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Sub-Acute Oral Toxicity of Aqueous Extract of Fresh Leaves of *Calotropis Procera* in Wistar Rats: Biochemical and Histopathological Evaluation

[1]Isuwa Bitrus Kashimbe, *[1]Ayuba David Telta, [1]Mohammed Bashir Tijani, [1]Mohammed Shuaibu Auwal, [1]Mohammed Hassan Gusi, [1]Emmanuel Solomon Musa, [1]Bukar Umaru, [1]Emmanuel Oluwasegun Adawaran, [1]Karagama Parnda Gadzama, [1]Shamaki B. U. and [2]Aliu Yahaya Gwoikura

[1]Department of Pharmacology and Toxicology, University of Maiduguri, Borno State, Nigeria.
[2]Veterinary Teaching Hospital, University of Maiduguri, Borno State, Nigeria.

*Corresponding authors' email: ayubatel@gmail.com Phone: +2348062545410

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ABSTRACT

Calotropis procera is a drought-tolerant medicinal plant with recognized toxic potential. This study evaluated biochemical and histopathological changes following repeated oral administration of its aqueous fresh leaf extract in Wistar rats. Fresh leaves were collected from the University of Maiduguri campus, authenticated by a taxonomist, processed, and extracted by aqueous decoction. Sixteen Wistar rats were divided into four groups (n = 4). The control received no extract, while treatment groups received 200, 400, or 800 mg/kg body weight orally once daily for 21 days. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, creatinine, total protein, and albumin were assessed. The liver, kidneys, lungs, heart, stomach, and brain were examined histopathologically. AST increased from 6.3 ± 0.7 U/L in controls to 8.0 ± 0.6 U/L at 800 mg/kg, while ALT increased from 6.3 ± 0.9 to 8.0 ± 0.0 U/L. ALP increased dose-dependently from 43.7 ± 8.0 to 88.3 ± 0.9 U/L. At 800 mg/kg, urea, total protein, and albumin decreased significantly, whereas creatinine remained relatively unchanged. Histopathological findings included renal tubular necrosis, glomerular atrophy, hepatocellular necrosis, pulmonary lesions, cardiac nuclear degeneration and necrosis, and gastric mucosal injury, generally more severe at higher doses. Mild cerebral oedema occurred at 800 mg/kg. Despite limited changes in some biochemical indices, repeated administration caused histopathological alterations in multiple organs. These findings highlight the limitations of biochemical parameters alone in detecting tissue injury and warrant caution regarding prolonged oral use of *C. procera* leaf preparations.

Keywords: *Calotropis Procera,* Aqueous Extract, Sub-Acute Toxicity, Biochemical Parameters, Nephrotoxicity, Wistar Rats.

INTRODUCTION

Traditional medicine, including the use of medicinal plants and herbal remedies, remains an important component of healthcare in many communities because of its accessibility, affordability, and cultural acceptability (WHO, 2025). However, the perception that preparations derived from natural sources are inherently safe may contribute to inappropriate or prolonged use without adequate toxicological evaluation. Assessment of both beneficial and adverse biological effects is therefore essential before medicinal plants can be recommended for sustained therapeutic use.

Calotropis procera (Aiton), is a perennial, drought-tolerant shrub belonging to the family Apocynaceae. It is widely distributed in tropical and subtropical regions of Africa and Asia and is commonly found in dry environments, roadsides, abandoned agricultural lands, wastelands, and overgrazed areas. In Nigeria, the plant is known by several local names, including Tumfafiya in Hausa and Kayôu in Kanuri.

Different parts of *C. procera*, including the leaves, latex, roots, bark, flowers, and seeds, have traditionally been used for the management of various ailments. They have been reported to have pharmacological activities such as analgesic, anti-inflammatory, antimicrobial, antipyretic, antiparasitic, antioxidant, anticancer, and antidiarrhoeal effects. These activities have been attributed to diverse secondary metabolites present in the plant.

Despite its medicinal relevance, *C. procera* has also been associated with toxic effects (Wadhwani *et al*., 2022). The plant contains cardiac glycosides and other biologically active compounds that may produce toxic effects depending on the plant part, preparation, dose, route of administration, and duration of exposure. Cardiac glycosides are particularly important because of their ability to inhibit Na^+/K^+-ATPase and produce effects resembling those of other cardiotonic glycosides. Previous experimental studies have associated exposure to *C. procera* with pathological alterations involving multiple organ systems, including the cardiac, hepatic, renal, respiratory and gastrointestinal systems (De Lima *et al*., 2011; Singh & Kaushik, 2023). The toxicological profile of plant preparations may vary considerably according to the extraction method, as different solvents can extract different groups and amounts of bioactive constituents. This is particularly relevant to *Calotropis procera*, for which aqueous, ethanolic and other solvent extracts have been reported to differ in their phytochemical profiles and biological activities. Habeeb *et al.,* (2024), in a review specifically examining the aqueous leaf extract of *C. procera*, noted that the solubility and extraction of bioactive compounds depend on the solvent used and that differences in extraction procedures may influence the resulting phytochemical composition and biological effects.

