

ORAL ACUTE TOXICITY AND RED BLOOD CYTOTOXICITY OF THE MEDICINAL HALOPHYTE *LIMONIASTRUM GUYONIANUM* LEAF EXTRACT

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Abstract

Limoniastrum guyonianum is widely used traditionally in North Africa as decoction or infusion to treat various types of infections. However, safety assessment of this medicinal halophyte is still lacking. The aim of this study was to investigate the safety of the aqueous leaves extract by determining the oral acute toxicity effect on adult mice and cytotoxicity on human red blood cells. No mortality or specific toxic signs were observed. The LD₅₀ can then be estimated to be greater than 5000 mg/kg. The recommended dose used in humans will be a maximum of 500 mg/kg. Histopathological examinations showed alterations that affected both renal and hepatic tissues, being the kidneys more sensitive. Indeed, glomerular necrosis appeared with the first ingested dose (1000 mg/kg bw). In contrast, focal hepatocyte necrosis was noticeable only at a concentration of 5000 mg/kg bw. The cytotoxic study showed a slight haemolysis of 13.52% which demonstrate that the extract does not express a haemolytic activity. Hence, the extract can be safely utilized in folkloric popular medicine as an oral anti-infective.

Rezumat

Limoniastrum guyonianum este utilizată tradițional pe scară largă în Africa de Nord, sub formă de decoct sau infuzie pentru tratarea diferitelor tipuri de infecții. Cu toate acestea, evaluarea siguranței acestei plante halofite nu a fost încă studiată. Scopul acestui studiu a fost de a investiga siguranța utilizării extractului apos de frunze prin determinarea toxicității acute la șoarecii adulți și a citotoxicității asupra globulelor roșii umane. Nu au fost observate semne toxice specifice. DL₅₀ estimată a fost mai mare de 5000 mg/kg. Doza recomandată la om se estimează la maximum 500 mg/kg. Examenle histopatologice au arătat modificări care au afectat atât țesuturile renale, cât și hepatice, rinichii fiind mai sensibili. Necroza glomerulară a apărut o dată cu prima doză ingerată (1000 mg/kg corp). În schimb, necroza hepatocitară focală a fost vizibilă doar la o concentrație de 5000 mg/kg corp. Studiul citotoxic a arătat o ușoară hemoliză, care demonstrează că extractul nu exprimă o activitate hemolitică. Prin urmare, extractul poate fi utilizat în siguranță ca antiinfecțios oral.

Keywords: *Limoniastrum guyonianum*, acute toxicity, cytotoxicity, human red blood cells

Introduction

Natural products play a dominant role in the development of novel drug leads for the treatment and prevention of diseases. Herbs have recently attracted attention as health beneficial foods and as source materials for drug development. They offer a potential natural health care approach that focuses on protecting and restoring health [33].

Limoniastrum guyonianum is medicinal halophyte, endemic to northern Sahara (Algeria, Tunisia), omnipresent in the salty lands of Sebkhass and Chotts and in the Sahara [9]. In Algeria, *L. guyonianum* is called "Zeïta". The traditional pharmacological uses of

L. guyonianum in the treatment of different pathologies are widely practiced in Algeria and other countries of North Africa. In Algeria, peoples of the southern "Aurès" region use the aerial part of the plant as a decoction against bronchitis and inflammation of the throat [1]. In the region of Ouargla (South-East), the plant is used either alone, or in combination with other plants in decoction, or powder to treat skin burns and wounds, metabolic and respiratory diseases [18]. The tannins of the galls have tonic properties associated with Henné [11], whereas roots decoction is used as depurative [12, 16]. In southern Tunisia, infusions of leaves and galls are used as anti-dysenteric and against bacterial (bronchitis) or parasitic infections

[9]. In the region of Tafilalet (Morocco), decoction of leaves and galls is also used for the treatment of diarrhoea [15].

A literature survey allowed us to identify the bio-active molecules of *L. guyonianum* responsible for the observed pharmacological activities, which can be associated to the traditional uses of plant. The active compounds are in most cases polyphenols, flavonoids, tannins and phenolic acids. These studies demonstrated antioxidant [14, 24, 41-43], antimicrobial [5, 7, 42], immunomodulatory [24], antimutagenic, antiproliferative, pro-apoptotic and anticancer activities [25-27, 45]. Furthermore, the aqueous extract of *L. guyonianum* proved to be more efficient than vitamin C pre-treatments in attenuating nickel induced haematological alterations [6].

Three powerful flavonoids with antioxidative property (gallic acid, epigallocatechin and epigallocatechin-3-O-gallate) were isolated from *L. guyonianum* [41]. Other phenolic compounds such as catechin and its derivatives, previously cited as antioxidant [40], were also detected [41]. Trabelsi *et al.* [43] have isolated a new dimer of phenolic acid amide with strong antioxidant activity which can be used in the pharmaceutical and food industries due to their antioxidant properties. Krifa *et al.* [24] have demonstrated the immunomodulatory effect of the aqueous extract of galls of *L. guyonianum*. This effect could be attributed, in part, to its cytoprotective effect exerted by some of its antioxidant components, such as flavonoids. These compounds can potentially modulate the functions of immune cells in physiological and pathological conditions.

