

## Full Length Research Paper

**Modification of biochemical and haematological parameters during 90-days subchronic toxicity assessment of *Carissa edulis* in Wistar rats****Osseni Razack<sup>1,2\*</sup>, Adanle Etienne<sup>1</sup>, Adjagba Marius<sup>2</sup>, Bonaventure Awede<sup>1</sup>, Hountohotègbè Tatiana<sup>4</sup>, Bigot André<sup>3</sup>, Raphaël Darboux<sup>2</sup> and Anatole Lalèyè<sup>2</sup>**

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A 90-days subchronic toxicity assay was evaluated on the aqueous extract of *Carissa edulis* leaves. Wistar rats were fed daily with oral doses of 125, 31.25 and 7.87 mg/kg of *C. edulis* leaves extracts. Toxic effects were assessed using physiological observation, body weight, relative organ weights, feed consumption, biochemical, haematological and histopathological parameters. Many changes of biological values were observed among treated rats versus controls. Although, alterations in biochemical parameters including aspartate amino transferase were observed, in the middle of treatment (day 45), the values were physiologically normalized at the end of the study. Changes were observed also among renal function parameters including increased levels of creatinine, urea and ions disorders. These results were related by the histopathological examinations. The haematological analysis showed an increase in erythrocytes count, haemoglobin, haematocrit (polyerythrocythemia) associated with an increase of MGv level (macrocytosis). These observations suggest that the long-term uses of *Carissa edulis* could alter some functions of the organism, especially the hepatic, renal and haematopoietic functions. Further specific toxicity studies must be investigated on the plant.

**Key words:** *Carissa edulis*, polyerythrocythemia, subchronic toxicity, Wistar rats.

**INTRODUCTION**

About 85% of the populations of developing countries continue using traditional resources in health care (Cunningham, 1993; Sheng-Ji, 2001). The main goal of ethnopharmacology is to identify novel compounds derived from plants and animals for use in indigenous

medical systems. This knowledge can be used in the development of new pharmaceuticals. Most of the literature in ethnopharmacology describes medicinal plants used by people who have lived in the same.

Ethnopharmacologists seek ways to improve

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the traditional medicine systems of the people whom they study by testing indigenous medicines for efficacy and toxicity (Farnsworth, 1988). Through this kind of work, they have contributed to the discovery of many important plant-derived drugs. *Carissa edulis* (CE) is a medicinal plant widely used for the treatment of many diseases.

*C. edulis* parts are used in ethnomedicine for a wide variety of illnesses, such as epilepsy, headaches, chest complaints, gonorrhoea, syphilis, rheumatism, rabies and often used as a diuretic (Ya'u et al., 2008; Nedi et al., 2004). Other folkloric uses of *C. edulis* include fever, sickle cell anaemia and hernia (Ibrahim et al., 2007). The plant is distributed in tropical Africa (Botswana, Cameroun and Benin) and Asia (Cambodge, Japan and Myanmar).

Despite all these pharmacological studies based on traditional uses of the plant, few toxicological data were available on the leaves of the plant. Preliminary toxicity investigations were carried out on the plant (Hajara et al., 2015; Ngulde et al., 2013). This first survey aims to provide the 90-days subchronic toxicity profile of *C. edulis* in Wistar rat.

## MATERIALS AND METHODS

### Preparation of extract

Fresh leaves of *C. edulis* (CE) were harvested at Abomey-Calavi a city close to Cotonou (South of Benin). A voucher specimen was authenticated at the National Herbal Center of Benin (No. AA6482/HNB). The leaves were cleaned and dried at the laboratory temperature between 16-18°C during three weeks. The dried leaves were pulverized and the powder was stored at room temperature until use. From the dried powder of *C. edulis*, 150 g were measured and extracted at 80°C with 500 ml of distilled water for 30 min. The decoction was filtered and then poured into an evaporating dish (RE-300) to evaporate the water over a water-bath at a temperature of 80°C. After evaporation of the filtered extract, a brown dried extract was obtained and stored in refrigerator at 4°C.

### Animals and design of 90 days subchronic oral toxicity test

Wistar albino rats weighing  $180 \pm 20$  g were used. They were maintained under standard environmental conditions (22 to 25°C, 12 h dark/light cycle, frequent air change) and had free access to tap water and food. They were separated by gender and housed five per cage. The subchronic toxicity test was carried out based on OECD 408 directive adopted in 1998 (OECD, 1998) with little modifications. Rats were preliminarily acclimatized during five days in the laboratory.