Aqueous preparations are particularly relevant because water-based preparations, including decoctions and infusions, are

commonly used in traditional medicinal practices. The use of aqueous preparations of *C. procera* has been documented in traditional medicine, while water is also considered an accessible and commonly used extraction medium. In addition, variation in the phytochemical profile of *C. procera* has been associated with agro-environmental and geographical conditions. Habeeb *et al*., (2024), specifically noted that differences in the chemometric profile of aqueous leaf extracts among studies could be attributed to the agro-environmental conditions of the locations from which the plant material was obtained. More recent evidence has further emphasized the influence of environmental and phytogeographical factors on the phytochemical composition and biological properties of *C. procera*.

Accordingly, factors such as geographical origin, environmental conditions, plant developmental stage and preparation procedures should be considered when comparing the toxicity of different *C. procera* preparations. Differences in these factors may alter the concentration and relative proportions of biologically active constituents and, consequently, may contribute to variation in pharmacological and toxicological responses.

Acute toxicity studies provide important information about immediate lethality but may not adequately predict adverse effects associated with repeated exposure. Repeated-dose studies incorporating biochemical and histopathological assessment are therefore necessary to identify target organs and subtle tissue injury that may occur in the absence of mortality.

This study investigated the effects of 21-day oral administration of an aqueous extract prepared from fresh leaves of *C. procera* on selected serum biochemical parameters and the histopathological architecture of major organs in Wistar rats. The study was designed to determine the effect of repeated exposure on hepatic, renal, cardiovascular, pulmonary, gastrointestinal, or neurological injury. It was hypothesized that repeated oral administration of aqueous fresh leaf extract of Calotropis procera for 21days would produce dose dependent alterations in selected serum biochemical parameters and histopathological changes in vital organs of Wistar rats, indicating potential hepatic, renal, cardiovascular, pulmonary, and gastrointestinal toxicity.

MATERIALS AND METHODS

Plant Collection and Identification

Fresh leaves of *C. procera* were collected from the University of Maiduguri campus, Maiduguri, Borno State, Nigeria. The plant was authenticated by a taxonomist in the Department of Botany, University of Maiduguri, Prof. M. A. Sunusi and a voucher specimen was deposited in the Veterinary Pharmacology laboratory University of Maiduguri under voucher number Species Vet. 209 A.

Preparation of Aqueous Leaf Extract

The fresh leaves of *C. procera* were washed with tap water, then cut into smaller pieces, and extracted using distilled water. Approximately 1600 g of fresh leaves were placed in a round bottom flask containing 2 L of distilled water and boiled at 100 °C for 3 h. Following cooling, the decoction was filtered through muslin cloth. The filtrate was spread in a tray and dried in a hot air oven at 40 °C for 24 h. The dried material yielded 40 g of extract, which was stored in an airtight container until administration. The yield of the aqueous leaf extract was calculated using the formula:

$$\% \text{ Yeild} = \frac{\text{Weight of the extract}}{\text{weight of the fresh leves}} \text{ x } 100$$

Experimental Animals and Ethical Approval

Sixteen Wistar rats were used for the sub-acute toxicity experiment. The animals were obtained from the Department of Pharmacy, University of Maiduguri, and were housed in the Department of Veterinary Physiology and Biochemistry Animal House. The animals were allowed to acclimatize for two weeks before commencement of the experiment. Ethical approval for animals used were obtained from the Animal Ethical Committee of the Faculty of Veterinary Medicine, University of Maiduguri with the approval number VPX/IAEC/1306-26, dated 30 April 2024.

Experimental Design and Treatment

The sixteen rats were randomly allocated into four groups (n = 4 per group). The control group received distilled water without the extract, while the treatment groups were administered 200, 400, and 800 mg/kg body weight of the aqueous leaf extract, respectively, by oral gavage once daily for 21 consecutive days. Blood samples were collected from the rats weekly throughout the 21-days treatment period, and the serum was separated and harvested for biochemical analyses. At the end of the treatment period, the animals were humanely sacrificed, and the liver, lungs, heart, kidneys, stomach, and brain were carefully harvested and processed for histopathological examination (OECD, 2025).

Biochemical Analyses

The serum samples collected were analyzed for selected biochemical parameters of hepatic and renal function. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using commercial Randox reagent kits based on the Reitman and Frankel colorimetric method, while alkaline phosphatase (ALP) activity was determined using a commercial Randox reagent kit according to the manufacturer's instructions. These analytical procedures are consistent with those reported in recent experimental studies involving serum biochemical assessment in laboratory animals (Egbung *et al.,* 2025; Hussaini *et al.,* 2025). Serum total protein was determined using the Biuret method, while albumin was estimated using the bromocresol green method. Creatinine concentration was determined using the Jaffe reaction, and serum urea was estimated using the urease-Berthelot method (Egbung *et al.,* 2025; Hussaini *et al.,* 2025).