L. guyonianum extracts exhibited an important and variable antibacterial activity. Inhibitory capacity against these pathogens was likely due to the richness of these extracts in phenolic compounds [42]. Methanolic, chloroform and petroleum ether extracts of *L. guyonianum* were found to be most potent against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [7].

Despite the widespread use of medicinal plants, few scientific studies have been undertaken to ascertain the safety and efficacy of traditional remedies [17]. Indeed, it is important to note that many medicinal plants have also been reported to be toxic to both humans and animals. Therefore, it should be emphasized that the traditional use of any plant for medicinal purposes, by no means, guarantees the safety of such plant. Evaluation of toxic properties of a substance is crucial when considering for public health protection. Toxicity screening models provide important preliminary data to help select natural remedies with potential health beneficial properties for future work [33]. However, there is no information or evidence available concerning the possible toxicity that *L. guyonianum* may cause to the consumers. Moreover, no toxicological studies on this medicinal plant have been performed. Considering both the ethnobotanical and pharmacological

applications of *L. guyonianum*, the aim of this study was to investigate the safety of the aqueous leaves extract of the plant by determining its possible oral acute toxicity on adult mice and cytotoxicity test on human red blood cells.

Materials and Methods

Plant material

L. guyonianum was collected from Bir-Alarbi, M'sila, Algeria, at the flowering stage. The plant was identified by Dr. Sarri Djamel, Department of Natural Sciences and Life, Faculty of Sciences, M'sila University, Algeria. The voucher was deposited at the same Department. The leaves were cleaned then separated and dried at room temperature until obtaining a constant weight. Dried leaves were ground to a fine powder and stored in air-tight bottles until use.

Aqueous extract preparation

Approximately 20 g of the powdered sample were added to 200 mL of boiling distilled water for 4 hours with continuous stirring [6]. The extract was filtered, and then evaporated to dryness at 55°C in ventilated oven. This operation was performed several times in order to obtain a manageable quantity of dry aqueous extract to carry out the toxicity study.

Oral acute toxicity

Animals

Acute oral toxicity test was performed as *per* OECD guidelines 420 [31]. Experiments were carried out using healthy young adult female Swiss albino mice, nulliparous, non-pregnant and weighing 26 - 32 g. These animals were obtained from Pasteur Institute of Algiers. Mice were placed in plastic cages with free access to food and water. Litter of wood chips was renewed every 2 days. The mice were acclimated to laboratory conditions for 15 days before experimentation. They were fed with croquettes from the National Company of cattle diet (ONAB) of El Kseur (Bejaia). Female mice were chosen because of their greater sensitivity to treatment [19]. All protocols used in this study were approved under the CNEPRU DO1-N01UN280120150001 by the Ethical Committee of Directorate General for Scientific Research and Technological Development at Algerian Ministry of Higher Education and Scientific Research.

Constitution of groups and treatment of mice

After acclimatization 4 groups of 10 mice each were constituted. The mice were deprived of food 18 h before the administration of the extract to stop any digestive food interaction. Three doses of the aqueous extract 1000, 2000 and 5000 mg/kg bw were administered as a single dose orally at a rate of 0.5 mL/100 g of body weight to the 3 groups of treated mice, the 4th group control received distilled water.

Symptomatic disorders

After treatment, the animals were maintained under the same conditions of temperature, lighting and feeding.

The treated mice were observed individually every 30 minutes for 6 hours, during the first day and every day for 14 days according to the guidelines of Conseil Canadien de Protection des Animaux [13] and Organization for Economic Co-operation and Development [30]. During this period, all the behavioural and symptomatic variations have been observed, with particular attention to external physical aspects (appearance and hair loss), behavioural changes (posture, scraping, aggressiveness, sensitivity noise and light, hyper-salivation, etc.), and measurable clinical signs (changes in heart and respiratory rhythms, abdominal contraction, diarrhoea).

Determination of lethal and tolerated doses

Among the aims of this study is the determination of the Maximum Tolerated Dose (MTD), which is the maximum dose with no observable toxic effect, the Minimum Mortal Dose (MDI) that kills an animal from the batch treated, the lethal dose for 50% of animals (LD_{50}) and the lethal dose for 100% of animals (LD_{100}). The value of the LD_{50} makes it possible to classify the extract on the toxicity scale of Hodge and Sterner [21] for mice and rats. The LD_{50} of the aqueous leaf extract of *L. guyonianum* is not yet determined.

Body and relative organ weights

Body weights were measured before starting the experimentation and every two days thereafter. At the end of the experiment, all surviving animals fasted overnight and sacrificed by decapitation. The organs such as liver, heart and kidneys were excised and weighed.

Serum biochemical parameters

In order to detect possible toxic effects of the extract, the blood of surviving mice after 14 days of experimentation was recovered to explore some biochemical parameters related to liver function (transaminases, alkaline phosphatase, serum total protein and albumin) and renal functions (creatinine, urea and uric acid). These analyses were performed at the central laboratory of the University Hospital Center of Setif using an automatic biochemistry analyser (Technicon, RA 1000).

Histological examinations

The excised livers and kidneys were washed in normal saline and fixed immediately in 10% formalin, dehydrated with upgraded ethanol series, embedded in paraffin, cut into 5 μ m thick sections and stained with haematoxylin-eosin dye for photo-microscopic observation. Histopathological examinations of the organs of the treated mice were compared with the control group.

