UTTAR PRADESH JOURNAL OF ZOOLOGY

43(21): 40-50, 2022
ISSN: 0256-971X (P)

TOXICO-PATHOLOGICAL ASSESSEMENT OF *Nerium oleander* ROOTS AND LEAVES ON WISTAR RAT

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Authors TM and FBS designed and written the manuscript. Authors TM, IO, AT and ZH realized the experiments. Author CA corrected the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.56557/UPJOZ/2022/v43i213210

Editor(s):
(1) Dr. Ana Cláudia Correia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.
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Received: 01 September 2022
Accepted: 04 November 2022
Published: 10 November 2022

Original Research Article

ABSTRACT

The aim of the study was to evaluate the toxic effects of *Nerium oleander* roots (R) and leaves (L) aqueous extract on Wistar rat. Serum chemistry, epididymal sperm parameters and histological examination of liver, kidney and brain were assessed after twelve consecutive day's oral administration of *N. oleander*. The extracts of R1, R2, L1 and L2 induced no treatment-related adverse effects with regard to general behaviors, hematological, serum chemistry, epididymal sperm parameters and histological profiles of liver, kidney and brain. However, the higher doses (R3, L3) led to, diarrhea, weakness, anorexia, frequent urination, and nasal hemorrhage, with no mortality. The hematological data revealed a significant increase in the levels of hematocrit and platelets in both L3 and R3 compared to the control. However, these two extracts have not affected the level of RBC, hemoglobin, WBC, and lymphocytes, while the L3 has reduced the concentration of glucose and increased that of urea. However, triglycerides, total cholesterol, alanine aminotransferease, albumin, conjugated bilirubin, potassium and calcium levels of L3 and R3 were not statistically significant compared to the control. Simultaneously bilirubin concentration was increased significantly in L3 and R3. There were no significant differences in epididymal sperm analysis when compared to the control. Adverse histological changes were observed in both liver and kidney as well as in the brain. To conclude, *N. oleander* extracts have disturbed the levels of certain vital variables in rats at higher dose, in which caution should be taken during the use of this plant.

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Keywords: *Nerium oleander*; hematological markers; serum chemistry; sperm parameters; histopathology.

1. INTRODUCTION

The use of herbal medicines in phytopharmaceuticals has increased in recent years as a refuge to treat many illnesses. The World Health Organization has recognized the contribution and the value of the herbal medicines used by a large segment of the world's population [1]. While reported incidents of intoxication due to intake of herbal preparation remains at a very low level, serious adverse reactions or even fatalities occur occasionally [1]. In Algeria, the majority of the population does not pay attention to theuseofmedicinal plants treatment for many ailments and serious diseases, such as diabetes for several considerations; historical, social, economic …etc. *Nerium oleander* is a species of shrubs belong to the Apocynaceae family. This species (*Nerium oleander*) is traditionally used as potent antidiabetic [2]. In the folk medicine, the juice of young leaves is effective in the cure of eye diseases. Oleander is native to Mediterranean regions and has been cultivated as ornamental shrubs throughout the tropical and subtropical regions [3].

Recently, substantial attention has been paid to oleander following the promising results of patented extracts, "Anvirzel", successfully tested on cancer patients [4]. Recent works has shown that oleandrin possesses selective cytotoxic potential against cancer cells [5]. The cell sensitivity to cardenolide is related to the expression pattern of Na/K-ATPase α isoform [6], in addition to antiviral activity that has been suggested to be used against different viruses, especially during the first two hours post infection [7]. Likewise, the observed decrease of cancer cells' invasion was established by the potential role of oleandrin and odoroside A for the inhibition of phospho-STAT-3 [8].

In addition to its therapeutic benefits *N. oleander* flowers, fruits, leaves and roots have been reported to contain poisonous compounds [9], where oleandrin is the most toxic among around 30 known cardenolides [10]. It was reported that the toxins of this plant, upon ingestion, can shut down the cardiovascular and nervous systems, after causing severe nausea, abdominal pain, and diarrhea [11]. Oleandrin can rapidly accumulate in the brain tissue of rats, causing important neurological depression in severe poisoning cases [12]. In addition, oleandrin and neriin have the capability to impair the plasma membrane Na/K-ATPase [13]. Thus, oleandrin along digoxin have been found in the blood of people intoxicated with *N. oleander* [1,14], in which the severity of toxicity was correlated with its blood level [15].