Histopathological Examination

Following the 21-day treatment period, the harvested organs, including the liver, kidneys, lungs, heart, stomach, and brain, were immediately fixed in 10% neutral-buffered formalin and subsequently processed for histopathological evaluation. Tissue sections from the control and treatment groups were examined microscopically for evidence of structural alterations, degeneration, necrosis, congestion, cellular infiltration, oedema, and other pathological changes. The fixation and histological evaluation procedures were consistent with established recommendations for veterinary histopathological examination (Chambers *et al.,* 2025; NIEHS, 2023)

Statistical Analysis

Data collected in these experiments were expressed as mean ± standard error of the mean (SEM). The differences among experimental groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was set at $p \leq 0.05$. The analyses were conducted using IBM SPSS Statistics version 24.

RESULTS AND DISCUSSION

Yield of the Extract

The total weight of *Calotropis procera* leaves used for extraction was 1600g. Following aqueous extraction, 40g of dried extract was obtained, resulting in a percentage yield of 2.5% w/v.

Repeated oral administration of the aqueous leaf extract produced increases in serum AST, ALT, and ALP activities. AST increased from 6.3 ± 0.7 U/L in the control group to 7.7 ± 0.3 U/L in both the 200 and 400 mg/kg groups and to 8.0 ± 0.6 U/L at 800 mg/kg. ALT increased from 6.3 ± 0.9 U/L in controls to 7.3 ± 0.3 U/L at both 200 and 400 mg/kg and to 8.0 ± 0.0 U/L at 800 mg/kg. The most pronounced change occurred with ALP. Mean ALP activity increased from 43.7 ± 8.0 U/L in controls to 50.7 ± 5.8 U/L, 77.0 ± 6.0 U/L, and 88.3 ± 0.9 U/L at 200, 400, and 800 mg/kg, respectively. The reported group differences were significant and indicated by different superscripts ($p < 0.05$) in table 1.

Table 1: Serum Liver Enzyme Activities of Rats Following 21-day Administration of Aqueous *C. Procera* Leaf Extract, Represented in Mean+Standard Error of Mean (X+SEM)

Liver Enzymes	Experimental Groups ((X±SEM))			
	Control	200mg/kg	400mg/kg	800mg/kg
AST (U/L)	6.3±0.7	7.7±0.3[a]	7.7±0.3[a]	8.0±0.6[b]
ALT (U/L)	6.3±0.9	7.3±0.3[a]	7.3±0.3[c]	8.0±0.0[b]
ALP (U/L)	43.7±8.	50.7±5.8[a]	77.0±6.0[b]	88.3±0.9[c]

Values bearing different superscript letters within rows indicate significant differences at $p \leq 0.05$.

Serum urea was 3.1 ± 0.20 mmol/L in the control group, compared with 3.3 ± 0.30, 3.0 ± 0.30, and 2.6 ± 0.23 mmol/L in the 200, 400, and 800 mg/kg groups, respectively. The decrease observed at 800 mg/kg was reported as statistically significant.

Creatinine concentrations remained relatively stable between the control, 200 mg/kg, and 800 mg/kg groups. The result reports 58.3 ± 2.33 µmol/L in controls, 59.3 ± 2.90 µmol/L at 200 mg/kg, 53.0 + 0.30 at 400 mg/ka and 55.7 ± 1.45 µmol/L at 800 mg/kg.

Total protein decreased from 66.3 ± 1.45 g/L in controls to 68.7 ± 2.60, 65.0 ± 1.20, and 62.3 ± 2.60 g/L at 200, 400, and 800 mg/kg respectively. The reduction at 800 mg/kg was reported as significant.

Albumin decreased from 36.0 ± 0.60 g/dL in controls to 35.3 ± 0.33 g/dL and 35.3 ± 1.86 g/dL at 200 and 400 mg/kg, respectively, and to 33.0 ± 0.57 g/dL at 800 mg/kg, with the superscription reported as statistically significant.

Table 2: Serum Biochemical Parameters of Rats Following 21-day Administration of Aqueous C. Procera Leaf Extract, Values are Reported in Mean ± SEM

Parameter	Control	200 mg/kg	400 mg/kg	800 mg/kg
Urea (mmol/L)	3.1 ± 0.20	3.3 ± 0.30	3.0 ± 0.30	2.6 ± 0.23[a]
Creatinine (µmol/L)	58.3 ± 2.33	59.3 ± 2.90	53.0 + 0.30	55.7 ± 1.45
Total protein (g/L)	66.3 ± 1.45	68.7 ± 2.60	65.0 ± 1.20	62.3 ± 2.60[b]
Albumin (g/dL)	36.0 ± 0.60	35.3 ± 0.33	35.3 ± 1.86	33.0 ± 0.57[a]

Different superscripts indicate significant differences at $p < 0.05$.

Histopathological Findings

Kidney

The kidneys of rats in control group exhibited normal histological architecture, with intact glomeruli and renal tubules. At 200 mg/kg, moderate renal tubular necrosis accompanied by mild interstitial haemorrhage was observed. At 400 mg/kg, mild glomerular atrophy and renal tubular necrosis were evident. The 800 mg/kg group showed more pronounced renal tubular necrosis. These findings indicate progressive renal structural injury following repeated exposure.