Cytotoxic study (Haemolytic test)

The cytotoxicity study was performed on human red blood cells (RBCs) from healthy donors. The haemolysis rate of RBCs was studied both by measuring the absorbance of released haemoglobin (Hb) and by

counting residual cells after haemolysis. To test the haemolytic effect of our extract, 7 concentrations (5, 10, 15, 25, 50, 100 and 200 mg/mL) were prepared in isotonic phosphate buffer solution (PBS) at pH 7.4. The blood of healthy volunteers (3 mL) was freshly collected in heparinized tubes. The RBCs were separated from the plasma by centrifugation at 4500 rpm for 5 min. The pellet was washed 3 times with 5 volumes of PBS. During the last wash, the erythrocytes were centrifuged at 4500 rpm for 3 min to obtain a packaged cell preparation. The washed cells were re-suspended in a final volume of 20 mL of PBS [46].

The haemolytic effect of the aqueous extract was evaluated according to the method described by Powell *et al.* [34]. Aliquots of 540 μ L of the erythrocyte suspension were mixed with 60 μ L of PBS solution containing the extract at different concentrations. The samples were mixed gently and incubated at 37°C for 35 min in a water bath with gentle agitation. The tubes were then cooled in an ice bath for 5 min and then centrifuged at 4500 rpm for 5 min. Volumes of 300 μ L of supernatant were diluted in 2700 μ L of PBS. The solution was used to monitor the leakage of haemoglobin by measuring the absorbances at 576 nm against a positive control (total haemolysis with distilled water). PBS was used as a negative control (0% lysis).

The haemolytic effect can also be evaluated by counting the residual RBCs after treatment with the extract. Cell count was performed on Thoma cell. After haemolysis, the pellet of each assay was suspended again in PBS for a second enumeration to determine the number of un-haemolysed RBCs [46].

The test was performed in triplicate for each concentration. The haemolysis rate (H_{Hb}) was calculated according to the following formula:

$$H_{Hb} (\%) = [A_t/A_c] \times 100,$$

where: A_t = absorbance of the test, A_c = absorbance of positive control.

The rate of cellular haemolysis (H_C) can also be calculated by cell count according to the following formula:

$$H_C (\%) = [C_i/C_f] \times 100,$$

where: C_i = number of cells before haemolysis, C_f = number of cells after haemolysis.

Statistical Analysis

All values are expressed as mean \pm SEM. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using Graph Pad. Prism 5 statistical software. A p value of < 0.05 was considered significant.

Results and Discussion

Oral acute toxicity

General signs

During this study it was not observed mortality or specific toxic signs after oral administration of the extract at all doses used apart from a hair changes and decreased activity of mice that received a dose of 5000 mg/kg bw and returned to normal soon thereafter. In addition, there were no respiratory, nervous, cutaneous or gastrointestinal symptoms. According to the toxicity scale of Hodge and Sterner [21] for mice and rats, the aqueous extract of *L. guyonianum* can be classified as a non-toxic substance because no mouse in the group receiving 5000 mg/kg was dead. This suggests that this dose represents the non observable toxic effect level (NOAEL), which is a primary criterion in clinical trials since it serves as a basis for calculating the first dose used in humans. This dose was calculated by dividing the NOAEL on the Safety Factor which is greater than or equal to 10 [2]. The recommended dose will be a maximum of 500 mg/kg. Considering the average human blood volume of 60 mL/kg, the dose circulating in the body will average 8.33 mg/mL.

Weight evolution

The variation of the mice body weight during the 14 days of experimentation was noted every 2 days and at the same hour which makes it possible to follow the weight evolution of the treated and control animals. On day 0, mice have homogeneous weights of 26 g to 32 g. During the 14 days the progress of weight seemed normal, demonstrating the insensitivity of animals to the extract. The ingestion of the extract caused no significant change in body weight of mice compared to controls throughout the test period (Figure 1).

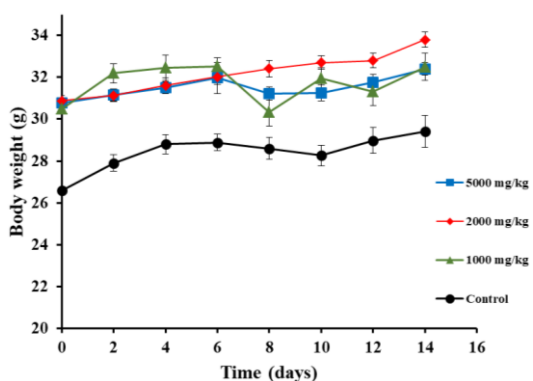


Figure 1.

Evolution of the body weight of the control mice and treated with the aqueous extract of the leaves of *L. guyonianum* at doses 1000, 2000 and 5000 mg/kg bw after 14 days of treatment

Each value represents the mean (n = 10) ± SEM

Relative weight of organs

After animal sacrifice, the liver, kidneys and heart were collected for macroscopic examination. The

size and shape of these organs were found to be normal and no morphological changes were observed, and this can be considered an index of lack of pathologies. Since the wellness of an organ can be correlated to its relative weight (weight of the organ/weight of the mouse × 100), being the hypertrophy of an organ means a beginning of dysfunction. No significant increase (p ≤ 0.05) in the relative weight of the kidneys and livers was observed for the 3 groups treated with 1000, 2000 and 5000 mg/kg bw (Figure 2).

