

In vivo Toxicological Assessment of the Aqueous Extracts of the Leaves of *Carissa edulis* (Apocynaceae) in Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RO, Stephen Akoha and BA designed the study, performed the literature searches and statistical analysis. Authors MA and Simon Azonbakin wrote the protocol and performed the histological slides. Authors AL, AB and RD managed the biological analyses and histological slides interpretation of the study. Author LL performed the extraction. Author AD revised wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To screen the acute and 28-days subchronic toxicity of the leaves aqueous extract of *Carissa edulis* (*C. edulis*).

Design: Experimental and observational study.

Place and Duration: Faculty of health sciences of Cotonou / Institute of Applied Biomedical Sciences of Cotonou, laboratories of Physiology/Human biology/Immunology/ biochemistry and molecular biology(pharmacognosy section) / toxicology and hydrology of Dakar (Senegal) between March 2014 and January 2015.

Methods: The aqueous extract was obtained from the powder of the leaves by a decoction method and evaporation using a rotavapor. In the acute toxicity study (Limit test), a single dose of 2000 mg/kg of the extract was orally administered to three female rats. Different parameters were assessed according to the Organisation for Economic Co-operation and Development (OECD) 423 guidelines for the Testing of Chemicals. During the sub-chronic toxicity, four groups of five rats of either sex received distilled water (control), 31,25,125 and 500 mg/kg of the extract daily for 28 consecutive days by oral gavage. The assay was conducted according to the OECD 407 guidelines.

Results: In both acute and sub-chronic toxicity test, neither mortality nor other toxicity symptoms were observed. This suggests that the median Lethal Dose 50 (LD₅₀) is superior to 2000 mg/kg. There were no significant differences in the body and organ weights between controls and treated animals of both sexes. Indeed, some biological parameters including erythrocytes (6,89±0,07), Haematocrit (41,6±1,69), and Haemoglobin (13,31±0,16) levels were significantly reduced when compared to the control group but this is not imputable pathologically. Histological structures of liver and kidney were normal in both two sexes.

Conclusion: The aqueous extract of *C. edulis* was found to be safe in acute and 28-days sub-chronic toxicity studies. Furthermore, aspects of the effects of this plant on haematopoiesis, liver and kidney function need to be gained and ascertained over longer periods of toxicity study.

Keywords: *Carissa edulis*; toxicity; Wistar rats; LD₅₀.

1. INTRODUCTION

Carissa edulis Vahl., is a plant which belongs to the family of Apocynaceae and is distributed in tropical Africa (Botswana, Cameroun, Benin) and Asia (Cambodge, Japan and Myanmar). It has been reported in traditional medicine to be used in managing disease conditions such as epilepsy, headache, toothache, cough, rheumatism, fever, sickle cell anaemia, gonorrhoea, syphilis, helminthiasis and rabies [1-3]. The roots' decoction is used against malaria, indigestion, post-partum pains and against chest pains. The roots' infusion is used against stomachache, Herpes simplex virus infection and cataract when used as an eye drop. In Benin, the roots are used as an aphrodisiac, against women sterility and hypertension. *Carissa edulis* is also used as a source of dye [4,5].

Despite all these several uses of the plant, few data are available in general on the leaves of the plant and especially on its toxicological studies.

The aim of this work was to evaluate the acute and 28-days sub-chronic toxicity of the aqueous extract of the fresh leaves of *Carissa edulis*.

2. MATERIALS AND METHODS

2.1 Animals and Plant Material

Wistars albino rats of either sex were used. They were acclimatized to an ambient temperature of 22±2°C, with a 12 h light-dark cycle, for at least five days prior to the start of the experiment. During the acclimatization and experimental periods, the rats had free access to tap water and food. All animals in the study were handled and cared for in accordance with the internationally accepted standard guidelines for use of animals.

Fresh leaves of *Carissa edulis* were collected in May 2014 at Abomey-Calavi (Southern Benin). Voucher specimen were identified and authenticated at the National Herbarium of Benin (N° AA6482). The leaves were dried sheltered of the sun between 20-25°C during three weeks. The dried leaves were ground into powder.