The test consists of a repeated administration of the leaves aqueous extract of *C. edulis* to three groups of 10 rats (5 males and 5 females). The treatment is based on a daily oral gavage of 125 mg/kg 31.25 and 7.87 mg/kg, respectively during 90 days. The control lot (5 males and 5 females) received distilled water. Individually, animals were clinically monitored quietly before the first exposure. Observations were focused on changes in the skin and fur, eyes, mucous, respiratory system, central nervous systems as well as somatomotor activity and behavioural patterns. From the 60<sup>th</sup> day of exposure, rats' observation was more particularly focused on their sensorial and auditive stimuli reactions. Body

weight, as well as food consumption, was recorded every week. At the 44<sup>th</sup> day, rats were starved overnight (12 h) but with free access to tap water; retro-orbitary blood and urine samples were collected on the 45<sup>th</sup> day for biological analysis. At the end, they were starved again overnight (12 h) but with free access to tap water. Then, they were anaesthetized with thiopental by intraperitoneal injection and blood samples were collected into tubes with or without EDTA for biochemical and haematological determinations. Rats were euthanized by a lethal dose of thiopental then liver and kidney of three rats per group were removed for histological examinations. Urine samples were analysed for glucosuria, proteinuria, ketonuria and haematuria using urinary reactive strips. Biochemical parameters including serum concentrations of glucose, total cholesterol, urea, creatinine, total proteins, potassium, chloride, sodium, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were performed using an automatic analyzer (MTN-658F) with specific kits. Haematological analyses were performed on blood using an automated haematology analyzer (Sysmex K x 21). These parameters included erythrocyte haematocrit (HCT), haemoglobin (Hb), mean corpuscular haemoglobin concentration (MCHC), leukocytes, mean corpuscular volume differential (MCV), mean corpuscular haemoglobin (MCH) and platelet count (PLT).

### Statistical analysis

The results shown are expressed as means  $\pm$  standard error of mean (S.E.M.). Body weights and food consumption values were compared by Student's t test while biological parameters data were analyzed by one-way ANOVA followed by Dunnett post hoc with Graph Pad Prism Version 6. Results were considered significant for a p-value less than 5% ( $p < 0.05$ ).

## RESULTS

Aqueous extraction was carried out from 150 g of powder of the leaves of *C. edulis*. The extraction yield was 16.55%.

### Animal general behavior, food consumption body and relative organ weight

At the end of this study, no death was observed due to the leaves aqueous extract of *C. edulis*. No significant clinically relevant changes were observed in general behavior. The only effect on treated animals was self-scratching just after gavage.

The body weights have progressively increased during the treatment in the different rats groups particularly in the control group (Figure 1). As shown in Figure 2, administration of *C. edulis* aqueous extract did not significantly affect food consumption. However, food intake increased during the treatment period in all groups. But no significant differences were observed between treated and control group throughout the test neither with the body weights nor with food consumption. Otherwise, administration of *Carissa edulis* extract decreased significantly organ relative weight when compared to control groups (Table 1).

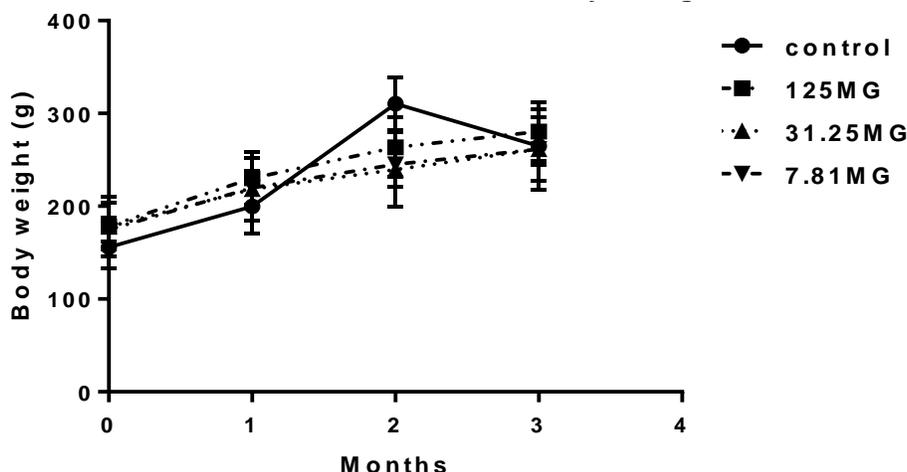


Figure 1. Evolution of rats' body weight during the test.