The lack of scientific data about the effectiveness and safety of medicinal plant is one of the major problems in the exploration of traditional medicinal preparations. This is likely linked to the lack of mechanism of action and toxicological profile of the plants studied.

The objectives of this study are to investigate the adverse effect of aqueous extracts of roots and leaves of *Nerium oleander var. Indicum* (Mill.) on Wistar rat.

2. MATERIALS AND METHODS

2.1 Preparation of the Plant Aqueous Extract

Matured leaves and roots of *Nerium oleander var. indicum*(Mill.). Deg. and Greenwell were collected personally from the rural region of Kherraza, Annaba city (36°50' N and 7°40' E) in mid-winter, and identified by Dr. Sihem Louhi Haou of Department of Biology, University of El -Taref, Algeria. Leaves (L) and roots (R) were washed with distilled and separated, shadow dried under room temperature before, powdered by an electric blender and then stored in sealed container. In order to obtain the aqueous extracts, 50 g of the powders of roots (R1, R2, R3) and leaves (L1, L2, L3) were infused in 500 ml of boiled distilled water for 24 h under agitation, and then a trouble solution was obtained after cooling and passing through a filter paper (Watman grade 1, Sigma).

2.2 Animals' Treatment

Males Wistar rats obtained from Pasteur Institute (Algiers) weighing (Mean ± SD) were divided equally into seven groups, with similar average body weight. Animals were placed in the animal house under standard laboratory conditions of temperature, light and humidity, in which standard food and water *ad libitum* were provided. The groups were divided into the control (vehicle saline serum NaCl 0.9%), R1 (0.5 ml/rat/day), R2 (1 ml/rat/day), R3 (1.5 ml/rat/day), L1 (0.5 ml/rat/day), L2 (1 ml/rat/day) and L3 (1.5 ml/rat/day). Aqueous extracts were administered daily by gavage in the morning for twelve consecutive days.

2.3 Samples' Collection

Animals were decapitated and blood was received into test tubes containing EDTAin order to evaluate red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), white blood cells (WBC), lymphocytes (Lym),

mean corpuscular haemoglobin concentration (MCHC) and platelets by using the automate instrument (BC 6800 Mindray, China). For serum chemistry, blood was received in dry test tubes, centrifuged at 3000 rpm/min for 15 minutes, and then analysed by the automate apparatus (Tokyo Boeki 50i) for the measurement of serum glucose, total cholesterol, triglycerides, urea, creatinine, alanine aminotransferase (ALT), albumin, bilirubin, unconjugated bilirubin (U-bilirubin), and conjugated bilirubin (C-bilirubin), while the automate apparatus (IlyteMedica) was used to measure calcium, sodium and potassium levels.

The epididymis was carefully separated from the testis, and the caudal epididymis was lacerated with scissors. Afterward, a drop of semen (about 1µl) was diluted with a physiological solution of 0.09% NaCl, and 5µL of the mixture was placed in an empty chamber slide (GoldCyto model). The slide was then placed on a Nikon Eclipse (Nikon E200-LED) microscope at the phase objective (x4). The concentration of sperm, motility, vitalitylinearity (VCL, VSL), velocity (VAP), the amplitude of lateralheaddisplacement (ALH) and the beat cross frequency (BCF) wereevaluated using Computer assisted semen analysis–Sperm Version CASA System (SCA®, Microptic, Barcelona, Spain).

Liver, kidney and brain were fixed in10% formol and embedded in parraffin. Sections at 5 µm were stained with hematoxylin and eosin (H and E) and examined under light microscopy. The photomicrographs of the organs' microscopic features were recorded.

2.4 Statistical Analysis

Results were expressed as means ± SD. Statistical analysis was performed using (MINITAB 17 Software ANOVA). The differences between two groups are considered statistically significant at equal or less than 0.05.