A decoction was made from 100 g of the powder with 750 mL of distilled water. The decoction was filtered three times on Whatman N^o1 paper and the same operation was repeated until extracting entirely the decoction extract. Then the filtered extract was evaporated with a rotavapor type RE-300 at 80°C. The dried extract obtained was stored at 4°C.

2.2 Acute Toxicity Test

The acute toxicity test was carried out based on the Organisation for Economic Co-operation and Development (OECD) guidelines for the Testing of Chemicals section 4-423- limit test, adopted in 2001 [6]. Rats were preliminarily acclimatized during five days in the laboratory and were left fasting 12 hours before the experimentation. Six (3) females' rats weighing 200±20 g were divided in two groups of 3 rats each. The experimental group was force-fed with a single dose of 2000 mg/kg of the aqueous extract while the control group was treated with distilled water. The animals were observed quietly and continuously for eight hours just after the administration and once the daily up to 14 days. The monitoring was based on general behaviour changes, body weight evolution, food and water consumption, mortality and any toxicity signs. At the end of the experience, 0.1 ml of Thiopental (100 mg/ml) was injected intraperitoneally to anesthetize the animals. Blood samples were collected in tubes without anticoagulant for biochemical examinations including blood glucose, urea, cholesterolemia, creatinine, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), sodium, potassium and chloride. Histological examinations were carried out on liver and kidney at the end of the experimentation.

2.3 Subchronic Toxicity Test

The subchronic toxicity test was carried out as described by OSSENI et al. [7]. Forty (40) rats including twenty (20) males and twenty (20) females weighing 180±20 g were used. Briefly, aqueous extract of the leaves of *Carissa edulis* was administrated to three groups of 10 rats (5 males and 5 females). The control groups of 10 rats (5 males and 5 females) receive only distilled water. Three different doses were administered to rats at 500 mg/kg, 125 mg/kg and 31.25 mg/kg during 28 days. Individually, animals were clinically monitored quietly before the first exposure. Observations were focused on changes in the skin and fur, eyes, mucous, respiratory system, autonomic and central

nervous systems as well as somatomotor activity and behavioural patterns. During the fourth week of exposition, rat observation was more particularly focused on their sensorial and auditive stimuli reactions. Body weights, as well as food consumption, were recorded every week. At the end, rats were starved overnight (12 hours) but with free access to tap water. They were then anesthetized with thiopental by intraperitoneal injection and blood samples were collected in tubes without anticoagulant (Serum) for biochemical and the whole blood in EDTA tubes for 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. 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. For haematological analysis, hematocrit (HCT), haemoglobin (Hb), Mean corpuscular haemoglobin concentration (MCHC), Erythrocytes, leukocyte count, mean corpuscular volume differential (MCV), mean corpuscular haemoglobin (MCH), platelet count (PLT), were performed on blood using Sysmex K x 21 automated haematology analyzer.

2.4 Statistical Analysis

The data were represented as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was performed to compare the differences between the means in the subchronic study while t-test was used to compare means of the results in the acute study. A mean difference was considered significant when * P = .05. Statistical analysis was performed using the GraphPad Prism 6 Demo.

3. RESULTS

3.1 Acute Toxicity Test

3.1.1 General observation

The limit acute toxicity test didn't cause either death among the rats, nor toxicity signs. All three rats were normal throughout the study and survived until the end of the fourteenth day experiment period. As shown in Fig. 1. there is no significant difference between the body weights of the two groups of rats after 14 days observation. This could be supposed that the leaves aqueous extract of *Carissa edulis* has no

effect on the body weight of Wistar rats. According to the globally harmonized system (GHS) classification, the aqueous extract of the leaves of *Carissa edulis* should be classified in category 5 since the median lethal dose (LD₅₀) is estimated to be over than 2000 mg/kg (low toxicity).

3.1.2 Body weight gain

Fig. 1 shows a slight increase of rats' body weight during the first week, but this gain is not significantly different from the control group.

As shown in Table 1, the biochemical markers were not modified treated rats. The values of Glucose, Cholesterol, creatinine and ionogram remained unaltered in the animals treated with the plant compared to the control group.