Table 1. Relative weights of rats' organs treated with CE.

Group (mg/kg)	Liver	Kidney
Control	3.01±0.02	0.68±0.02
7.81	2.04±0.03*	0.43±0.02*
31.25	1.9±0.12*	0.40±0.03*
125	2.88±0.07*	0.66±0.01*

Values represent means ± SEM. \* p<0.05: Significantly different from the control group.

### Biochemical parameters

On day 45, significant differences were observed in the biochemical parameters. Indeed, urea, creatinine, transaminases, cholesterol, potassium and chloride values were increased while glucose level decreased dose dependent manner (Table 2). Only the transaminases (ASAT) values were worrisome. On day 90, the biochemical parameters values showed that *C. edulis* extract intake induced a significant decrease of transaminases (ASAT and ALAT) and glucose plasma levels at the dose of 125 mg/kg and chloride dose dependent manner. In the other hand, significant increase was observed with urea, creatinine, sodium, potassium and proteins levels (Table 3)

### Haematological parameters

Table 4 shows, a polyerythrocythemia on day 45 at 31.25 mg/kg associated with a slight hyperchromicmacrocytosis at 7.81 mg/kg. On day 90, changes in haematological parameters still showed a tendency to a polyerythrocythemia (dose dependant increases of erythrocytes haemoglobin, haematocrit values) but not significantly and a slight macrocytosis (Table 5).

### Urine parameters

Qualitative analysis revealed no presence of red blood cells, glucose and ketone bodies in the urine samples. However, proteinuria was observed in all the groups (treated and control).

### Histopathology

The histopathological photography showed abnormalities in livers as well as the kidneys of treated rats at high doses. Pyknotic nuclei were seen in livers, whereas the renal cortex showed a thickening of glomerular interstitium.

### DISCUSSION

*C. edulis* is a medicinal plant used to treat many diseases including hypertension. Pharmacological screening and toxicological studies assessment are considered as a crucial step before use of drugs. This 90-days subchronic toxicity test is a continuity of the toxicological studies on leaves aqueous extract of the plant (Osseni et al., 2016) since hypertension is a long duration treatment disease.

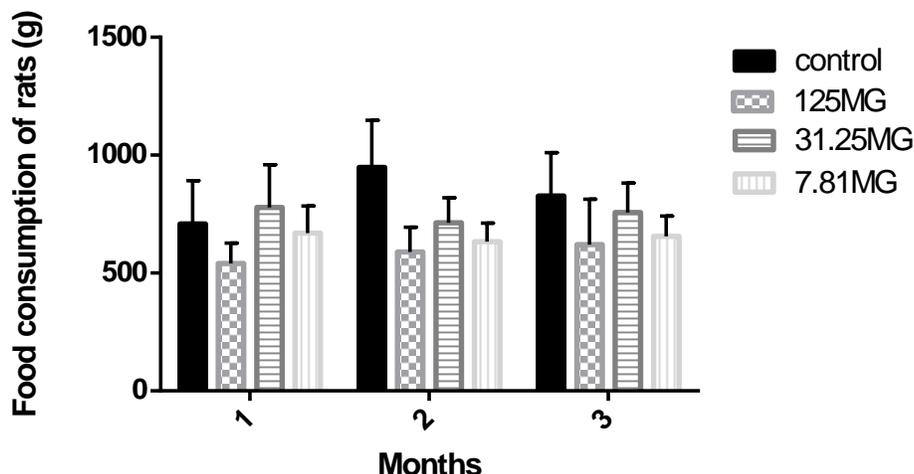


Figure 2. Evolution of rats' food consumption during the test.

Table 2. Biochemical parameters on day 45.