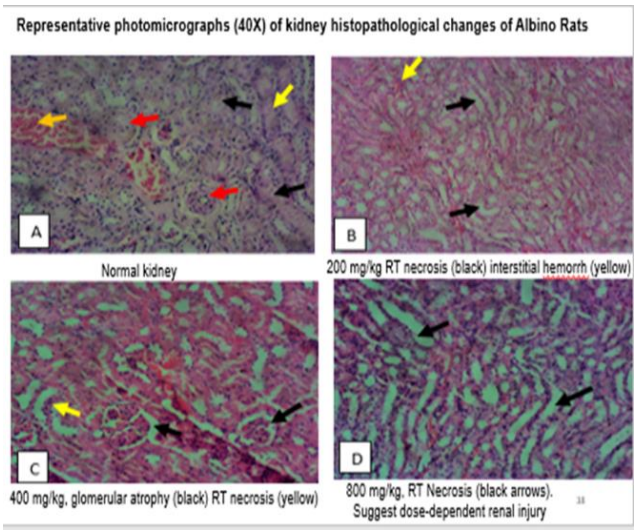


Figure 1: Photomicrographs of kidney

Liver

Control animals showed normal hepatic architecture, including intact hepatocytes and clearly defined central veins. Mild hepatocellular necrosis with cellular infiltration around the central vein was observed at 200 mg/kg. At 400 mg/kg, mild hepatocellular necrosis and cellular infiltration around the portal triad were present. The 800 mg/kg group exhibited more pronounced, moderate hepatocellular necrosis, consistent with increasing hepatic tissue injury.

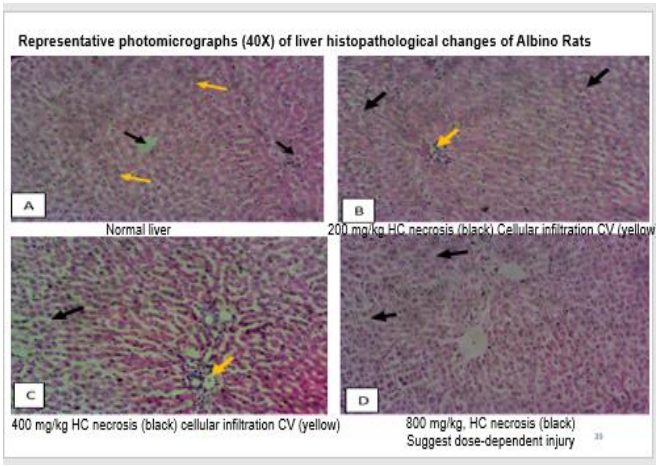


Figure 2: Photomicrographs of liver

Lungs

The lungs of control rats showed normal bronchioles, blood vessels, and alveolar architecture. At 200 mg/kg, loss of bronchiolar epithelium and thickening of the interalveolar spaces were observed. The 400 mg/kg group showed vascular congestion and moderate necrosis of the interalveolar septa. At 800 mg/kg, extensive necrosis of the interalveolar septa and loss of bronchiolar epithelium were evident.

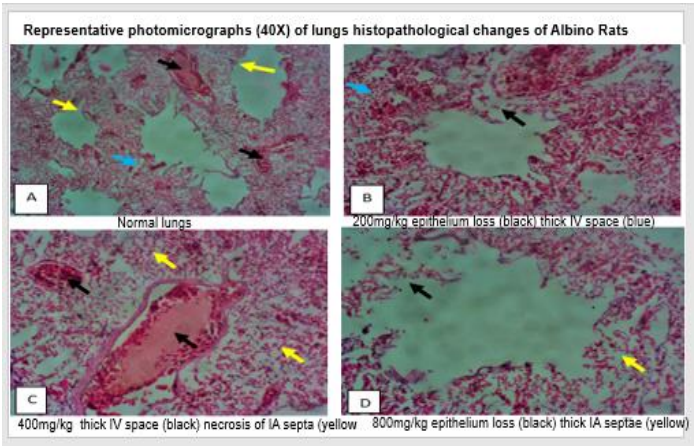


Figure 3: Photomicrographs of lungs

Heart

Control cardiac tissue exhibited normal striated muscle fibres and well-defined nuclei. At 200 mg/kg, the striated muscle fibres remained largely preserved, although mild nuclear necrosis was observed. At 400 mg/kg, nuclear degeneration and necrosis accompanied by mild congestion were evident. Extensive nuclear necrosis and degeneration were observed in the 800 mg/kg group.

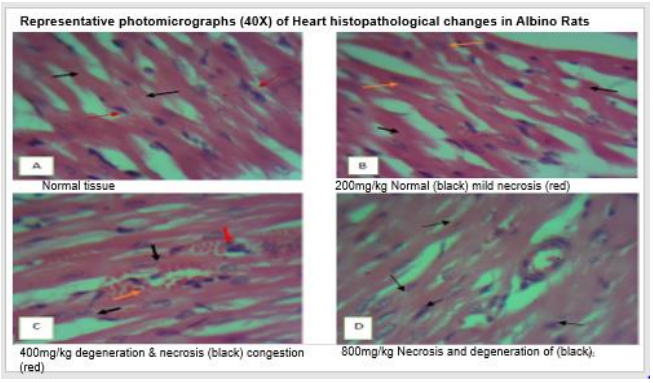


Figure 4: Photomicrographs of heart

Stomach

The control group displayed normal gastric architecture, including intact gastric mucosa, lamina propria, gastric pits, and mucosal glands. The 200 mg/kg group retained generally normal gastric architecture. At 400 mg/kg, mild necrosis of the gastric mucosal epithelium and widening of the lamina propria were observed. At 800 mg/kg, extensive mucosal epithelial necrosis and marked widening of the lamina propria were evident.