3. RESULTS AND DISCUSSION

3. 1 Poisoning Symptoms

The lack of scientific data about the effectiveness and safety of medicinal plant is one of the major problems in the use of traditional medicinal preparations. This is probably due to the non-evaluation of toxicological data and mode of action of these plants. Results revealed that treated groups with R1, R2, L1 and L2 did not produce any behavioral changes during the study period. Though, in the groups that received the highest dose of roots R3 (1.5 ml/rat/day) and leaves L3 (1.5 ml/rat/day), signs of poisoning started to appear in the sixth day of the experiment that included diarrhea, weakness, anorexia frequent urination, nasal hemorrhage and irritation. However, during necropsy, liver and heart did not show any alteration. Furthermore, no mortality was recorded in all groups during the twelve days. Other investigations reported that giving leaves of *N. oleander* in different concentrations to sheeps and mice induced intoxication symptoms [16,17]. Hence, changes in general behaviors have been used as the critical indicators for toxicity evaluation [10]. Further study revealed the presence of oleandrin in the serum of individuals supplemented with *N. oleander* [1].

3.2 Hematological Parameters

The obtained data presented in Table 1 showed no significant change in the level of RBC, haemoglobin, WBC, and lymphocytes of the treated rats compared to the control, while that of haematocrit and platelets were significantly raised in both R3 and L3 groups. Initially the increase in hematocrit is probably an indication of haemoconcentration, where water quantity decreases (hypovolaemia) in the blood [18]. As of result, total body water and sodium are low, because of the increased ADH secretion seen in hypovolaemic state, causing increased water reabsorption [19]. It has been reported that hypovolemia is one of the consequences of hyponatremia observed in our study; especially rat's ingested *N. oleander* had experienced diarrhea and digestive difficulties, a sign of intoxication [11].

The effect of oral administration of R3 and L3 on platelet counts is remarkably high. These findings are in accordance with other results [20]. Previously, it was reported that certain glycoproteins mainly produced by liver, kidney and bone marrow, are responsible for the production, proliferation and the differentiation of megakaryocytes into large numbers of platelets [21]. Therefore, the chemical components of R and L extracts might enhance the thrombopoietin in rats administered with *N. oleander* extract for twelve days. The Arachidonate 12-lipoxygenase gene was proved to modulate platelets' production and aggregation [22]. Therefore, further investigations are needed to identify the active compounds of *N. oleander* extracts that perhaps responsible for the thrombopoiesis activation.

Table 1. Hematological variations (mean ± SD) of rats treated with aqueous roots and leaves extract of *N. oleander* for 12 days

Markers	Control	ROOTS			LEAVES		
		R1	R2	R3	L1	L2	L3
RBC (10^6/µl)	7.85±1.74	7.35±0.15	8.05±1.15	8.06±0.29	8.00±0.95	8.15±1.05	8.58±0.53
Hb (g/dl)	12.9±2.04	13.64±0.07	13.04±1.27	13.9±0.68	12.64±0.07	13.94±0.07	14.3±0.76
Ht (%)	36.27±6.40	38.63±1.22	37.33±3.32	45.40±3.02*	40.01±4.22	37.13±4.07	45.68±2.44*
WBC (10^3/µl)	9.16±3.31	10.48±2.03	8.08±1.03	8.67±1.66	10.28±2.13	9.38±1.93	11.10±2.50
Lymph (10^3/µl)	7.71±2.94	7.27±0.43	6.17±0.66	5.82±0.86	6.21±3.23	6.27±0.47	6.52±2.25
MCHC (pg)	16.48±0.84	16.35±01.54	16.25±1.22	17.10±0.37	17.25±1.04	16.95±1.77	16.72±0.28
Platelets (10^3/µl)	593.4±65.5	600.83±30.70	610.66±19.50	817±120.5*	609.33±20.2	588.08±25.20	872±103.5*

**: Statistically significant when compared to the control*

3.3 Serum Chemistry

Biochemical profile is known to provide important information about the internal environment of a given organism. In the present investigation, the variations of the sero-biochemical markers of *N. oleander* extracts were presented in Table 2. Results revealed no changes in glucose levels in all treated groups compared to the control, except in L3 group, in which it was decreased significantly, confirming the anti-diabetic benefit of this plant as reported elsewhere [2]. Similar results were observed in other studies after oral administration of *N. oleander* leaves extract to diabetic rats [4]. The *N. oleander* extract was reported to possess inhibitory effect on both the primary enzymes of polysaccharides catabolism, and thereby it causes delay in the breakdown of carbohydrates to monosaccharides [23]. In these cases, *N. oleander* could be utilized as an anti-nutritional supplement to reduce the digestion of carbohydrates and decrease intestinal glucose absorption.