The biochemical parameters did not show any marked changes in the treated group compared with the controls except for the Urea, ASAT and ALAT which are significantly increased.

3.1.3 Histology during acute toxicity test

The histopathology of liver and kidney at the end of the acute toxicity test did not show any abnormality. Figs. 2a,b and 3a,b related respectively the microscopy of a control and treated rats' liver and a control and treated rats' kidney.

3.2 Days Subchronic Toxicity Test

3.2.1 General signs

No deaths were observed; no significant clinical relevant changes were observed in general behavior and other physiological activities in the present study.

3.2.2 Food consumption, relative organs weight and body weight

Administration of *Carissa edulis* did not alter either food consumption or body weight gain of treated rats, when compared with control group (Figs. 4 and 5). A decrease in food consumption was observed during the first week however there were no significant differences between treated rats and the control group. Table 2 relates liver and kidney weights after of all rats at the end of the experimentation. The results show no differences between treated and control groups.

Table 1. Biochemical parameters during acute toxicity test

Parameters	Control	Treated
Glucose (mmol/l)	6.72 ±1.28	9.10 ±1.89
Urea (mmol/l)	10.49 ±0.50	12.15 ±0.50*
Creatinine (µmol/l)	76.64 ±5.13	73.64 ±5.13
Cholesterol (mmol/l)	1.68 ±0.49	1.91 ±0.18
ASAT (UI/L)	280.00 ± 9.54	439.00 ±18.97*
ALAT (UI/L)	197.00 ±36.10	438.33 ±38.88*
Sodium (mmol/l)	140.33 ±1.53	142.33 ±1.15
Potassium (mmol/l)	5.70 ±0.75	7.23 ±3.97
Chloride (mmol/l)	97.00 ±1.73	99.00 ± 1.00

Values represent Average ± SEM. ASAT: Aspartate Amino-Transferase, ALAT: Alanine Amino Transferase. * P=0.05: Significantly different from the control group

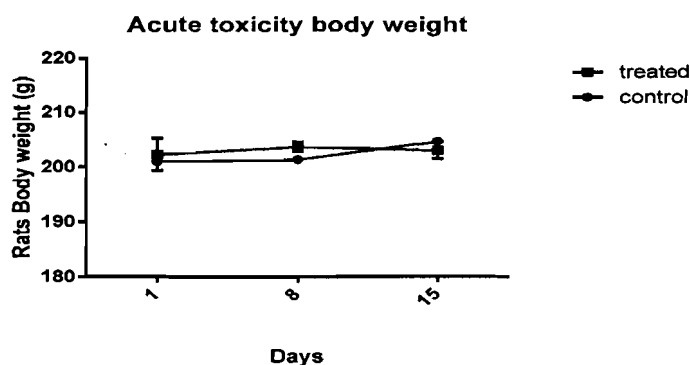


Fig. 1. Changes of rats body weight during the acute toxicity experimentation

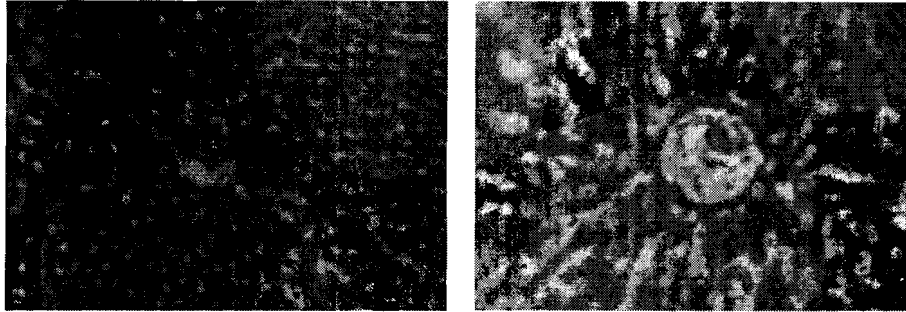


Fig. 2. Liver a. Control rats (HE x 40): General vision of Hepatic lobule showing hepatocytes disposed radially around centrilobular vein (V).

Liver b. Rat treated at 2000 mg/kg (HE x 40): Global architecture of the liver is conserved.



