80 ± 1.71 μ mol/l) and E (43.24 ± 2.25 μ mol/l) compared to A (30.50 ± 1.31 μ mol/l) (Table 5). Additionally, the plasma levels of ALT were 78 % and 51.6 % significantly ($p<0.05$) higher respectively in Groups D (425.60 ± 25.64 U/l) and E (362.52 ± 27.01 U/l) compared to A (239.10 ± 22.50 U/l).

There were also 67.7 % and 70.2 % significant ($p<0.05$) increase in AST in Groups D (348.60 ± 21.45 U/l) and E (353.85 ± 16.84 U/l) respectively, compared to A (207.90 ± 19.67 U/l) as well as 138 % significant ($p<0.05$) increase in CK level in Group D (1516.1 ± 150.15 U/l) compared to A (636.8 ± 45.81 U/l) (Table 5).

Histological evaluation of the samples from the varying groups of mice with administration of different doses of CELE and distilled water daily for 90 d showed significant ($p<0.05$) differences in the lesion score between the different treatment groups as determined by Kruskal-Wallis H test (Table 6). Pairwise comparisons test showed a moderate eosinophilic cytoplasm (fig. 2B and fig. 2D) in Group E (2.15 ± 0.20) compared to A (0.00 ± 0.00), moderate cytoplasmic vacuolation (fig. 2D) in Group E (2.20 ± 0.15) and moderate pyknosis (fig. 2B and fig. 2D) of the hepatocytes in Group E (2.05 ± 0.26) compared to A (0.00 ± 0.00) (Table 6). There was also mild to moderate karyolysis of the hepatocytes in Group E (1.85 ± 0.22) compared to A (0.00 ± 0.00). There was significant mild to moderate activated Kupffer cells in Group E (1.85 ± 0.22) compared to A (0.00 ± 0.00), hepatic regeneration in Group E (1.90 ± 0.07) compared to A (0.55 ± 0.24). Moreover, Group E showed significant $p<0.05$ mild cellular infiltrations (fig. 2C and fig. 2D) (1.60 ± 0.21)

TABLE 5: BIOCHEMICAL PARAMETERS (MEAN \pm SEM) OF FEMALE ICR MICE IN SUB-CHRONIC TOXICITY STUDY OF CELE

| Parameters | A | B | C | D | E |
|---------------------------|--------------------|-------------------|--------------------|----------------------|--------------------|
| Urea (mmol/l) | 7.03 \pm 0.25 | 9.54 \pm 0.62 | 9.36 \pm 0.88 | 8.02 \pm 0.41 | 10.22 \pm 0.74* |
| Creatinine (μ mol/l) | 30.50 \pm 1.31* | 41.80 \pm 1.17 | 44.40 \pm 0.99 | 42.80 \pm 1.71 | 43.24 \pm 2.25 |
| ALT (U/l) | 239.10 \pm 22.5 | 326.30 \pm 15.1 | 336.20 \pm 27.7* | 425.60 \pm 25.6* | 362.52 \pm 27.0* |
| AST (U/l) | 207.90 \pm 19.7* | 304.30 \pm 19.0 | 304.60 \pm 15.3 | 348.60 \pm 21.5 | 353.85 \pm 16.8 |
| CK (U/l) | 636.8 \pm 45.81 | 574.0 \pm 52.27 | 648.9 \pm 70.86 | 1516.1 \pm 150.15* | 716.5 \pm 74.90 |
| TP (g/l) | 57.58 \pm 4.20 | 58.24 \pm 1.65 | 55.22 \pm 1.52 | 58.46 \pm 2.19 | 59.73 \pm 2.35 |
| ALB (g/l) | 32.05 \pm 2.01 | 31.34 \pm 0.76 | 32.08 \pm 1.44 | 31.19 \pm 0.89 | 34.27 \pm 1.79 |
| Globulins (g/l) | 25.53 \pm 2.66 | 26.90 \pm 1.30 | 23.14 \pm 1.00 | 27.27 \pm 1.88 | 25.47 \pm 2.55 |