However, the one-way ANOVA revealed a no significant difference in serum triglycerides, total cholesterol, ALT, creatinine, conjucated bilirubin and albumin concentration of R3 and L3 groups. Contrary, R3 and L3 have showed significant elevation of serum total bilirubin concentration, whereas, unconjugated bilirubin level has increased in L3 group only. It was reported that the elimination of *N. oleander*, oleandrin occurs mainly through faeces, suggesting enterohepatic circulation (EHC) [24], where oleandrin possibly reabsorbed into the liver and enter the systemic circulation in the terminal ileum [25], which may be the reason of increased serum biliribun. Such results are in line with other studies carried out on *N. oleander* using animal models [10]. Hyperbilirubinaemia is a very sensitive test to demonstrate the functional integrity of liver and severity of hepatocyte necrosis [26]. Biliribun may accumulate in the blood and extracellular fluid when the hepatocytes are damaged [27]. The observed hyperbilirubinaemia after exposure to *N. oleander* possibly is due to the induction of heam oxygenase that plays an important role in haem catabolism [28]. It has been suggested that the increase in total and unconjugated bilirubin of extract-treated animals may suggest cholestasis [27]. The one-way ANOVA revealed a no significant difference in the serum K and Ca of all treated rats. Similarly, there was a non-significant decrease of Ca concentrations of R and L group alike. Noticeably, the concentration of Na was significantly decreased at higher doses of R3 and L3 groups. Serum Na level is often considered as reliable marker for renal function, where hyponatremia usually occurs in patients with chronic renal failure, because of decreased glomerular filtration that leads to decreased delivery of fluid to the nephron distal diluting segment [29]. Remarkably, reduced plasma volume caused by fluid excretion during hyponatremia can result to high hematocrit level [30]. Generally, the mechanism by which cardenolides affect cardiac contractility is known to be mediated by a highly specific inhibition of the Na/K-ATPase, leading to variations in the intracellular levels of electrolytes [31]. Since *N. oleander* is rich in cardiac glycosides oleandrin and oleandrigenin, it was suggested that these molecules might inactivate the Na/K-ATPase pump of cardiac cells [16], reducing sodium extrusion from cardiac muscle. The increase in intracellular Na reduces the extrusion of Ca from the cell via the Na/Ca exchanger [32]. This raises the intracellular calcium content and cause calcium release from the sarcoplasmic reticulum, which results in an increase in cardiac contraction [16]. This probably why serum Ca level has decreased in rats' administered with the higher doses of L3 and R3. In contrast, the level of K has not been affected by *N oleander* extracts, although hyperkalemia was observed during the inhibition of the Na/K-ATPase pump, which impairs the entry of K to cells [33].

Table 2. Varations in serum chemistry (mean ± SD) of rats treated with aqueous roots and leaves extract of *N. oleander* for 12 days