Fig. 3. Kidney a): Control rat (HE x40): Partial vision of renal cortex showing renal glomerulus (G) and tubules (T).

Kidney b): Rat treated at 2000mg/kg(HE X40): There is no structural abnormality.

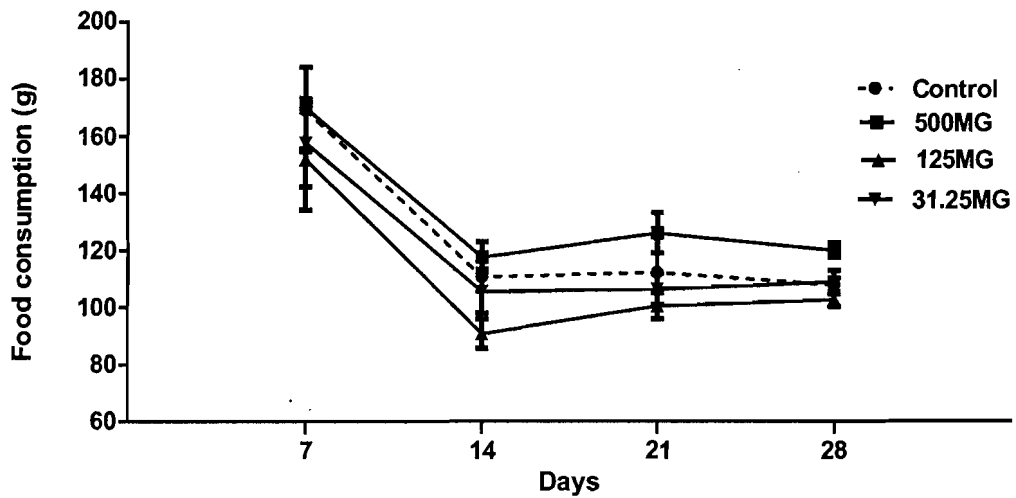


Fig. 4. Rats food consumption during the 28-days subchronic test

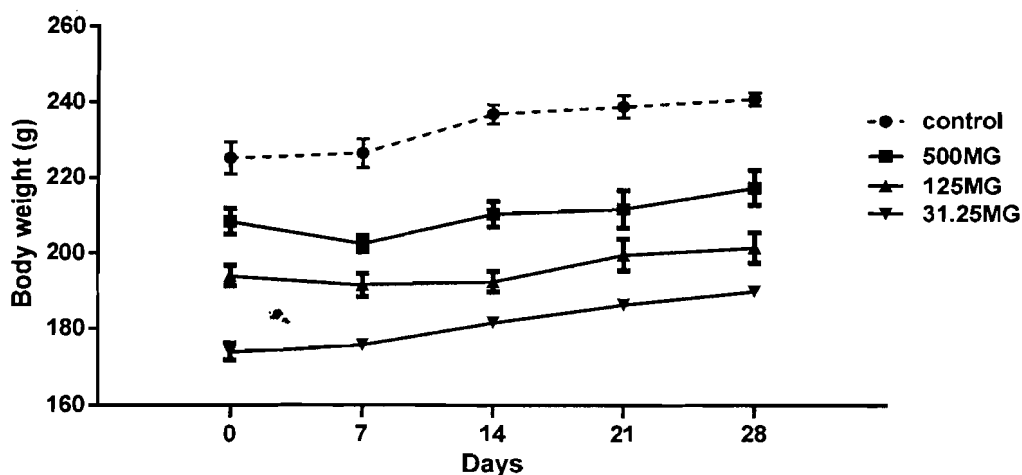


Fig. 5. Rats body weight during the 28-days subchronic test

Organ weight revealed that *Carissa edulis*, at the doses used did not produce organ swelling, atrophy or hypertrophy (Table 2).

3.2.3 Biochemical and haematological parameters

After 28-days of treatment with *Carissa edulis* extract, there were no significant changes in the serum biochemical levels of the different parameters doses (Tables 3 and 4). A significant increase ($p < 0.05$) in glucose levels were only observed in the female of treated rats at 125 mg/kg and 31.25 mg/kg as well as potassium, chloride and proteins values in male rats. All haematological parameters were within normal, although the significant decrease was noted in red blood cell, haematocrit, haemoglobin of male and female rats (Tables 5 and 6).