Note: CELE-C. *nutans* ethanolic leaf extract; A-Control; B-125 mg/kg CELE; C-250 mg/kg CELE; D-500 mg/kg CELE; E-1000 mg/kg CELE; values in the same row with asterisk differ significantly ($p<0.05$)

TABLE 6: LESION SCORES (MEAN \pm SEM) OF THE LIVER OF FEMALE ICR MICE IN SUB-CHRONIC TOXICITY STUDY OF CELE

| Lesions | A | B | C | D | E |
|-------------------------|-----------------|------------------|-----------------|-----------------|------------------|
| Hydropic degeneration | 0.35 \pm 0.18 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.30 \pm 0.20 | 2.20 \pm 0.15* |
| Eosinophilic cytoplasm | 0.00 \pm 0.00 | 0.10 \pm 0.10 | 0.30 \pm 0.15 | 0.40 \pm 0.28 | 2.15 \pm 0.20* |
| Pyknosis | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.35 \pm 0.24 | 2.05 \pm 0.26* |
| Karyolysis | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 1.85 \pm 0.22* |
| Sinusoidal dilatation | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.15 \pm 0.15 | 0.85 \pm 0.32* |
| Activated Kupffer cells | 0.00 \pm 0.00 | 0.15 \pm 0.15 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 1.85 \pm 0.22* |
| Inflammation | 0.10 \pm 0.10 | 0.45 \pm 0.19 | 0.30 \pm 0.15 | 0.60 \pm 0.21 | 1.60 \pm 0.21* |
| Regeneration | 0.55 \pm 0.24 | 1.75 \pm 0.21* | 0.95 \pm 0.17 | 1.30 \pm 0.19 | 1.90 \pm 0.07* |

Note: CELE-C. *nutans* ethanolic leaf extract; A-Control; B-125 mg/kg CELE; C-250 mg/kg CELE; D-500 mg/kg CELE; E-1000 mg/kg CELE; *-significantly different at $p<0.05$