Markers	Control	Roots			Leaves		
		R1	R2	R3	L1	L2	L3
Glucose (g/l)	1.35±0.15	1.25±0.12	1.30±0.15	1.2±0.13	1.27±0.22	1.29±0.15	1.00±0.19*
Cholesterol (g/l)	0.64±0.07	0.60±0.10	0.59±0.1	0.54±0.09	0.56±0.02	0.59±0.05	0.63±0.09
Triglycerides (g/l)	0.63±0.22	0.60±0.99	0.59±0.32	0.61±0.24	0.58±1.22	0.60±1.02	0.59±0.17
ALT (IU)	66.35±11.54	60.55±10.54	56.35±10.24	55.59±5.47	60.15±9.54	59.35±9.84	64.92±10.74
Albumine (mg/l)	27,26 ±5,10	25,51 3,47	27,38 4,26	28,37 ±3,97	27,99 3,66	27,48 3,05	32,68 ± 6,07
T-Bilirubin (mg/l)	0.88±0.20	0.86±0.70	0.95±0.20	0.99±0.20*	0.94±0.90	0.94±0.50	1.15±0.17*
C-Bilirubin (mg/l)	0.01±0.00	0.01±0.04	0.01±0.00	0.01±0.00	0.01±0.04	0.01±0.02	0.01±0.00
U-Bilirubin (mg/l)	0.86±0.20	0.88±0.70	0.98±0.20	0.99±0.20	0.87±0.40	0.98±0.30	1.77±0.20*
Urea (g/l)	0.48±0.03	0.46±0.13	0.50±1.15	0.46±0.05	0.49±0.33	0.51±0.53	0.59±0.08*
Creatinine (g/l)	5.27±0.43	4.97±0.48	5.07±0.93	4.99±0.41	5.01±1.43	4.87±0.63	5.50±0.38
Ca (mg/l)	95.43±5.22	90.43±6.02	92.73±4.22	89.04±4.30	92.33±3.33	96.93±2.22	88.75±6.72
Na (mEq/l)	150.40±2.09	152.40±1.00	145.70±2.09	136.70±6.06*	153.33±1.99	147.60±2.11	136.50±4.76*
K (mEq/l)	7.16±1.18	6.46±1.48	7.06±2.00	6.37±0.41	7.00±1.08	6.96±0.98	6.91±0.77

**: Statistically significant when compared to the control*

3.4 Sperm Parameters

Results of oral administration of aqueous extract of *N. oleander* on epididymal spermatozoa count, motility, vitality, linearity (VCL, VSL), velocity (VAP), the amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF) showed no statistically significant differences between experimental groups (Fig. 1). Our results are in contradiction with other results [34], which indicated that *N. oleander* flower extract has some spermicidal effect such as immobilizing the sperms, clumping of sperms in fresh sheep testes. In addition, these results are not in line with that reported elsewhere [35], which demonstrated antifertility of the triterpenoids from *Nerium oleander* 100 and 200mg/kg body in male albino rats.

3.5 Histological Study

3.5.1 Liver

The biochemical finding was confirmed through by histological observations. The histopathological assessment of the liver shows clear sinusoids and hepatocytes, and well outlined central vein in the control (Fig. 2, A) as well for the other sections of R1, R2, L1 and L2 (Fig. 2, B, C, E, F). However, in R3 and L3 groups, liver histology of male rats revealed certain lesions characterized with mostly distorted histoarchitecture. Histological features of R3 showed the presence of significant congestion in the hepatic veins (Fig. 2, D), but that of L3 demonstrated mild inflammatory cells, infiltration in the portal triads

