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Nephrotoxic Effect "Focal Segmental Glomerulosclerosis" In Wistar Rats Of *Ruta chalepensis L* Aqueous Extract Used In Traditional Moroccan Pharmacopoeia.

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ABSTRACT

Kidney damage following the use of a nephrotoxic herb is a serious condition that is often associated with significant morbidity and mortality. Because of its rich vascularization (25% of cardiac output), the kidney is indeed an organ particularly vulnerable to the toxicity of drugs present in the body. In addition, the existence of a cortico-medullary osmotic gradient promotes the interstitial accumulation of toxic agents in the papillae and in the medullary zone. Several floristic and ethnobotanical studies of medicinal plants used by the Moroccan population have made it possible to draw up catalogs of some medicinal species used in indigenous therapeutic recipes. The toxicity of several of them has been frequently reported. Our research on the possible nephrotoxic effects of the aqueous extract of *Ruta Chalepensis* L areal part, a medicinal plant commonly used in traditional Moroccan pharmacopoeia, aims to study their harmful effects on the physiology and function of the renal system. Performing a subchronic toxicity study, an evaluation of the toxicity of the aqueous extract of the aerial part of *Ruta Chalepensis* L in single daily doses was carried out in male and female rats in accordance with OECD guidelines 423 and 407 for chemical testing. As a single dose of 10g/kg in 24h of the aqueous extract of *Ruta Chalepensis* L areal part administered intraperitoneally during 2 months. A significant difference was noted in the mean weight lost in treated males and females. Also mean relative weights of kidney is reduced ($p<0.05$) in females and males, and mean relative weights of spleen were significantly reduced ($p < 0.001$) in females. The urinary volume shows a chronic polyuria mainly in males compared to females. Histological sections of the kidneys show segmental lesions (part of the glomerulus) or global lesions (the whole glomerulus) and focal lesions (some glomerulus), Sclerosis (or fibrosis) is also observed, which is a cicatricial accumulation of collagenous material replacing the entire destroyed glomerulus (sclerotic glomerulus) or part of the destroyed glomerulus (segmental sclerosis). Subchronic toxicity study of the aqueous extract aerial part of *Ruta Chalepensis* L in single daily doses of 10g/kg administered intraperitoneally during 2 months induces a nephrotoxic effect, a common glomerular pathologies: the Focal segmental glomerulosclerosis (FSGS) in wistar rats under the conditions of our experiment.

Keywords: FSGS; Nephrotoxic; subchronic toxicity; polyuria; lead; Wistar Rats.

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INTRODUCTION

The interest of populations in herbal medicine has continued to grow over the years. This is understandable when we consider the many positive aspects that it presents. These include: its diversity, flexibility, availability in many parts of the world, low cost, low level of technological participation, and growing economic importance (WHO, 2002). However, this obvious enthusiasm for herbal medicine does not take into account some aspects of it which still appear to the scientific community as challenges to be overcome. This is the case, for example, with its harmlessness and effectiveness. Indeed, the evidence collected which supports these 2 aspects, such as for example the use over the centuries to a large number of practices recommended by traditional medicine in general and the experience transmitted from generation to generation, are considered too insufficient both on the both quantitative and qualitative plan to meet the criteria required to support its use on a global scale. Scientific research is still needed to support these findings (WHO, 2000). It is in this context that the Environment and Health Laboratory of the University of Moulay Ismail has specialized in the study of the pharmacological and toxicological properties of medicinal plants.

Ruta Chalepensis L It is a Mediterranean species, relatively common throughout Morocco (BABA AISSA, 1999), in northeast Africa, southern Europe and southwest Asia (MIOULANE, 2004).

Aleppo Street An ornamental plant for gardens, the street is considered to be honey-bearing and its presence keeps vipers away. It repels insects (LE MOINE, 2001) and is used against scabies and parasites of the head (BONNIER, 1999).

Cultivated in Central Europe since the 10th century, it served as an anti-poison. The Greeks consider that it improved eyesight (LE MOINE, 2001; BALCH and STENGLER, 2004) by using the essence extracted from the fresh plant (SCHAUENBERG and PARIS, 1977).

It contains alkaloids, flavonoids, vitamin C and furo-coumarins. It was formerly an emmenagogue and abortive plant (LE MOINE, 2001) indeed the rue exerts a clear excito-motor action on the uterus (MERAD CHIALI, 1973). Its sap irritates sensitive skin. It is used for eye problems and as a gargle for sore throats. The leaves treat phlebitis and varicose veins. Its use is not recommended for pregnant women (LE MOINE, 2001).

Several species of *Ruta* are sources of various classes of natural products with activities: antifungal, phytotoxic and antivenom (OLIVA et al, 2003).

Other activities cited by (DUKE et *al*, 2008; GUTIERREZ-PAJARES et *al*, 2003; KONG et *al*, 1989; CHIU and FUNG, 1997; FOSTER and TYLER, 1999):

Abortif; Analgesic; Anti fertility; Anti-inflammatory; Antiseptic (against: Bacillus, Candida, Escherichia, Microsporium, Pseudomonas, Staphylococcus); Antispasmodic; Aphrodisiac; Arachnifuge; Bactericide; Candidicide; Cardiotonic; Decongestant; Digestive; Emetic; Embryotoxic; Emmenagogue; Febrifuge; Immunomodulator; Insect repellent; Molluscicide; Sedative; Stomachic; Sudorific; Vermifuge; Vulnerable; antipyretic, antiparasitic, anthelmintic; aqueous extracts from rue has hypotensive activity through a direct effect on the cardiovascular system.