Table 2. Relative weights of removed organs

Doses (mg)	Liver (gram)	Kidney (gram)
Control	2.99±0.17	0.28±0.01
500	3.20±0.57	0.32±0.01*
125	3.06±0.11	0.32±0.04*
31,25	2.72±0.44	0.31±0.00

3.2.4 Histopathology

Microscopic examination of the liver and kidney obtained from rats after 28 days administration of the aqueous extract showed normal cells structure and formation when compared with the control. There was no gross abnormality or damage neither in liver nor in kidney histological structure when viewed under the light microscope (Figs. 6 and 7)

Table 3. Biochemical parameters of males' rats during 28-days sub-chronic test

Parameters	Control	mg/kg		
		31,25	125	500
Glucose (mmol/l)	2.70±0.10	3.20±0.30	2.90±0,20	2.70±0,30
Urea (mmol/l)	13.60±0.70	13.40±0,70	14.10±0,30	13.10±0.80
Creatinine (µmol/l)	139.00± 60.00	138.00±80.00	150.00±30.00	132.00±90.00
ASAT (UI/L)	241.20±23.50	185.60±20.31*	174.40±17.90*	245.75±36.71
ALAT (UI/L)	197.20±11.57	181.80±14.50	186.80±8.05	151.50±25.91*
Total cholesterol (mmol/l)	1.71±0.10	1.60±0.08	1.81±0.05	1.47±0.18*
Sodium (mmol/l)	149.08±1.49	149.22±3.01	145.02±0.89*	144.10±3.34*
Potassium (mmol/l)	4.82±0.35	5.81±0.27	6.45±0.54*	5.13±1.28
Chloride (mmol/l)	97.64±4.37	109.22±0.58*	108.88±0.98*	96.35±4.07
Proteins (mmol/L)	41.80±1.93	37.20±0.86*	36.20±0.58*	36.75±1.38*

Values represent Average ± SEM. * P = .05: Significantly different from the control group

Table 4. Biochemical parameters of females rats during 28-days sub-chronic test

Parameters	Control	mg/kg		
		31,25	125	500
Glucose (mmol/L)	3.55±0.06	4.55±0.56	4.55±0.17*	4.22±0.28*
Urea (mmol/L)	14.32±0.50	13.65±0.33	14.15±0.33	13.82±0.67
Creatinine (mg/L)	88.40±4.86	83.10±3.54	83.10±4.51	79.56±04.00*
ASAT (U/L)	225.00±5.21	223.60±18.18	220.40±7.67	242.80±24.27
ALAT (U/L)	53.20±3.89	59.40±1.60*	65.40±4.72*	62.60±2.73*
Total cholesterol (mmol/L)	1.55±0.08	1.47±0.10	1.62±0.16	1.53±0.05
Sodium (mmol/L)	145.00±0.71	143.20±0.73*	145.40±0.68	145.20±0.73
Potassium (mmol/L)	5.20±0.11	5.57±0.17*	5.31±0.15	5.40±0.09
Chloride (mmol/L)	102.80±0.66	101.60±1.44	103.80±0.97	102.40±0.68
Proteins (g/L)	34.80±0.73	35.60±0.87	36.20±0.58*	36.60±0.68*

Values represent Average ± SEM. *P = .05: Significantly different from the control group

Table 5. Haematological parameters of male rats during the 28-days sub-chronic test