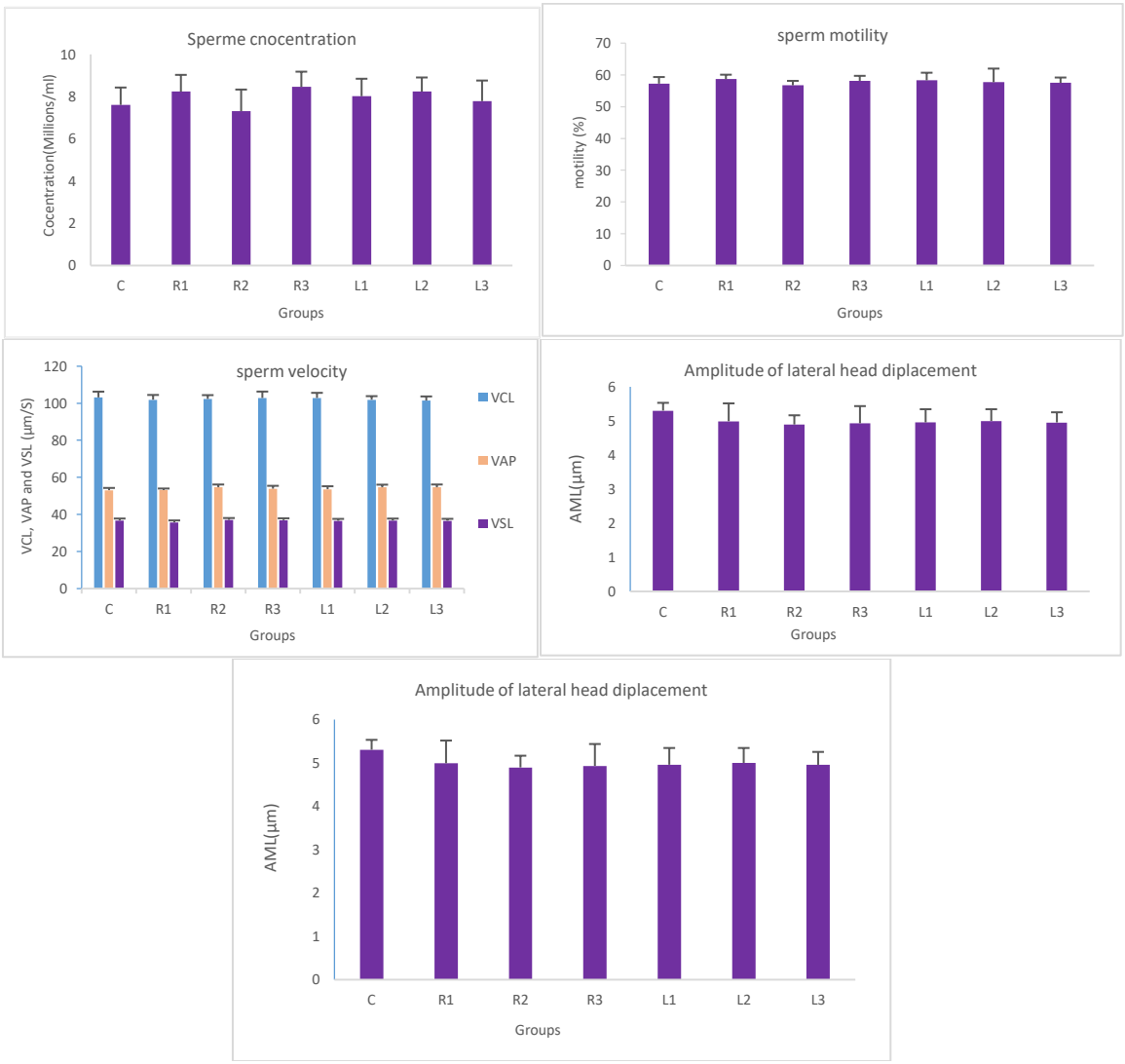

Fig. 1. Evaluation of sperm concentration (Millions/ml), sperm motility percentage (%), velocity (VCL, VSL and VAP), sperm amplitude lateral head displacement (ALH), sperm beat cross frequency (BCF) of rats treated with aqueous roots and leaves extracts of *N. oleander* for 12 days