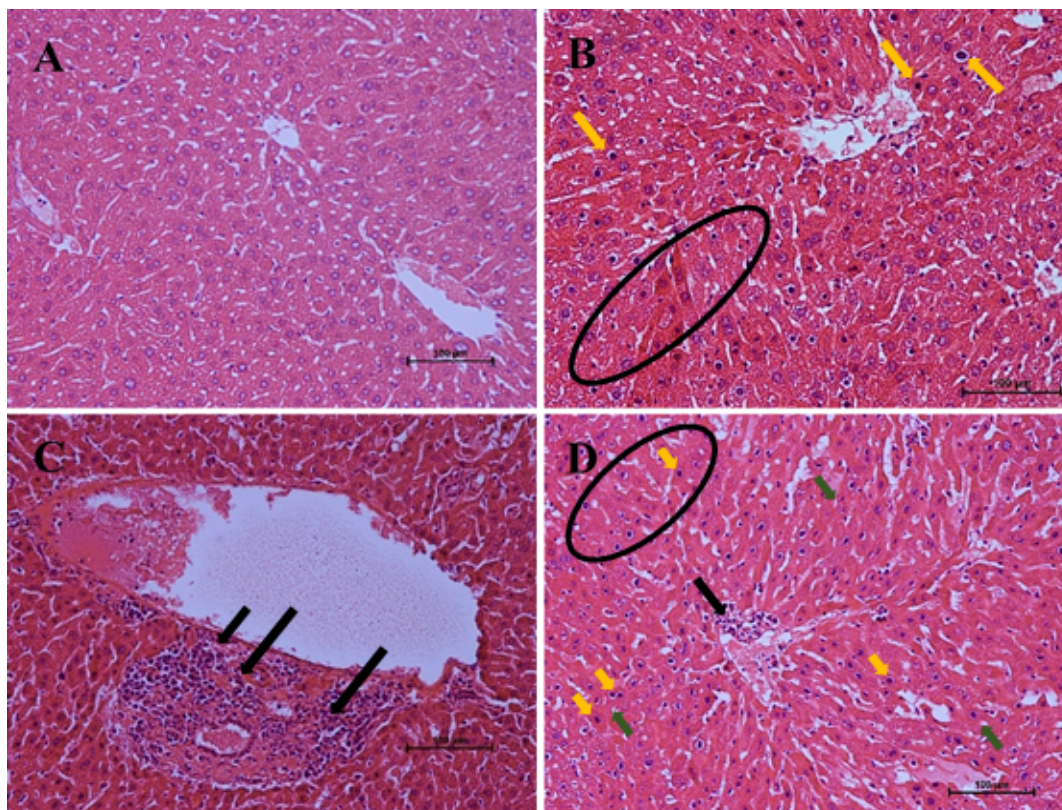


Fig. 2: Effects of repeated oral administration of CELE for 90 d on the histology of liver of female ICR mice. (A) Photomicrograph of a liver section (H&E stain 200x) from a mouse in Group A (control) showing normal architecture of liver; (B) In Group D (500 mg/kg CELE) showing eosinophilic cytoplasm (encircled) and pyknosis of the hepatocytes (yellow arrow); (C) In Group D (500 mg/kg CELE) showing cellular infiltration (black arrows); (D) In Group E (1000 mg/kg CELE) showing vacuolation (green arrows), eosinophilic cytoplasm (encircled) and pyknosis of the hepatocytes (yellow arrows) as well as cellular infiltrations (black arrows); H&E-Haematoxylin and Eosin; CELE-*C. nutans* ethanolic leaf extract; Scale bars represent 100 μ

compared to Group A (0.10 ± 0.10). These findings correspond to those of plasma biochemistry, where there were statistically significant ($p < 0.05$) differences in the liver parameters between the control and treatment groups (Table 5).

The histopathologic effects of oral administration of different doses of CELE daily for 90 d on the kidneys of female Institute of Cancer Research (ICR) mice are presented on Table 7. Kruskal-Wallis H test showed statistically significant ($p < 0.05$) differences in the lesion score between the different treatment groups (Table 7). Pairwise comparisons test revealed a mild to moderate renal cytoplasmic vacuolation (fig. 3B and fig. 3D) in Group E (1.80 ± 0.08) compared to A (0.00 ± 0.00). There was moderate renal necrosis characterised by significant ($p < 0.05$) eosinophilic cytoplasm (1.75 ± 0.24) in Group E (fig. 3C) compared to A (0.00 ± 0.00). Furthermore, there was mild ($p < 0.05$) pyknosis (0.90 ± 0.27) and karyolysis (0.60 ± 0.26) of the renal tubules in Group E compared to A (0.00 ± 0.00). Furthermore, there was mild ($p < 0.05$) inflammatory cellular infiltration (fig. 3B and fig. 3D) in Groups D (0.90 ± 0.21) and E (0.75 ± 0.25) compared to A (0.00 ± 0.00) (Table 7).

Evaluation of toxic effects of plants is very essential in determining its safety in both human and animal subjects. Toxicological studies are usually performed in animals to establish the effects of a chemical or substance on their biological system, to predict the possible effects and/or doses of the same substance in humans^[33,34]. Liver and kidney are the main organs for consideration in oral toxicity study. This is because compounds administered orally are metabolized by the liver and excreted by the kidney^[35]. Acute toxicity study may possibly provide significant information that could help in identifying the targeted organs of the test substances following acute exposure^[35]. However, sub-acute and sub-chronic toxicity studies assess the adverse effects of repeated administration of a chemical or substance on certain parameters of experimental animals. This is to generate evidences on the target-organ toxicity and bioaccumulation potentials of the substance, which are essential in determining the No-Observed-Adverse-Effect Levels (NOAELs), in order to establish doses for human exposure^[15]. The relative internal organ weights provide information as whether or otherwise the organs are affected by the administration of the test substance.