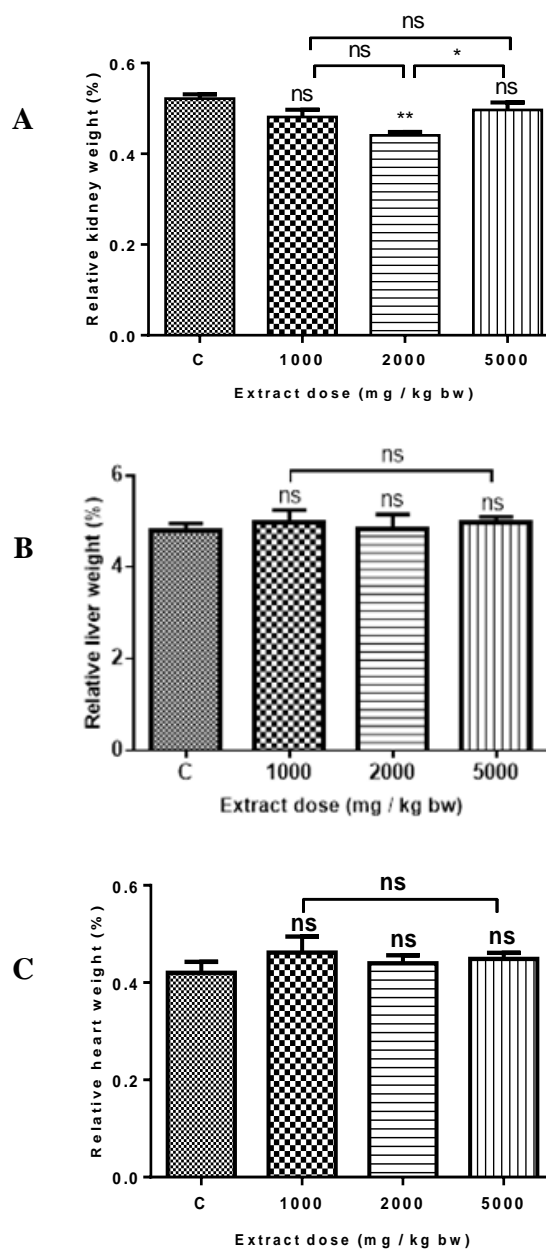


Figure 2.

Variation of the relative weights of kidneys (A), liver (B) and heart (C) of the control mice and treated with 1000, 2000 and 5000 mg/kg bw of the aqueous extract of leaves of *L. guyonianum* after 14 days of treatment

Each value represents the mean (n = 10) ± SEM

The clinical signs facilitate the interpretation of the results of the toxicological studies. Indeed, our mice never showed signs of toxicity or a decline in zootechnical performance. According to the globally harmonized classification system of the OECD [30] and since the LD₅₀ has been estimated at more than 5000 mg/kg bw, the aqueous extract of leaves of *L. guyonianum* can be classified under category 5, therefore considered a non-toxic oral substance.

Biochemical parameters

The assessment of the extent and severity of poisoning by medicinal plants or their extracts is primarily based on the prospection of liver and renal markers. To ensure non-toxicity of the aqueous extract, various biochemical parameters were measured at the end of the experiment (D14) and compared to controls. The results obtained are listed in Table I.

Table I

Variation of the biochemical parameters of control mice and treated with the aqueous extract of leaves of *L. guyonianum* at doses 1000, 2000, 5000 mg/kg bw after 14 days of treatment

Biochemical markers	Extract dose (mg/kg bw)			
	Controls	1000	2000	5000
ALAT (U/L)	65.31 ± 1.94 ^a	51.80 ± 5.42 ^a	46.75 ± 3.63 ^a	48.80 ± 6.17 ^a
ASAT (U/L)	275.35 ± 8.93 ^a	251.80 ± 23.00 ^a	213.33 ± 13.38 ^a	230.75 ± 12.73 ^a
PAL (U/L)	77.75 ± 8.05 ^a	68.40 ± 6.47 ^a	65.10 ± 9.52 ^a	60.87 ± 2.01 ^a
Total Proteins (g/L)	63.08 ± 5.55 ^a	53.66 ± 3.48 ^{ab}	44.43 ± 6.49 ^{ab}	44.76 ± 1.07 ^b
Albumin (g/L)	29.60 ± 0.29 ^a	29.21 ± 0.86 ^a	29.00 ± 0.31 ^a	30.82 ± 0.97 ^a
Uric acid (mg/L)	84.57 ± 4.75 ^a	65.40 ± 3.20 ^{ab}	61.41 ± 7.44 ^b	60.6 ± 2.16 ^b
Urea (g/L)	0.49 ± 0.02 ^a	0.47 ± 0.04 ^a	0.51 ± 0.04 ^a	0.42 ± 0.02 ^a
Creatinine (mg/L)	4.32 ± 0.05 ^a	4.47 ± 8.93 ^a	5.14 ± 0.07 ^b	5.64 ± 0.03 ^c

Each value represents the mean (n = 10) ± SEM (p < 0.05, Tukey test). Values with different letters on the same line are significantly different.

Markers of hepatic function

Hepatocyte involvement causes the release of enzymes into the extracellular compartment, then some liver enzymes have been tested, namely alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and alkaline phosphatase (ALP). The increase in transaminases reflects cell damage, particularly in the liver [22, 36].