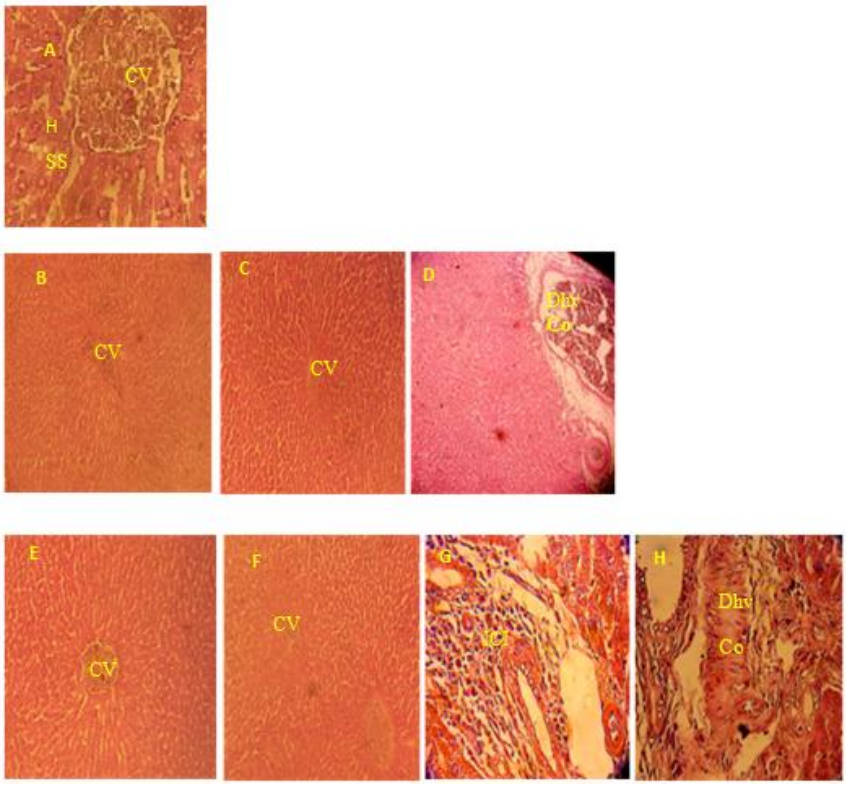

Fig. 2. Microscopic observation of rat liver sections (hematoxylin and eosin). (A): Control group: Hepatocyte (H), sinusoidal spaces (SS), central vein (CV); B and C: R1 and R2 treated group, respectively; D: R3 treated group: Dilated hepatic veins, significant congestion (Dhv, Co); E and F: L1 and L2 treated group, respectively; Group H: L3 treated group: Inflammatorycells infiltration in the portal triads (ICI), (magnification: (G, H) = 40x),. Slides A, B, C, E and F demonstrated normal histological structure (magnification: 10x)

(Fig. 2, G), and dilated hepatic veins with significant congestion (Fig. 2, H). Our observations are in agreement with other investigations [17] that observed the presence of leukocytes' infiltration, diffuse fatty vacuolation and hepatocyte necrosis in sheep treated with single dose (250 mg/kg) of *N. oleander* leaves. In addition, pharmacokinetic studies in mice have shown that oleandrin is absorbed in the stomach and binds to plasma proteins, with high affinity for albumin. It is metabolized in the liver or there is an enterohepatic cycle, suggesting hepatobiliary excretion [14]. Thus, oleandrin was reported in the liver and cerebrospinal fluid of poisoned individuals [16]. It has been well established that free radicals elicit an inflammatory response at the site of injury [36]. Thus, toxic elements may induce pro-inflammatory cytokines by activating Kupffer cells [37]. The TNF-α, an important pro-inflammatory cytokine that is noticeably concerned in the progression or initiation of inflammatory response [38] and also stimulate the triggering of inflammatory mediators like IL-1, IL-6, TGF-β and prostaglandins [39] that facilitate liver injury [40].

3.5.2 Kidney

At the level of the Histoarchitecture of kidneys, the renal histoarchitecture of the control (Fig. 3, A), R1 (Fig. 3, B), R2 (Fig. 3, C), L1 (Fig. 3, G) and L2 (Fig. 3, F) groups showed normal morphology of parenchyma with well-defined glomeruli and tubules. Remarkably, the kidney of R3 group (Fig. 3, D, E) revealed the presence of inflammatory cells infiltration and glomerular atrophy, while rats treated with L3 (Fig. 3, H, I) revealed dilated blood vessels with significant congestion and enlarged bowman's space. These results are similar to other researchers [41] who found wide dilated cystic and very thin bowman's space in mice. Furtheremore, severe injury in glomeruli and renal tubules, in rat was noted [42]. These alterations observed are perhaps generated from oxidative stress produced by toxic substances that can be isolated from all parts of the *N. oleander*, which are similar to digitalis toxin [2]. These toxins can lead to an imbalance in the level of production/consumption of reactive oxygen species [20], especially it was confirmed the presence of

considerable amount of oleandrin in renal tissue [1]. The disagreement in the results is possibly linked to differences in the susceptibility of different animal species or to the concentration of cardenolide present in the plant, which may vary depending on plant variety and stage of growth [7].