TABLE 7: LESION SCORES (MEAN±SEM) OF THE KIDNEY OF FEMALE ICR MICE IN SUB-CHRONIC TOXICITY STUDY OF CELE

| Lesions | A | B | C | D | E |
|------------------------|-----------|-----------|-----------|------------|------------|
| Hydropic degeneration | 0.00±0.00 | 0.35±0.24 | 0.00±0.00 | 0.80±0.27 | 1.80±0.08* |
| Eosinophilic cytoplasm | 0.10±0.10 | 0.40±0.28 | 0.00±0.00 | 1.20±0.27 | 1.75±0.24* |
| Pyknosis | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.90±0.27* |
| Karyolysis | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.60±0.26* |
| Nephritis | 0.30±0.15 | 0.20±0.13 | 0.15±0.15 | 0.90±0.21* | 0.75±0.25* |
| Protein casts | 0.65±0.18 | 0.10±0.10 | 0.20±0.13 | 0.30±0.15 | 0.40±0.16 |
| Cellular casts | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.10±0.10 |
| Granular casts | 0.00±0.00 | 0.10±0.10 | 0.00±0.00 | 0.10±0.10 | 0.20±0.13 |

Note: CELE-*C. nutans* ethanolic leaf extract; A-Control; B-125 mg/kg CELE; C-250 mg/kg CELE; D-500 mg/kg CELE; E-1000 mg/kg CELE; *-significantly different at $p < 0.05$