Parameters	Control	mg/kg		
		31,25	125	500
Erythrocytes (T/L)	7.32±0.07	6.89±0.07*	6.32±0.24*	7.14±0.11
Haemoglobin (g/L)	145.50±1.10	133.2±1.6*	114.1±4.8*	140.0±3.9
Haematocrit (%)	47.40±0.51	44.60±1.21*	41.60±1.69*	46.00±0.91
MCV (fL)	65.20±0.80	66.00±1.05	64.40±0.60	64.25±0.48
MCH (Pg)	19.00±0.32	18.40±0.24*	18.80±0.37	18.75±0.25
MCHC (%)	30.80±0.37	30.00±0.55	31.20±0.37	30.50±0.29
Leucocytes (G/L)	3.04±0.21	2.82±0.36	2.34±0.45	5.25±1.63
Neutrophils (%)	34.60±4.09	46.40±5.91	45.60±4.25	38.00±10.46
Eosinophils (%)	0.20±0.31	0.60±0.40	0.40±0.40	0.50±0.29
Lymphocytes (%)	64.20±4.33	49.80±5.83*	49.00±4.07*	59.75±10.04
Monocytes (%)	1.20±0.37	3.20±0.58*	5.00±1.30*	1.75±0.48
Platelets (G/L)	592.60±22.27	594.20±15.07	545.40±22.52	575.25±70.63

Values are mean ± SEM (n=5). MGCV: Mean globular volume, MCH: Mean Concentration Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration. *significantly different from the control, *P = .05 using one way ANOVA.

Table 6. Haematological parameters of female rats during the 28-days sub-chronic test

Parameters	Control	mg/kg		
		31,25	125	500
Erythrocytes (T/L)	6.84±0.12	6.73±0.14	6.31±0.12*	6.67±0.09
Haemoglobin (g/L)	141.40±0.32	134.40±0.52	124.00±0.37*	130.60±0.22*
Haematocrit (%)	47.12±1.21	45.60±1.36	42.60±1.03*	45.80±1.07
MCV (fL)	68.80±0.86	67.46±0.69*	67.60±0.87	69.00±0.77
MCH (Pg)	19.80±0.37	19.00±0.45	18.80±0.37*	19.00±0.00*
MCHC (%)	30.00±0.55	29.80±0.49	28.80±0.37*	28.40±0.51*
Leucocytes (G/L)	1.72±0.26	4.40±0.78*	3.24±0.69*	2.94±0.52*
Neutrophils (%)	7.00±1.38	9.80±2.40	5.60±0.93	8.60±2.56
Eosinophils (%)	00.00±00.00	00.00±00.00	00.00±00.00	2.40±1.802.4*
Lymphocytes (%)	90.60±2.11	85.80±3.25	92.20±1.20	86.80±4.37
Monocytes (%)	2.40±0.93	4.40±0.93*	2.20±0.37	2.20±1.11
Platelets (G/L)	603.20±8.95	629.00±35.33	634.80±45.19	606.80±30.3

Values are mean ± SEM (n=5). MGCV: Mean globular volume, MCH: Mean Concentration Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration. *significantly different from the control, *P = .05 using one way ANOVA

3.2.4.1 Histology of liver

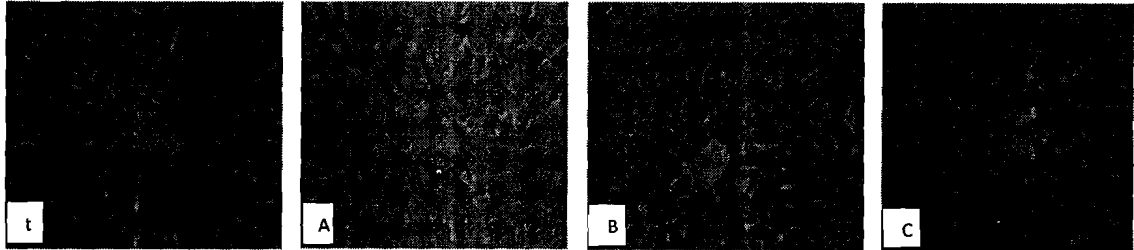


Fig. 6. Liver of control (t), and treated rats with the aqueous extract of *Carissa edulis* at 500 mg/kg (A), 125 mg/kg (B) et 31,25 mg /kg (C) body weight (HE × 400)rats (HE x 40) showing general vision of hepatic lobule relating hepatocytes disposed radially around centrolobular vein (V)