Parameter	Control	7.81 mg/kg	31.25 mg/kg	125 mg/kg
Glucose (g/L)	1.27±0.25	1.05±0.15	0.77±0.07*	0.71±0.14*
Urea (g/L)	0.6±0.05	0.76±0.09*	0.55±0.08	0.53±0.02
Creatinine (mg/L)	8.67±0.58	12±1.00*	9±1.73	8.67±0.58
ASAT (UI/L)	149.33±44.29	261.33±29.14	352.33±37.9*	369±95.69
ALAT (UI/L)	65±16.00	153.33±15.27*	26±18.68	43.67±1.15
Total Cholesterol (g/L)	0.89±0.20	0.74±0.06	0.77±0.12	0.62±0.10*
Sodium (mEq/L)	141.33±1.53	142.67±5.13	142.33±1.53	142.67±1.15
Potassium(mEq/L)	4.61±0.35	9.57±1.70*	4.43±0.06	4.6±00
Chloride (mEq/L)	97±3.00	103.33±4.72	104.33±2.31*	106.33±0.58*
Proteins (g/L)	79.67±9.61	72.33±3.51	75.67±2.08	70±2.64

Values represent means ± SEM. ASAT: aspartate amino-transferase. ALAT: alanine amino transferase. \* p<0.05: Significantly different from the control group.

After 90 days of treatment, no death was recorded in either the control or treated groups. The animals did not show any changes in general behavior. The body and relative organ weights of the rats treated with the extracts of *C. edulis* are given respectively in Figure 1 and Table 1. There were no significant differences in the body weight between control and treated animals of both sexes. The relative organ weights analysis showed a significant reduction of liver and kidneys weights treated with the extract in all groups as compared to control. One could, therefore conclude that the extract is toxic to the two organs but further investigations are required to certify the hypothesis. No macroscopic changes (colour or appearance) were observed. This decline in the relative organs weight suggests that long period uses of *C. edulis* extract could induce an atrophy of the organs and therefore alter their functions. A decrease in food consumption could be considered as a negative effect of the plant extracts on the rats' development caused by alteration of glucids, lipids and proteins metabolism

(Ezeonwumelu et al., 2011). Generally, the reductions in body weight gain and internal organ weights are simple and sensitive indices of toxicity after exposure to toxic substances (Raza et al., 2002; Teo et al., 2002). The toxic effect is manifested by significant changes in the color, appearance and relative organ weight (Amresh et al., 2008).

As one of the biochemical parameters analyzed, transaminases (ASAT, ALAT) are normally found in the cytoplasm and mitochondria of many cells, primarily in cardiac muscle, liver and skeletal muscle. Its concentration is much lower, however, in the kidney, pancreas and erythrocytes (Costa- Silva et al., 2008). Therefore, an increase in the serum levels of ALAT, ASAT, indicates hepatic toxicity. These changes occur in the blood when the hepatic cellular permeability is changed or when necrosis and cellular injury occur.

In this study, biochemical analysis of the treated and control rats showed that there were significant differences in the levels of transaminases especially at the middle of

**Table 3.** Biochemical parameters on day 90.

Parameter	Control	7.81 mg/kg	31.25 mg/kg	125 mg/kg
Glucose (g/L)	1.32±0.23	1.08±0.09	1.28±0.21	0.70±0.16**
Urea (g/L)	0.58±0.05	0.37±0.01*	0.65±0.10	0.81±0.01*
Creatinine (mg/L)	7.48±0.66	9±1.49	12.33±1.15*	14±2.64
ASAT (UI/L)	127.66±39.07	133.33±15.27	83.33±9.45	5.66±3.78*
ALAT (UI/L)	72.33±14.15	106.33±8.50	121±29.82	7.66±2.89*
Total Cholesterol (g/L)	0.69±0.05	0.8±0.09	0.81±0.13	0.91±0.23
Sodium (mEq/L)	136±2.00	142±2.00	145±1.00*	127.33±2.08
Potassium(mEq/L)	4.71±0.23	5.47±0.81	5.12±0.16*	3.76±0.87
Chloride (mEq/L)	108±2.00	103.67±2.52*	87.67±1.15*	95.67±0.58*
Proteins (g/L)	68.33±1.15	67.33±0.58	83±7.21	76±1.00*

Values represent means ± SEM. ASAT: aspartate amino-transferase. ALAT: alanine amino transferase. \* p<0.05: Significantly different from the control group.