ALAT is cytosolic localization, while ASAT is mainly mitochondrial [3]. Given its specificity and the availability of the assay, ALAT is the reference for biomarkers of liver damage [32]. Increased serum ALAT activity is typically associated with cell membrane damage and leakage of hepatocyte enzymes [44]. A moderate increase (2 times) is more in favour of a chronic condition or cholestasis [28]. Because of its presence both in the liver, heart and skeletal muscle, plasma concentration increase of ASAT activity can be linked to muscle inflammation. However, because of its predominantly mitochondrial distribution in hepatocytes and its very short half-life, it is often considered a reliable marker of the severity and persistence of hepatic necrosis [28].

The analysis of transaminase levels in experimental mice showed a downward trend, suggesting that the extract did not affected these liver parameters. This extract probably has no hepatotoxic effect on mice.

The ALP is another enzyme that can be used to evaluate liver function. In addition to liver damage (hepatitis, abscess, etc.), the ALP assay can detect cholestasis and biliary obstructions. ALP is a not very specific enzyme, given the existence of numerous isoenzymes in bone, placental and renal tissues [32]. The results showed a decrease in its activity for the

3 doses tested, but compared to the control group this decrease was not significant. The absorption of the aqueous extract of *L. guyonianum* would not cause cytolysis, and the plant appears to be well tolerated. In addition to its role of detoxification, the liver is also the site of the synthesis of several proteins (90% of total proteins) essential to the general metabolism, in particular albumin [8]. Following cell injury, the synthesis capacity is reduced and the concentration of these constituents decreases. A decrease in protein levels could mean severe liver dysfunction (cirrhosis). Our study revealed that the extract at 1000, 2000 and 5000 mg/kg bw in single doses did not show a significant difference for total proteins. Similarly, administration of the extract caused no significant change in serum albumin levels in mice compared to their controls. The fluctuations noted are minimal or even negligible. The results of the protein assay reflect a normal protein metabolism. In conclusion, the biochemical markers of liver function have been slightly modified indicating the absence of hepatotoxicity of the extract.

Markers of kidney function

Blood urea and creatinine are considered to be excellent markers of renal function. The extract did not induce any significant changes in plasma urea concentration in the treated mice compared to the control group. Instead, mean values of serum uric acid concentrations were significantly different and ranged from 84.57 mg/L in control mice to 65.4, 61.4 and 60.6 mg/L in mice treated with 1000, 2000 and 5000 mg/kg bw of extract, respectively. In parallel, the extract has a significant effect in increasing serum creatinine for

doses of 2000 and 5000 mg/kg bw. In sum, the results showed that the extract did not appear to be nephrotoxic. Creatinine and uric acid were selected as serum biomarkers of renal function, and their levels increased in a dose-dependent manner in the serum. The mice were “able” to reveal these metabolic disruptions, without presenting clinical signs, at least during the 14 days of study. In some cases, metabolic disruptions affecting the target organ may take longer to set in, or may show greater amplitude, resulting in disruption of the body's homeostatic balance. This can then result in clinical or biochemical manifestations that may appear later. Sometimes these metabolic disruptions remain tenuous and are unable to cause clinical disorders.

Histopathological study

The histopathological study occupies a preponderant place and constitutes an unavoidable step in the study of the toxicity. The liver and kidneys are the preferred targets in these studies. Histological examination was performed under photonic microscopy at 100x magnification.

Liver histopathology

Figure 3 shows histological sections of the liver of treated animals. The control group exhibits overall normal liver architecture marked by the presence of hepatocyte trabeculae arranged radially around a centrilobular vein, a portal vein, a bile duct and a hepatic artery (Figure 3A). In contrast, the hepatic architecture of the mice treated with 1000 mg/kg bw is altered with irregular and bloated hepatocytes associated with sinusoidal congestions and the portal system (Figure 3B). The mice treated with 2000 mg/kg bw of extract showed, in addition to the ballooning of the hepatocytes, a congestion of the portal vein associated with peria-centro-lobular dilatation of the sinusoidal capillaries (Figure 3C). It is important to note that alterations of liver tissue were more apparent and more marked in the group treated with the extract at 5000 mg/kg bw (Figure 3D). In these mice, we observed a severe sinusoidal dilatation around the centrilobular vein associated with distended hepatocytes and hepatocellular focal necrosis.