3.2.4.2 Histology of kidney

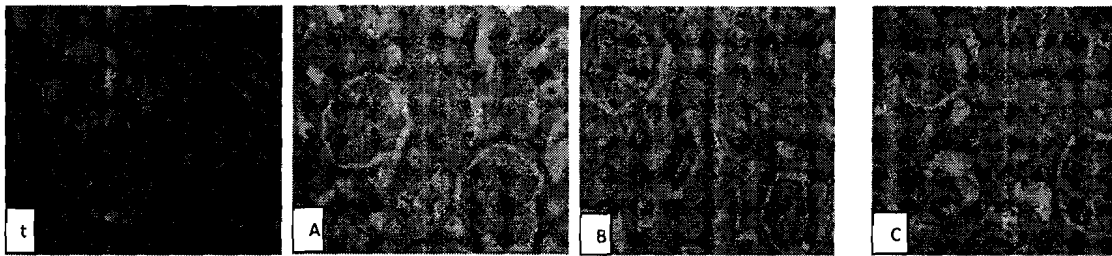


Fig. 7. Kidney of control rat (t)(HE × 400) showing the renal cortex with normal glomeruli (G) and tubules (T)and treated rats with the aqueous extract of *Carissa edulis* at 500 mg/kg (A), 125 mg/kg (B) et 31,25 mg /kg (C) of body weight (HE × 400).

4. DISCUSSION

The use of herbal medicines as alternative treatments has been increasing worldwide and gaining popularity in developing countries [8]. Although *Carissa edulis* is widely used for the treatment of many ailments in traditional medicine and biological efficacy studies [9-11] carried out on the plant, the potential toxicity effect of the plant has not been well established. To the best of our knowledge, there are no published studies on *Carissa edulis* toxicological profile following subacute exposure. In this study, we assessed the acute and the 28 days sub-chronic toxicity of *Carissa edulis* aqueous extract in rats. The results show that the minimum lethal dose (DL50) is higher than 2000 mg/kg since there was no mortality among the animals at that dose. Yau et al. [12] have reported the same result with the ethanol extract of *Carissa edulis* at the same dose. Changes in body weight and food consumption have been used as an indicator of adverse effects of drugs and chemicals [13]. Since no significant changes were observed in the general behavior, food consumption and body weight in the treated

groups as compared to the control group after the acute and subacute toxicity test. This suggested that at the chronic doses administered, the aqueous extract of the leaves of *Carissa edulis* had no effect on the normal growth of rats.

Analysis of blood parameters is relevant for risk evaluation, as any changes in the hematological and biochemical systems have a higher predictive value for human toxicity when data are translated from animal studies [14]. The hematopoietic system is one of the most sensitive targets for toxic compounds and an important index of physiological and pathological status in man and animal [15]. Among the hematological parameters of both genders of rats, daily oral administration of the extract for 28 days decreased slightly red blood cells, hematocrit and hemoglobin in treated group at 31.25 mg/kg and 125 mg/kg. Nevertheless, the values were not pathologic. Lymphocytes values were also decreased in male rats at 125 mg/kg when compared with the control group. The white blood cells (WBC) of female rats and monocytes of male' rats were in the other hand slightly

increased. This could justify its antibacterial and antiviral properties. [16,17]. Koffuor et al. [18] have demonstrated that the ethanol extract of the roots stem bark could stimulate erythropoiesis in phenylhydrazine treated rats.

The biochemical parameters were not in general affected. Nevertheless, a slight but acceptable rising of blood glucose was noted in female rats (See table III and IV) whereas the male rats showed a significant increase of chloride and potassium values comparing to the control group. There is any argument in favor to the toxic effect of the plant. However all these modifications of biological parameters are not dramatic, a very careful monitoring is needed during the repeated administration of the plant.

Finally, the histopathological evaluation (macroscopic and microscopic) examination was carried out on liver and kidney of both the treated and control rats. 28-days subchronic oral ingestion of the aqueous extract of *C. edulis* shows the normal architecture of the histological structure, suggesting no detrimental changes.

5. CONCLUSION

The aqueous extract of the leaves of *C. edulis* was found to be safe in acute and 28-days sub-chronic toxicities studies. But Furthermore, aspects of the effects of this plant on hematopoiesis, liver and kidney function need to be gained and ascertained over longer periods of toxicity study.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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