**Table 4.** Haematological parameters on day 45.

Parameter	Control	7.81 mg/kg	31.25 mg/kg	125 mg/kg
Erythrocytes (T/L)	7.3±0.48	6.92±0.59	8.67±0.60**	7.71±0.29
Haemoglobin(g/dL)	12.47±1.15	13.13±1.30	15.03±0.47*	13.63±0.64
Haematocrit (%)	43.67±4.51	47.67±3.51	50.33±2.08*	45.33±3.05
MGV (fL)	59.67±3.05	69±1.73*	58±2.00	59±2.00
MCH (pg)	17±1.00	18.67±0.58*	17.33±0.58	18±00
MCHC (%)	29±2.00	27.67±0.58	30±00	30±1.00
Leukocytes(G/L)	11.67±2.89	6.23±1.30*	14.73±5.52	9.5±4.29
neutrophils (G/L)	1.39±1.21	0.8±0.09*	1.61±0.49	1.96±0.26
Eosinophiles (G/L)	0±00	0±00	0±00	0±00
Lymphocytes (G/L)	9.78±1.54	5.39±1.38*	12.98±5.05	7.46±4.57
Monocytes (G/L)	0±00	0.04±0.07	0.14±0.24	0.07±0.08
Platelets (10 <sup>3</sup> /μL)	712.33±244.68	885.33±18.58	919.67±111.13	826.33±347.92

the treatment (day 45). This result was demonstrated in the 28-days toxicity study but at the end of the study (day 90) the level of transaminases was normalized (Table 3). Throughout this survey, the glucose level was reduced dose-dependent manner (Table 2 and 3) and this reinforces the antidiabetic use of *C. edulis*.

The levels of creatinine, urea, sodium, potassium or chloride, are good indicators of kidney function (Marcelo et al., 2002). The biochemical analysis revealed a tendency to hypercreatininemia and hyperuremia. A hyperkalemia associated with a hyponatremia was also observed. This result evokes the mechanism action of potassium-exclusion diuretic. Moreover, Nedi et al., (2004), had already notified that different extracts of *C. edulis* increased diuresis. Rats treated with the lower dose (7.85 mg/kg), have shown a significant increase in urea, creatinine and potassium levels at day 45 and this suspect a real kidney failure. But this failure seems transient because at day 90, the values of these parameters are normalized. All these data indicate that

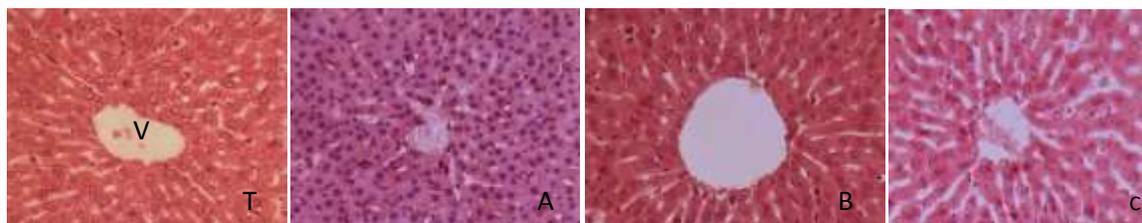
the aqueous extract of *Carissa edulis*, induced damage to the liver and kidneys. This point was further confirmed by histopathological analysis and microscopic examination on these organs (Figures 3 and 4).

Haematological studies easily reveal anomalies in body metabolic processes, and the blood profile usually provides vital information (Yakubu et al., 2007) on the response of the body to injury, deprivation and/or stress. Moreover, this analysis is relevant in risk assessment since changes in the hematopoietic system have a high predictive value for toxicity in humans when data are transferred from animal studies (Olson et al., 2000). In this study, the main effect revealed by administration of aqueous extract of *C. edulis* was the tendency to polyerythrocythemia (increase in erythrocytes count, haemoglobin and haematocrit level) associated with a slight macrocytosis (Tables 4 and 5). No significant changes were noted in the other parameters between treated and the control rats groups. These effects could be explained by the presence in the extract substances

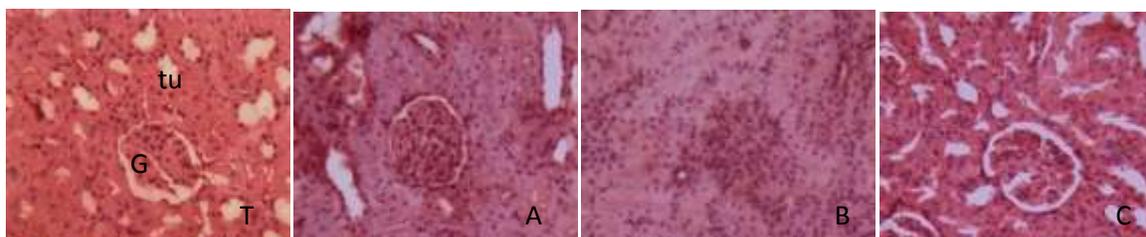
**Table 5.** Haematological parameters on day 90.