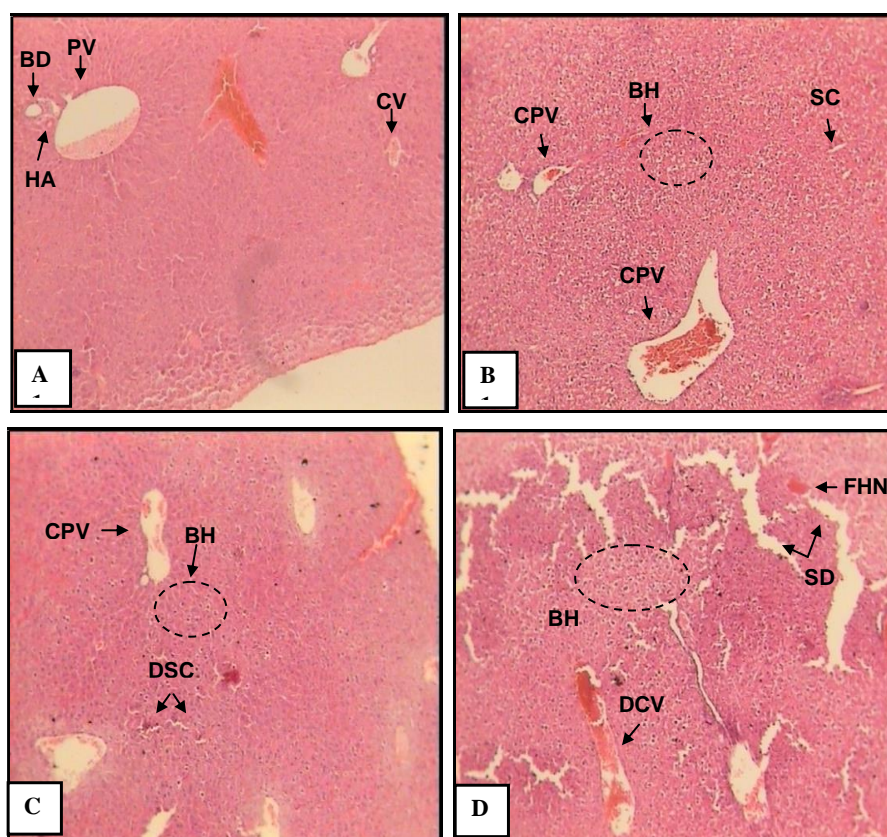


Figure 3.

Histological comparison of hepatic tissues in normal and *L. guyonianum* - treated mice. Photomicrographs showing sections from the centre of the largest liver lobes tissues of control mice (A) and experimental animals treated with aqueous leaves extract of *L. guyonianum* at doses 1000 (B), 2000 (C) and 5000 mg/kg bw (D). Examinations were carried out at 100x magnifications with haematoxylin-eosin's stain. HA = Hepatic artery; BD = Bile duct; CV = Centrio-lobular vein; DCV = Dilated centrio-lobular vein; PV = Portal vein; CPV = Congestion of portal vein; DSC = Dilation of sinusoidal capillaries; SD = Sinusoidal dilatation; SC = Sinusoidal congestion; BH = Ballooning of hepatocytes; FHN = Focal hepatocytes necrosis

Histopathology of the kidneys

Microscopic observation of the renal tissue of control group mice (Figure 4A) allowed us to see normal cortical and medullary areas with the presence of well-defined uniform membrane glomeruli associated with normal tubules. The glomeruli appear as dense rounded structures, surrounded by a clear narrow space, Bowman's space. A large number of tubes, mainly proximal convoluted tubes, surround these glomeruli. The mice treated with 1000 mg/kg bw of extract (Figure 4B) revealed an altered renal architecture in which a relatively small number of glomeruli are dilated or even necrotic. In addition, we observed vascular congestions, a tubular necrosis in phase of installation

(degeneration of tubular epithelial cells with the loss of the brush border of the proximal tubes) and the presence of cellular debris in the damaged tubules. At higher extract dosage (the mice treated with 2000 mg/kg bw) (Figure 4C), the dilated glomeruli increased in number and shape, similarly acute tubular necrosis was more pronounced giving lesions characterized by the absence of epithelium. However, the basement membrane remains intact and maintains its integrity. In mice treated with 5000 mg/kg bw (Figure 4D), congestion with leukocyte infiltration associated with degeneration and tubular necrosis were observed, in addition to the presence of several foci of glomerular necrosis.

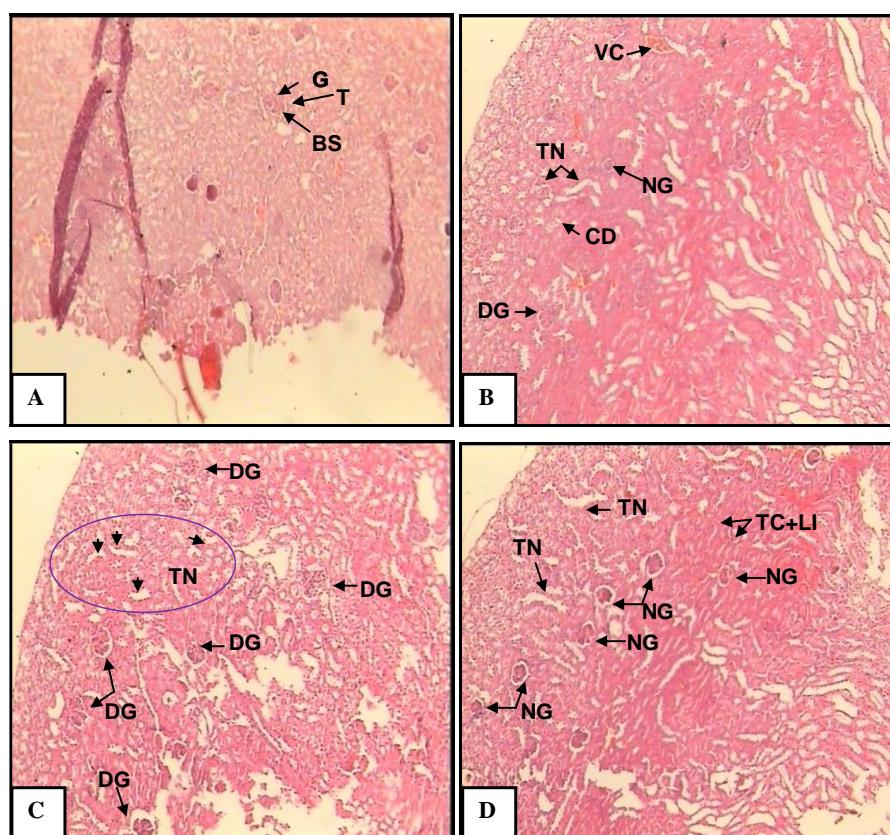


Figure 4.