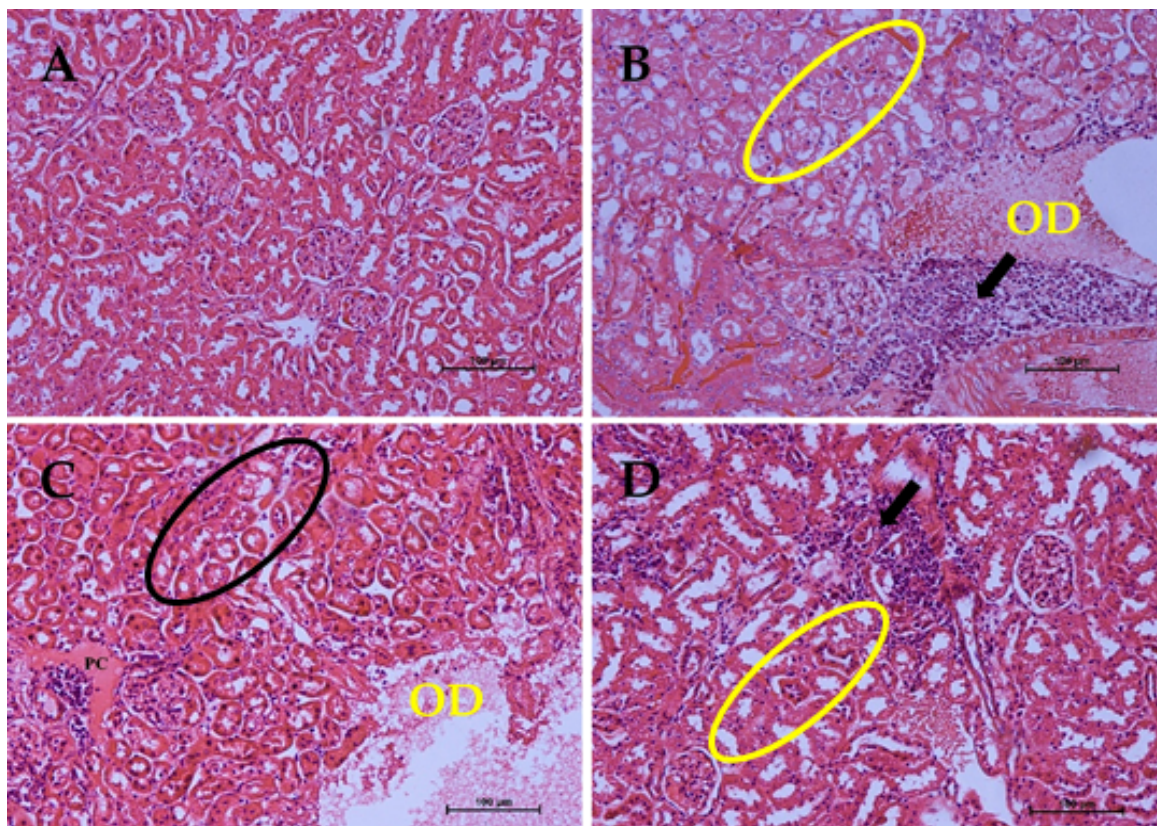


Fig. 3: Effects of repeated oral administration of CELE for 90 d on the histology of kidney of female ICR mice. (A) Photomicrograph of a kidney section (H&E stain, 200x) from a mouse in Group A (control) showing normal structure of kidney; (B) In Group C (250 mg/kg CELE) showing vacuolation of the renal tubules (yellow encircled), cellular infiltration (black arrow) and oedema (OD); (C) In Group E (1000 mg/kg CELE) showing eosinophilic cytoplasm (black encircled) and oedema (OD); (D) In Group E, showing cellular infiltration (Black arrow) and vacuolation of the renal tubular cells (yellow encircled); H&E-Haematoxylin and Eosin; Scale bars represent 100 μ

Repeated oral administration of CELE daily for 90 d in this study showed certain abnormalities in the mice. The significant reduction in weight gain observed in the mice treated with 1000 mg/kg CELE daily for 90 d, could suggest that the extract affected the mice's feed intake^[10,36] or has resulted in the reduction in the deposition of fats^[37,38]. Decreased body weight gain in animals has been associated with toxicity following exposure to potential toxic chemical or substances^[39,40]. These findings are in agreement with the report of

Chavalittumrong *et al.*^[41], where a significant decrease in body weight of male rats treated with 1000 mg/kg of *C. nutans* ethanolic extract daily for 90 d compared to the control group was observed. Similarly, Aliyu *et al.*^[10] reported a significant decreased in the body weight gain of mice administered with repeated doses of *C. nutans* daily for 28 d at 500 and 1000 mg/kg doses^[10]. However, the result is contrary to the report of Zakaria *et al.*^[15], who reported that repeated oral administration of methanolic extract of *C. nutans* daily for 28 d did not affect the body weight of the mice even

at the highest dose of 2500. This perhaps may be due to shorter duration of exposure^[39,40] compared to the present study.

Previous study from our group by Aliyu *et al.*^[10] has shown that oral administration of high dose of CELE at 2000 mg/kg once and repeated oral administration of lower doses of the extract at 125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg daily for 28 d did not affect the haematological parameters of ICR mice^[10]. However, administration of the extract for longer duration of 90 d in the current study resulted in abnormal haematological values. The significant increase in total White Blood Cells (WBCs) and differential counts, particularly the neutrophilia and monocytosis observed in the mice administered 1000 mg/kg CELE daily for 90 d, indicates some ongoing inflammatory conditions^[42] in the mice, perhaps due to some adverse effects of the extracts on some vital organs. The neutrophilia and increased lymphocytes and eosinophils counts observed in this study were contrary to the earlier reports by Sajjaratul *et al.*^[5], Zakaria *et al.*^[15] and P'ng *et al.*^[16], who found that repeated administration of *C. nutans* extracts did not affect the haematological parameters of both rats and mice. The differences could be due to the shorter duration of administration^[15,16], different experimental animals^[5,16] and/or different extraction solvents used^[9,43-45].

The results of plasma biochemical parameters showed that daily oral administration of CELE for 90 d at 500 and 1000 mg/kg doses has effect on some plasma biochemical parameters. The significant increase in urea observed at 1000 mg/kg dose and increase in creatinine level seen at 125, 250, 500 and 1000 mg/kg for 90 d in this study, suggested that the extract especially at higher doses of 500 and 1000 mg/kg may possess some adverse effects on the kidneys of the ICR mice^[46-48]. This is because creatinine, which is the product of creatine metabolism, is solely excreted by the kidneys, hence any injury to the kidneys may result in hypercreatinemia and therefore creatinine is considered as an important marker of renal injury^[49]. These findings were similar to the report of Zakaria *et al.*^[15] and Aliyu *et al.*^[10], where there was significant increase in the level of creatinine in both male and female mice treated with both 500 and 1000 mg/kg *C. nutans* repeatedly for 28 d. However, Chavalittumrong *et al.*^[41] reported a significant decrease in the creatinine levels in the male rats treated with 1000 mg/kg *C. nutans* ethanolic extract daily for 90 d compared to the control. The notable elevation in the levels of ALT, AST and CK in the group treated