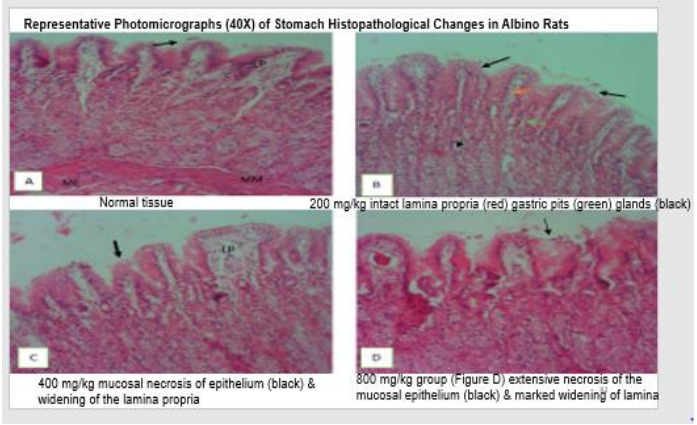


Figure 5: Photomicrographs of Stomach

Brain

The control brain showed normal neuronal and vascular architecture. At 800 mg/kg, mild cerebral oedema was observed, although neuronal cells appeared relatively preserved.

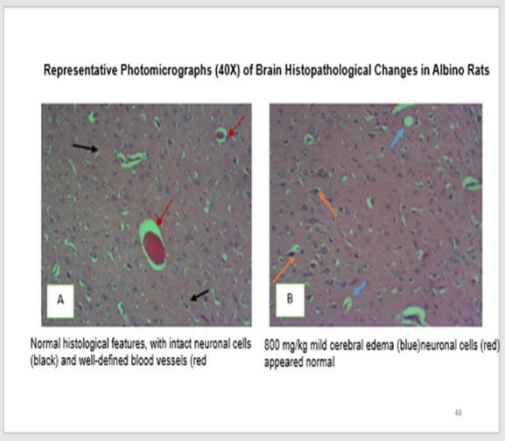


Figure 6: Photomicrographs of Brain

Discussion

The study demonstrates that repeated oral exposure to aqueous extract fresh *C. procera* leaves can produce measurable biochemical changes and structural changes in the treated organs of the experimental rats.

The serum transaminases (AST and ALT) showed slight increases across the treatment groups compared with the control group. A more pronounced increase was observed in ALP activity, particularly at the highest dose of the extract, suggesting a possible alteration in hepatic enzyme activity following extract administration. These biochemical alterations were accompanied by progressively more evident hepatocellular necrosis on histopathological examination, suggesting that repeated exposure to the extract produced hepatic tissue injury. The present findings are consistent with the findings of Mohammed *et al.,* (2012), in a 60-day study of aqueous *C. procera* leaf extract in Wistar rats, he reported no significant alterations in serum ALT, AST, ALP, albumin, total protein, or bilirubin; however, histopathological examination revealed mild vascular degenerative changes and necrosis in the liver, kidney, and heart. This observation is particularly relevant to the present study because both investigations demonstrate that histopathological changes may occur even when serum biochemical markers show relatively little alteration**.** The present study extends this observation by demonstrating a clearer dose-related increase in hepatic enzymes, particularly ALP, together with progressively more severe hepatocellular necrosis.

Similarly, Sarkodie *et al.,* (2022) investigated aqueous and ethanolic leaf extracts and latex of *C. procera* in Sprague-Dawley rats. Although their hematological and biochemical indices did not indicate marked toxicity, histopathological examination revealed mild alterations in liver architecture, leading the authors to advise caution with chronic consumption. The histopathological findings in the present study therefore agree with their observation that tissue level hepatic alterations may be detectable despite relatively unremarkable routine biochemical indices. In contrast, evidence of more pronounced hepatic and systemic toxicity has been reported following exposure of fresh *C. procera* latex in a study conducted by De Lima *et al*., (2011), that reported hepatocellular vacuolization and multifocal coagulation necrosis in rats while sheep administered *C. procera* leaves developed pathological changes including

pulmonary oedema, hepatic haemorrhage and cardiac lesions. The authors concluded that *C. procera* possesses cardiotoxic and hepatotoxic potential. Although that study used latex in rats and leaf material in sheep rather than the aqueous fresh-leaf extract used in the present experiment, its findings support the biological plausibility of the hepatic lesions observed in our study.

The apparent differences among these studies may be explained by variations in plant part, extraction solvent, dose, duration of exposure, animal species/strain, geographical origin and phytochemical composition. Aqueous extracts may contain a different spectrum and concentration of bioactive constituents compared with ethanolic extracts or latex. Likewise, fresh leaves may differ chemically from dried plant material because drying and processing can alter the concentration and stability of certain phytochemicals. Therefore, the absence of toxicity reported for one preparation of *C. procera* should not necessarily be extrapolated to other preparations.