Histological comparison of renal tissues in normal and *L. guyonianum*-treated mice. Photomicrographs showing sections from the renal cortex tissues of control mice (A) and experimental animals treated with aqueous leaves extract of *L. guyonianum* at doses 1000 (B), 2000 (C) and 5000 mg/kg bw (D).

Examinations were carried out at 100x magnifications with haematoxylin-eosin's stain. **G** = Glomerulus; **T** = Tubule; **BS** = Bowman's Space; **DG** = Dilated glomerulus; **NG** = Necrotized Glomerulus; **VC** = Vascular congestion; **TN** = Tubular necrosis; **CD** = Cell Debris; **TC+LI** = Tubular congestion + Leukocyte infiltration

Histopathological examinations showed alterations affecting both renal and hepatic tissue. However, the kidneys were more sensitive to the extract. Indeed, glomerular necrosis appeared with the first ingested dose (1000 mg/kg bw). In contrast, focal hepatocyte necrosis was noticeable only at a concentration of 5000 mg/kg bw. These attacks appear dose-dependent and are the result of short exposure to high doses of extract.

Toxicity study of this medicinal halophyte is still lacking. In addition, there are few works devoted to studying the toxicity of neighbouring species. Therefore, little is known about their toxicity and safety issues. The hydroacetic extract of *L. brasiliense* rhizomes did not cause any apparent clinical changes in the mice during the 14 days of observation. In addition, the calculated LD₅₀ was 4800 mg/kg [4]. *L. sinense* root extracts expressed hepatoprotective activity [39].

The ethanolic extract of *Plumbago rosea* has been studied to evaluate its acute toxicity in mice. The LD₅₀ (after 24 hours) values of the extract were 239.88 and 1148.15 mg/kg bw for the intraperitoneal and oral routes, respectively. Oral doses above 1250 mg/kg bw produced severe diarrhoea [37]. The results of the acute oral toxicity study of the non-alcoholic extract of *Plumbago zeylanica* roots showed no deaths and no clinical signs of toxicity after increasing doses up to 5000 mg/kg bw in Swiss albino rats. LD₅₀ was found to be greater than 5000 mg/kg bw [35]. Similarly, the study of the orally acute toxicity of the aqueous extract from the leaves of *Guibourtia tessmannii* in mice showed no death and no clinical signs of toxicity during the fourteen days of study.

Cytotoxic study

The cytotoxicity test of the aqueous extract was performed on a universal model of human red blood cells (RBCs) from healthy donors. The amount of haemoglobin released and by extension the proportion of affected RBCs can be determined spectrophotometrically. The test was confirmed by counting residual RBCs after treatment with the extract.

The haemolytic potential test was controlled by the blood without haemolytic agent and blood completely haemolysed by a known haemolytic agent. In the case of our study, distilled water was used as a positive control allowing total haemolysis. PBS was used as a negative control (0% lysis). The haemolysis test was performed for a wide range of concentrations ranging from 5 to 200 mg/mL.

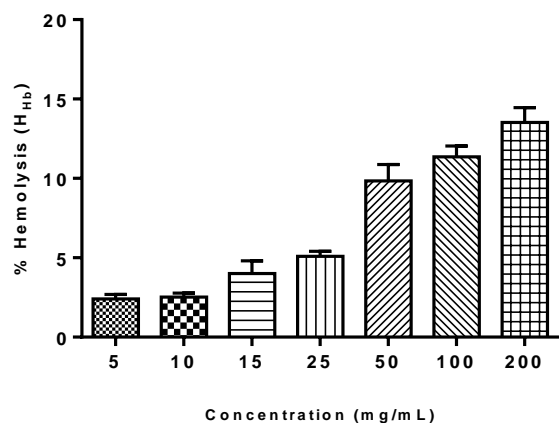


Figure 5.

Haemolytic effect of aqueous leaf extract of *L. guyonianum* measured by H_b release (absorbance at 576 nm)

Different letters represent significant differences $p < 0.05$ (Tukey test)

These results (Figure 5) proved that the aqueous extract induced RBCs haemolysis in a dose-dependent manner. At the lowest concentrations (5, 10 and 15 mg/mL), the extract caused slight haemolysis < 5% close to the negative control (2.4, 2.5 and 4.0% respectively). At

the concentration of 50 mg/mL, the haemolysis rate did not exceed 9.8%. At the highest used dose (200 mg/mL), the haemolysis rate increased slightly but remains low and did not exceed 13.52%.