with 500 and 1000 mg/kg CELE daily for 90 d, could suggest that the extract at these high doses might have affected the normal function of liver and/or muscles in the treated mice^[47,50,51]. This was further confirmed by the results of the histopathological evaluation of liver in this study. Increased serum activities of AST and CK, with a corresponding increase in Lactate Dehydrogenase (LDH) activity are associated with muscle injury, increased muscle activity or myocardial infarction^[52]. However, the significant elevation in the levels of AST in this study may be associated with the release of the enzymes from other organs of the body, including skeletal muscles, kidneys, erythrocytes, lungs, pancreas, brain, lungs and heart^[21,50].

The histopathological evaluation of liver and kidney of the experimental mice in this study, revealed that daily oral administration of CELE for 90 d at 1000 mg/kg dose resulted in various hepatic and renal lesions. The significant hepatic degeneration and necrosis observed histopathologically in the group of mice administered with daily oral doses of CELE at 1000 mg/kg for 90 d were suggestive of toxic or adverse effects^[5,53,54] of the extract on the animals, as earlier observed in the results of plasma biochemical parameters for hepatic injury markers (ALT and AST). Similarly, the renal tubular degeneration and necrosis observed in this study further justified the elevation of AST level, which also corroborate the increase in the plasma creatinine levels. These also provide more indication that administration of the extract at 1000 mg/kg is not safe^[53,54] in the treated mice. Severe hepatic damage has been reported to be associated with oxidative stress and depletion of Adenosine triphosphate (ATP) leading to necrosis of hepatocytes^[55,56]. Nevertheless, the significant increase in the kidney injury marker observed in the groups treated with 125 and 250 mg/kg of the extract did not tally with the histological findings. This may suggest that the increased plasma concentration of urea and creatinine at these lower concentrations of the extract could be due to pre or post renal azotaemia resulting from either muscle injury, increased protein catabolism or blockage in the lower urinary tract^[47,57].

The interstitial oedema observed in this study could suggest further that the extract might have induced renal injury in the treated mice and also indicated by the hypoalbuminaemia observed in the CELE treated groups of mice^[58], which might have resulted from Protein Losing Nephropathy (PLN). Decreased ALB levels in the treated mice could affect the effective colloid osmotic pressure gradient negatively, thereby

causing excess filtration of fluid from the intravascular to interstitial spaces^[59,60] portrayed by oedema of the renal interstitium (interstitial oedema) in the treated mice. Alternatively, the decreased level of ALB observed in the treated mice, could be due to decrease feed intake^[37,38] and/or liver disease^[47,50,51], as depicted by the decreased body weight gains of the mice and the significant alterations in the liver injury markers (ALT and AST). Renal interstitial oedema may also be as a result of renal inflammation^[61], as indicated by the significant neutrophilia, monocytosis and cellular infiltrations in the group of mice treated with 500 and 1000 mg/kg of the extract. Tanaka and Nangaku^[61] reported that tubular interstitial nephritis is characterized histologically by inflammatory changes including interstitial oedema, leukocyte infiltration, accumulation of extracellular matrix proteins, tubular dilation and renal atrophy in the tubulointerstitial compartment. The inflammation observed in this study, might have caused increase vascular permeability leading to escape of fluid into the renal interstitium^[59]. Similarly, the ongoing inflammatory process might have triggered the release of inflammatory mediators causing vasodilation and inducing leukocytes infiltration^[59].

It is concluded from this study that, repeated oral doses of CELE for 90 d induced significant hepatic and renal degeneration and necrosis at 1000 mg/kg in female ICR mice. Hence, the plant extract at this dose may be considered unsafe for medicinal purposes and/or food supplementation.

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Conflict of interests:

The authors declared no conflict of interest.

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