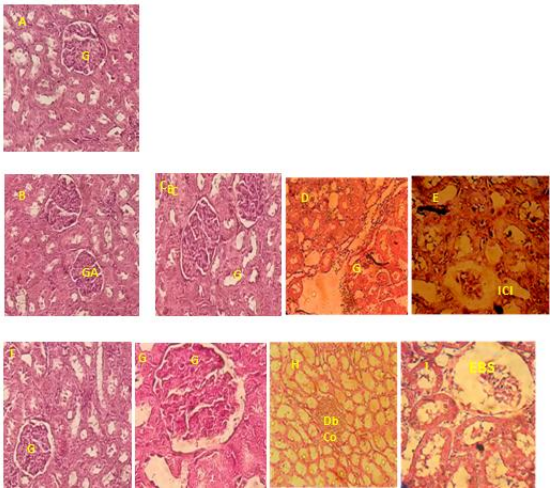

Fig. 3. Microscopic observation of rat kidney sections (hematoxylin and eosin). (A): control group, (magnification: (A) = 10x), Normal renal architecture, Glomerulus (G);(B, C): R1 and R2 treated group respectively, (magnification: (B, C) = 10x), Normal structure; (D, E): R3 treated group, (magnification: (D, E) = 10x). Inflammatory cells infiltration (ICI), Glomerular atrophy (GA); (F, G): L1 and L2 treated group respectively, (magnification: (F) = 10x;(G) = 40x), Normal structure. (H, I): L3 treated group, (magnification: (H) = 10x, (I) = 40x); Dilated blood vessels with significant congestion (Db, Co), enlarged bowman's space (EBS)

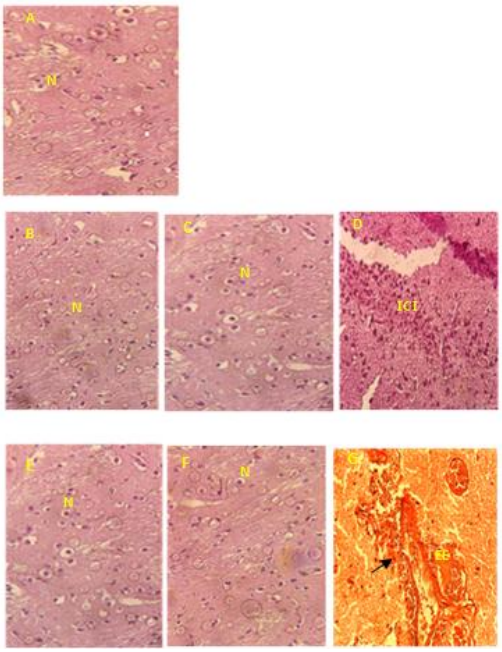

Fig. 4. Microscopic observation of rat brain sections (hematoxylin and eosin) at magnification of 10x. (A): control group: neuron (N); B and C: R1 and R2 treated group, respectively: B and C: L1 and L2 treated group, respectively; D: R3treated group; D: Inflammatory cells infiltration (ICI) (magnification: (D) = 40x); E and F: L1 and L2 treatedgroup, respectively; G: L3 treated group; G: Cellular infiltration (black arrow), extravasated erythrocytes (EE) (magnification: (G) = 40x). Slides A, B, C, E and F demonstrated normal histological structure

3.5.3 Brain

The histological sections of the brain disclose a normal morphology of the control (Fig. 4, A) as well as of R1, R2, L1 and L2 (Fig. 4, B, C, E, F) treated groups. Interestingly, the brain of R3group showed inflammatory cells infiltration (Fig. 4, D), but for rats treated with L3 showed cellular infiltration and extravasated red blood cells (Fig. 4, G). It has been reported that the nerioleanderoside (1) at the dose of 15 mg/kg provoked CNS depressant activity in swiss mice [43]. Many studies in mice have shown that oleandrin was rapidly absorbed after oral administration, with a bioavailability of about 30%, and biotransformed to oleandrigenin possibly by an enzymatic process [15]. It has also been shown that oleandrin can cross the blood-brain barrier and accumulate in brain tissue; these data suggest that other components of *N. oleande*r extract may enhance the transport of oleandrin to cross the blood-brain barrier [14,34].

4. CONCLUSION

In conclusion, the administration of weak doses of roots and leaves of *N. oleander* aqueous extract to rats for twelve consecutive days have resulted in no adverse effect on serum and sperm biological markers studied. In contrast, the higher doses have altered the vital organs; liver, kidney and brain reflected by the significant variations in most biochemical, and histopatological indices. In conclusion, the aqueous extract of *N. oleander* at higher doses is not safe as an oral remedy, in which caution should be taken when used for treatment of certain diseases.

ETHICAL APPROVAL

The experimental procedures were carried out according to the National Institute of Health Guidelines for Animal Care and approved by the Ethics Committee of our Institution.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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