Generally, the haemolytic potential test results in a 50% haemolytic concentration (IC₅₀). However, in our case it was not possible to determine this IC₅₀ for all the concentrations used, since the percentage of haemolysis did not exceed 13.52%. It is important to note that the results of haemolysis levels measured by cell count (H_C) match well with those obtained by spectrophotometric measurement as shown in Figure 6 and Figure 7. Based on these data, we can say that our extract is not cytotoxic to RBCs.

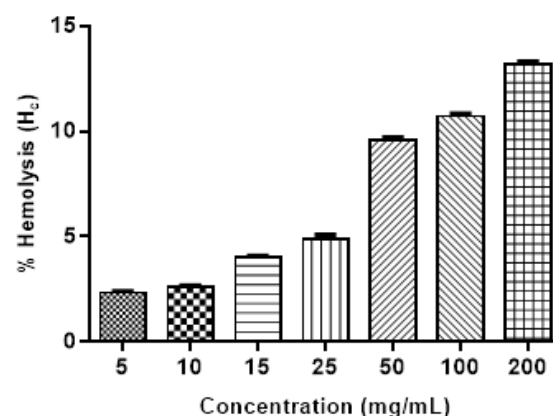


Figure 6.

Haemolytic effect of aqueous leaf extract of *L. guyonianum* measured by cell count (H_C)

Different letters represent significant differences $p < 0.05$ (Tukey test)

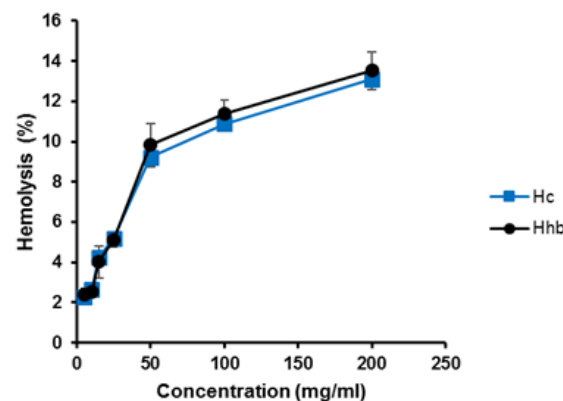


Figure 7.

Evolution of haemolysis percentages measured by spectrophotometry (H_{Hb}) and cell count (H_C)

Similar studies on the haemolytic potency of the methanolic extracts of 9 plants (*Calotropis procera*, *Paronychia argentea*, *Paronychia chlorothyrsa*, *Morettia canescens*, *Haloxylon scoparium*, *Thymelaea hirsuta*, *Tamarix aphylla*, *Daphne gnidium* and *Arthrophytum schmittianum*) harvested from arid zones and sub-arid regions of Algeria have been carried out [29]. The range of concentrations of the aforesaid extracts ranged

between 50 and 500 µg/mL. The highest haemolytic activity was observed with the Saharan species *M. canescens* (14.80%). On the other hand, no haemolytic activity was observed with *P. chlorothyrsa* extract. Our results are comparable to those of Chaouche *et al.* [10] for the methanolic extract of the root bark of *Juniperus oxycedrus*, which for concentrations of 5, 10, 15 and 20 mg/mL, showed percentages of haemolysis of 2.40, 2.94, 4.09 and 4.37%, respectively. In addition, the incubation of the previous 4 extracts of the root bark of *J. oxycedrus* with human erythrocytes for one hour led to haemolytic activities between 2.05% and 4.37% [10].

The results of the haemolytic effect of the various racquet extracts of *Opuntia ficus indica*, showed that this plant has a low toxic effect against isolated erythrocytes compared to total haemolysis. The doses causing 50% haemolysis ranged from 0.389 (acetone extract) to 1.587 mg/mL (butanolic extract), whereas the dose of the aqueous extract was 0.606 mg/mL [20]. Zubair *et al.* also reported that the haemolytic potential of the various leaf extracts of *Smilax macrophylla* (*Smilacaceae*) was low and did not exceed 8.48% [46]. Most recently, Surendra *et al.* reported that *Moringa oleifera* skin extract had no toxicity to RBCs [38]. RBCs were chosen as a cellular model for the *in vitro* study of cytotoxicity because of their isolation facilities and simplicity. Therefore, the results of the haemolytic study showed that *L. guyonianum* aqueous leaf extract has a very low or negligible toxic effect against the human RBCs. However, further studies on a wider range of cells will be needed to confirm the results of this research.

Conclusions

This study provides important data on the acute toxicity profile and cytotoxicity test of the aqueous extract of *L. guyonianum*. In the acute toxicity assays, no mortality or specific toxic signs were observed in female mice after oral administration of the extract at all tested doses. The extracts did not produce any remarkable histopathological signs or serum biochemical alteration. The LD₅₀ can then be estimated to be greater than 5000 mg/kg bw. This value is relatively high inducing a safety use of this extract, which can be considered a non-toxic substance. The recommended dose used in humans will be a maximum of 500 mg/kg bw. The cytotoxic study showed a slight haemolysis of 13.5% obtained for an extreme concentration of 200 mg/mL, showing no cytotoxic activity of the extract against the RBCs. Hence, the extract can be used without any health risk both in folkloric medicine as an oral anti-infective and for pharmaceutical formulations. However, further toxicity studies should be done to further ascertain the safety of the users of this plant. Finally, our results should be very useful for

any future *in vivo* and clinical study of this medicinal halophyte plant.

Conflict of interest

The authors declare no conflict of interest.

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