The hepatic effects observed in the present study may be related to the complex phytochemical composition of the aqueous fresh-leaf extract of *Calotropis procera*. Previous phytochemical investigations have established that *C. procera* contains several major classes of secondary metabolites, particularly flavonoids, terpenoids, cardenolides, steroids, glycosides and phenolic compounds. Wadhwani *et al.*, (2021), in a comprehensive review, reported that flavonoids and their glycosides are important constituents of the leaves, whereas cardenolides and steroids are particularly prominent in the latex. They also documented the occurrence of terpenoids, hydrocarbons, saturated and unsaturated fatty acids, fatty acid esters and other compounds identified by chromatographic techniques. The biological effects of the extract are therefore likely to reflect interactions among multiple constituents rather than a single compound. Consequently, the hepatic alterations observed in the present study are likely to reflect the combined biological effects of multiple constituents rather than the activity of a single phytochemical**.** The concurrence of a chemically diverse extract with dose-related increases in hepatic enzymes and progressive hepatocellular necrosis provides evidence of a possible relationship between the phytochemical composition of the extract and its observed toxicological effects. Nevertheless, this relationship should be regarded as associative rather than causal because the present study did not individually quantify the detected phytochemicals or establish which specific constituents were responsible for the hepatic lesions.

The present study revealed a reduction in serum total protein and albumin concentrations, particularly at the highest dose of the aqueous extract of fresh *Calotropis procera* leaves following 21 days of oral administration. These decreases may suggest a possible effect of the extract on protein synthesis, metabolism, or nutritional and hepatic functions. These reductions occurred concurrently with histopathological evidence of hepatocellular necrosis, suggesting that repeated exposure to the extract may have adversely affected hepatic protein homeostasis and possibly hepatic synthetic function. However, the observed reduction should be interpreted cautiously because serum albumin and total protein can also be influenced by nutritional status, hydration, inflammatory processes and other systemic factors. The present finding differs from the study of Mohammed *et al*., (2012)**,** that investigated the effects of aqueous *C. procera* leaf extract in Wistar rats administered 500, 1000 and 1500 mg/kg for 60 days. Mohammed et al, reported no significant changes in serum total protein, albumin or globulin in the treated animals compared with controls. Interestingly, despite the absence of significant biochemical alterations, mild vascular degenerative changes and necrosis were observed histologically in the liver, kidney and heart. This discrepancy is important because both studies used aqueous leaf extracts in rats. It suggests that biochemical responses to *C. procera* may vary according to the characteristics of the plant material and extract, experimental dose, treatment duration and other experimental conditions.

The present observation is also different from the findings of Habeeb *et al*., (2024) which was revealed in a 14-day study in female rabbits in which administration of aqueous *C. procera* leaf extract at 20, 40 and 80 mg/kg produced no statistically significant changes in serum albumin or total protein**.** These differences may partly reflect the substantially lower doses used in the studies compared with the 800 mg/kg dose in the present experiment.

This study demonstrated histopathological evidence of renal injury following repeated oral administration of aqueous extract of fresh *Calotropis procera* leaves. Although serum creatinine remained relatively stable and serum urea decreased at the highest dose, renal histopathology revealed tubular necrosis in the treated groups, with mild glomerular atrophy observed at 400 mg/kg. The occurrence of renal lesions in the absence of corresponding elevations in conventional serum markers suggests that the renal injury may have been relatively early, mild or insufficiently severe to cause a measurable reduction in overall glomerular filtration during the 21-day exposure period. Thus, the findings highlight the importance of combining serum biochemical indices with histopathological examination in the assessment of repeated-dose nephrotoxicity. The renal histopathological observed in this experiment is consistent with observations involving *C. procera* by Mohammed, (2012).

Cardiac histopathology revealed a dose-related progression from mild nuclear necrosis at 200 mg/kg to extensive nuclear degeneration and necrosis at 800 mg/kg. This observation is particularly important because *C. procera* contains cardenolide/cardiac glycoside constituents. The dissertation identified cardenolides and cardiac glycosides during qualitative phytochemical screening. Cardiac glycosides are pharmacologically active compounds with a recognized capacity to influence cardiac function through Na^+/K^+-ATPase inhibition. The cardiac histological changes observed in the present study are therefore biologically plausible in the context of the phytochemical profile, although direct mechanistic attribution would require measurement of individual cardenolides and cardiac functional parameters

Cardiac histopathology in this study demonstrated a dose-related progression of myocardial injury, characterized by mild nuclear necrosis at 200 mg/kg and progressively more extensive nuclear degeneration and necrosis at 400 and 800 mg/kg. This dose-related pattern suggests that repeated oral exposure to the aqueous extract of fresh *Calotropis procera* leaves adversely affected cardiac tissue. The finding is consistent with the study conducted by Ahmed *et al.,* (2016)**,** that investigated the cardiac and testicular toxicity of *C. procera* latex and ethanolic leaf extract in male albino rats. They reported a dose- and time-dependent alterations in cardiac histology, including oedema, inflammatory-cell infiltration, degeneration and necrosis of cardiac myocytes. They also observed significant increases in serum CK-MB, AST and LDH, together with increased lipid peroxidation and depletion of antioxidant defenses in cardiac tissue. These findings closely support the present observation because both studies demonstrate progressive cardiac structural injury

following repeated exposure to *C. procera* and indicate that the severity of injury increases with increasing exposure.