Parameter	Control	7.81 mg/kg	31.25 mg/kg	125 mg/kg
Erythrocytes (T/L)	7.15±0.63	7.4±0.48	7.74±0.52	7.71±0.62
Haemoglobin(g/dL)	12.4±0.87	13.63±0.76*	13.97±0.66*	13.4±0.87
Haematocryt (%)	41±3.46	48.67±3.78*	47.33±3.21*	46.33±3.21*
MGV (fL)	57.33±0.57	66±2.64*	60.67±1.53*	60.33±0.58*
MCH (pg)	17.33±0.57	18.67±0.58	18.33±0.58	17.33±0.58
MCHC (%)	30.33±0.57	28±1.00	29.67±0.58	29±1.00
Leukocytes(G/L)	9.53±1.56	7.73±3.79	8.87±1.25	7.73±4.46
neutrophiles (G/L)	0.67±1.19	2.71±1.76	1.02±0.74*	0.87±0.54*
Eosinophiles (G/L)	0±00	0.04±0.07	0±00	0±00
Lymphocytes (G/L)	8.37±1.81	4.86±2.13*	7.77±0.53	6.47±4.15
Monocytes (G/L)	0.49±0.28	0.12±0.06*	0.07±0.12*	0.18±0.07*
Platelets (10 <sup>3</sup> /μL)	794±139.82	607.33±133.09	739.33±9.71	833.67±62.53

Values represent means ± SEM. MGV: Mean globular volume, MCH: mean concentration haemoglobin, MCHC: mean corpuscular haemoglobin concentration, \*p<0.05: Significantly different from the control group.



**Figure 3.** Livers histopathology of rats treated with *C. edulis* extract. Liver of control rat (T) (HE 400 x), with a hepatic lobule showing hepatocytes disposed radially around the centrolobular vein (V); treated rats at 125 mg/kg (A); 31,25 mg/kg (B) et 7,81 mg/kg (C) b/w (HE x 400). (A) and (B) shows hyperchomatic nucleus of cells around the centrolobular vein while (C) is a normal hepatic lobule.



**Figure 4.** Kidneys histopathology of rats treated with *C. edulis* extract. Kidney of control rat (HE 400x). Cortex of kidney showing a normal glomerulus with tubules in the urinary chamber. A and B showed a thickening of the glomerular interstitium.

which are able to stimulate erythropoiesis. These observations confirm the results obtained by Koffuor (Koffuor et al., 2012); who showed that the ethanol extract of the root bark of *C. edulis* corrected anemia in rats pretreated with phenylhydrazine. This effect could also be due to the diuretic effect of *C. edulis* since the diuretic effect could cause a hemoconcentration due to polyerythrocythemia. The standard toxicity studies are

very useful to understand the effects of a substance on the immune system (International Conference on Harmonisation, 2006). Any malfunction of the immune system both quality and quantity could be manifested by changes in hematological type of leukocytosis or leucopenia, lymphopenia or lymphocytosis; an increase in the incidence of infection but also by the occurrence of tumors. A slight reduction was noted in the values of

leukocytes, neutrophils, lymphocytes and monocytes. In view of the above, it could be stated that the extract is toxic to immune cells. The histological pictures showed abnormalities both in livers and kidneys treated with high doses (31.25 and 125 mg/kg) of the extract. The modifications include hyperchromatic or pyknotic nuclei of the hepatocytes around the centrolobular vein (Figure 3) and a thickening of the glomerular interstitium (Figure 4). These changes suggest a beginning of necrotization of hepatic cells and the hyalinization of glomerular interstitium which relates glomerular nephritis since proteinuria test was positive.

## Conclusion

This subchronic toxicity study was necessary for the safety assessment of *C. edulis*. However, in this study, alterations in biochemical parameters such as transaminases were highlighted in exposed animals. Fortunately, they were transitory because after 45 days of exposure, the values were normalized at the end of the study. However, some parameters remain altered among the renal function parameters as well as haematological ones. Further organo-specific analyses would be required to provide evidential support for the safety uses of this plant.

## Conflict of Interests

The authors have not declared any conflict of interests.

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