The findings of De Lima *et al*., (2011) provided evidence supporting the cardiotoxic potential of *C. procera* in their experimental study, where rats administered fresh *C. procera* latex developed multifocal coagulation necrosis of cardiac muscle fibres at the highest dose. Similarly, sheep administered *C. procera* leaves developed tachycardia and transient cardiac arrhythmias, while histological examination revealed cardiac lesions ranging from mild to moderate, including myocardial necrosis. The authors concluded that *C. procera* should be regarded as a cardiotoxic and hepatotoxic plant. Although their experimental preparation differed from the aqueous fresh-leaf extract used in the present study, the occurrence of myocardial necrosis in both investigations provided important comparative evidence that *C. procera* constituents can adversely affect cardiac tissue.

Pulmonary lesions were also evident in the present study. Loss of bronchiolar epithelium, thickening of the interalveolar spaces, vascular congestion, and progressive necrosis of the interalveolar septa were observed with increasing doses of the aqueous fresh-leaf extract. The progressive nature of these lesions suggests a dose-related effect of repeated exposure on the respiratory system and indicates that the toxicity of *C. procera* was not restricted to the liver and kidneys. These findings are consistent with reports of pulmonary alterations following exposure to *C. procera* by Jato *et al*., 2016, that reported mild pulmonary oedema and peribronchial lymphocytic infiltration in female rabbits administered an aqueous extract of *C. procera* leaves for 14 days. Similarly, Pouokam *et al.,* 2011 observed pulmonary damage in rabbits following repeated administration of an aqueous leaf extract, including thickened interalveolar septa associated with oedema and widespread haemorrhage. Although the animal species and experimental conditions differed from those of the present study, the occurrence of interalveolar and pulmonary vascular changes provides supporting evidence that the respiratory system may be a target of *C. procera*-associated toxicity. More recently, De Lima et al., (2011) also reported pulmonary oedema and congestion in sheep following experimental administration of Calotropis procera leaves, further supporting the pulmonary toxic effects previously described by Mahmoud *et al.,* (1979). The same study also documented hepatic necrosis, renal tubular degeneration and cardiac haemorrhage, demonstrating that exposure to the fresh leaves can produce multisystem toxicity. De Lima *et al.,* (2011), similarly reported pulmonary oedema and tracheal exudates in sheep administered fresh *C. procera* leaves repeatedly at 60 g/kg/day for 10 days; pulmonary congestion was also observed histologically. These findings are particularly relevant to the present study because both investigations involved repeated exposure to fresh leaves rather than isolated compounds, although the route, dose, species, and extract preparation differed.

The gastric tissue exhibited a dose-dependent pattern of histopathological alterations following administration of the extract. The changes ranged from mild epithelial damage at lower exposure levels to more pronounced mucosal epithelial necrosis and widening of the lamina propria at higher exposure levels.

The progressive severity of these lesions with increasing dose suggests that repeated oral administration of the aqueous fresh leaf extract may adversely affect the gastric mucosa, either through direct mucosal irritation or indirectly through disruption of gastric mucosal defence mechanisms.

The present findings differ from the reports by Al-Taweel *et al*., 2017 where they described gastroprotective properties of *C. procera* ethanolic extracts of the leaves, flowers and fruits in gastric ulceration in rats. The leaf extract administered to the rat at the dose of 200 and 400 mg/kg significantly reduced gastric lesion indices produced by ethanol, sodium hydroxide, sodium chloride and indomethacin. Histopathological examination further showed that pretreatment with the leaf extract ameliorated ethanol induced gastric mucosal congestion and necrosis. The authors also reported increased gastric wall mucus and favourable changes in glutathione and malondialdehyde levels following administration of the leaf extract, suggesting antioxidant and mucosal protective effects. The apparent discrepancy between the present findings and those of Al-Taweel *et al.,* (2017) may be explained by important differences in experimental design. AL-Taweel *et al.,* 2017, employed an ethanolic leaf extract for pretreatment in rats with experimentally induced gastric lesions, whereas the present investigation involved repeated daily administration of an aqueous extract prepared from fresh leaves to otherwise healthy Wistar rats for 21 days. Thus, the gastroprotective effects reported by Al-Taweel *et al.,* 2017 should not necessarily be interpreted as evidence that prolonged exposure to a crude aqueous fresh-leaf preparation is devoid of gastric toxicity. Indeed, the present findings suggest that the biological effects of *C. procera* on the gastrointestinal tract may be strongly dependent on dose, preparation method and exposure conditions. The gastrointestinal effects observed in the present study may be related to the complex phytochemical composition of *C. procera*. In a recent review of aqueous leaf extracts by Habeeb *et al*., 2024, they reported that *C. procera* contains diverse secondary metabolites, including flavonoids, tannins, alkaloids and other bioactive constituents, whose concentrations can vary according to extraction conditions and environmental factors. Although some of these compounds may contribute to gastroprotective and antioxidant activity at appropriate concentrations, their combined effects at higher or repeatedly administered doses may differ. Therefore, the presence of potentially beneficial phytochemicals does not preclude tissue toxicity under particular exposure conditions.

The brain was examined and found to have lesions only at the group treated with the highest dose of the extract at 800 mg/kg. Mild cerebral oedema was observed, while the neuronal architecture remained relatively preserved. Compared with the more pronounced lesions observed in the kidney, liver, lungs, heart and stomach, the relatively limited cerebral changes may suggest that the brain was less severely affected. However, this interpretation should be made cautiously because histological examination precludes assessment of a dose response relationship.

The relatively mild cerebral changes observed in the present study contrast with an earlier report of more pronounced neurological and cerebral effects associated with *C. procera* by More recently, El Badwi and Bakhiet (2010) reported pathological changes associated with *C. procera* latex exposure in goats, further supporting the toxic potential of the plant latex in small ruminants. The animals also developed nervous signs, dyspnoea, tachycardia and other manifestations of systemic toxicity. These findings indicate that the nervous system can be involved in *C. procera* toxicity, although the study used latex rather than an aqueous leaf extract and involved different animal species and routes of administration. Consequently, the findings cannot be directly equated with the mild cerebral oedema observed in the present study. The potential neurological

effects of *C. procera* have also been highlighted in phytochemical and toxicological reviews by Wadhwani *et al.,* (2021) described the latex of *C. procera* as having irritant, neurotoxic and anticholinergic properties and noted neurological manifestations such as salivation, pupillary dilation, convulsions and collapse following toxic exposure. The authors also summarized evidence of brain congestion among toxic effects reported in experimental models. More recently, Habeeb *et al.,* (2024) reviewed the toxicity and pharmacological properties of the aqueous leaf extract of *C. procera*, emphasizing that the biological effects of the aqueous extract depend on its phytochemical composition and dose.

The relatively mild cerebral oedema observed at 800 mg/kg in the present study may therefore represent an early or comparatively limited neurological response to repeated exposure rather than evidence of complete neurological safety. The preservation of neuronal architecture is reassuring, but it does not exclude functional or biochemical effects that may precede obvious neuronal degeneration. Therefore, the present findings suggest that the brain was comparatively less affected morphologically than several peripheral organs under the experimental conditions employed. Nevertheless, the occurrence of cerebral oedema at 800 mg/kg, together with previous reports of neurological manifestations and cerebral changes following exposure to *C. procera* preparations, indicates that potential neurotoxicity deserves further investigation. Differences between studies also emphasize that the neurological effects of *C. procera* should be interpreted in relation to the plant part, extraction method, dose, route, duration of exposure and animal species.

An important finding of this study is the difference between biochemical and histopathological outcomes. The serum biochemical profile did not show dramatic evidence of organ failure, yet microscopic examination revealed lesions in several organs. This supports the use of histopathology as an important complementary endpoint in repeated dose plant toxicity studies. The study similarly concluded that histopathological evaluation demonstrated clearer dose-dependent organ injury despite relatively stable biochemical findings.

The limitations of this study include; No quantitative histopathological scoring was reported, meaning that the assessment was primarily descriptive, the cardiac biomarkers, oxidative stress markers, and specific cardiac functional assessments were not considered in this research and also the individual toxic constituents, particularly cardenolides, were not quantitatively determined. Despite these limitations, the consistency of dose related tissue changes across several organs provides important evidence that repeated exposure to the aqueous fresh leaf preparation can produce organ level injury that may not be fully reflected by routine serum biochemical indices.

Conclusion

Twenty-one days of repeated oral administration of aqueous extract prepared from fresh leaves of *Calotropis procera* produced dose related histopathological alterations in the kidneys, liver, lungs, heart, and stomach of Wistar rats, with mild cerebral oedema observed at the highest dose. Serum biochemical changes were comparatively modest but included increases in AST, ALT, and particularly ALP, together with reductions in urea, total protein, and albumin at the highest dose.

The findings of this study indicate that, the apparent biochemical stability of some conventional organ function markers should not be interpreted as evidence of complete tissue safety. Histopathological examination revealed multi organ injury, particularly at 800 mg/kg. Therefore, repeated or prolonged oral use of aqueous *C. procera* leaf preparations should be approached cautiously until their safe exposure limits and toxicological mechanisms are better established. Further studies are therefore recommended to investigate the chronic toxicity of aqueous *C. procera* leaf extract using larger sample sizes, incorporate quantitative histopathological scoring, determine circulating and tissue concentrations of individual cardenolides and cardiac glycosides, and include cardiac biomarkers and electrocardiographic assessments to clarify the observed cardiac lesions; additional investigations should explore oxidative stress and inflammatory pathways associated with tissue injury, compare the toxicological profiles of fresh and dried leaf preparations, assess the elemental composition of plant materials from different environments, and establish dose–response relationships and no-observed-adverse-effect levels (NOAELs) before any consideration of prolonged therapeutic use.

Declarations

Ethics Approval

The study received approval from the Institutional Animal Ethical Committee under approval number VPX/IAEC/1306-26 dated 30 April 2024.

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