Popular use in Morocco: The rue is widely used for various purposes, Febrifuge, local antivenom, against nausea and vomiting, in constipation, in malaria, to treat anemia (MERAD CHIALI, 1973), rheumatism, against gastric pains, intestinal worms (BABA AISSA, 1999), in difficult deliveries, eye and ear ailments, in asthma, neuroses (MERAD CHIALI, 1973).

Studies have already had to justify the presumed pharmacological properties of alcoholic extracts my absence of studies on the aqueous extract of *Ruta Chalepensis L*, thus relating to the traditional use of the plant prescribed by traditional healers and also the absence of data on the toxicity of the plant. This failure prompted the implementation of this work, which consisted in studying the toxicity of the aqueous extract of the aerial part of *Ruta Chalepensis L* in rats, with the main objective of highlighting the potential dangers for human health associated to its consumption. More specifically, to obtain information on the possible harmful

effects due to the daily consumption of the product via a subchronic toxicity test and mainly the nephrotoxic effects.

MATERIELS AND METHODS

Preparation of plant material extracts

The plant was harvested, dried and stored in the sun at room temperature for about 6 weeks it has been identified by RAHOU Abdelilah Botanist of the department of Biology of the Faculty of Sciences of Meknes.

In order to be in accordance with the traditional form of use of the *Ruta chalepensis L* as recommended by traditional therapists, we proceeded to an aqueous decoction to obtain study extracts. 80 g of dry matter (areal parts plant blossoms and leaves) were decocted in a flask containing 2000ml of distilled water, the decoction was kept under continuous reflux for two hours, at the end of this operation, the decoction obtained after cooling was filtered through a funnel containing a cotton wool and centrifuged 2500tours/min. The solution obtained was adjusted with distilled water to a final concentration of 10g/kg the dose administered, particularly, intraperitoneally at the dose of 10 g/kg induce significant acute effect on urinary output of water and electrolytes in normal wistar rats (Bellahmar et *al*, 2020).

Evaluation of acute toxicity

To determine the acute toxicity parameters of the aqueous extract of *Ruta chalepensis L* on white males mice divided into several batches treated with different doses of the extract. The results obtained at the end of the experiment give a Lethal Dose 100 (LD100), 30g/kg/vi body weight, a lethal dose 50 (LD50) of 19.6g/kg/vi body weight and a Maximum Tolerated Dosage (DMT) of 4.5g/Kg/vi of body weight.

The value of the Lethal Dose 50 obtained makes it possible to deduce that the total aqueous extract of *Ruta chalepensis L* is relatively harmless in Swiss albino white mice (Bellahmar et *al*, 2019).

Evaluation of subchronic toxicity

Experimental animals

Adult wistar rats 15 animals (weight: 84-113 g for the 08 females and 105-134 g for the 07 males; age: 6-7 weeks) bred in the animal house of the Department of Biology, Faculty of Sciences, Meknes, Morocco, were housed under standard environmental conditions (22±2 ◦C) with a 12 h light–dark cycle.

The animals were given corn as food and drinking water as drink adlibitum. Among the animals, 12 apparently healthy young rats of both sexes were selected, and randomly assigned to the different control and test groups. The selected females were nulliparous and not pregnant. None of these animals had been subjected to previous experiments. The experiments were conducted in accordance to internationally accepted standard procedure for animal use. The animals are divided into individual metabolism cages. They were given free access to food and water (at will).After an adaptation period of one week in the metabolic cages, these animals are divided into control groups and treated:

The Controls Rats: 5 in number (3 females and 2 males) and subjected to the standard laboratory regime and daily intraperitoneally injections of 1ml of distilled water as a single dose in 24h.

The Experimentals Rats: 10 in number (5 females and 5 males) subjected to daily intraperitoneally injections of 1 ml of aqueous extract from the aerial part of *Ruta Chalepensis L* at 10 g/kg as a single dose in 24h.

Analytical techniques

Body weight: In order to monitor weight, rats are weighed regularly each day before injection. All animals were weighed shortly before treatment began, then every 7 days, and finally on the day of sacrifice. Weekly weight changes were calculated and recorded.

The rats were sacrificed by decapitation after the experimental period and the different organs (spleen, liver, kidneys) were removed, cleaned, washed with distilled water, dried on blotting paper and then weighed using a scale.

Urinary volume: Metabolic progress is followed by urine collection.

Sample analysis and statistical calculations: Each experimental value is given with its arithmetic mean ± standard error of mean (ESM). The comparison of two means is made according to the Student's test. The differences were considered significant for $p < 0.05$.

Observations: The observation period lasted 37 days. At least twice a day, at the start and at the end of each day, all animals were examined for symptoms of morbidity and mortality. Observations were made on all the animals at least once before the first exposure (in order to be able to make comparisons on the same individual), and then once a week. Symptoms noted included, but are not limited to, changes in skin, fur, breathing, gait, stereotypes (excessive body grooming, animals circling repeatedly) and bizarre behavior (self-harm, walking backwards). From the fourth week of exposure, sensory responsiveness to auditory and proprioceptive stimuli, grip strength, and motor activity were assessed daily:

- Hearing was assessed by assessing the reaction of animals to a sudden clap of the hands;
- Proprioception by appreciating their reaction to a bite of their paws;
- Grip strength by appreciating their ability to grip the edges of the cage when trying to get them out.

At the end of the experiment (02 months), the animals are sacrificed after anesthesia with 22% urethane (0.4ml / 100g). A sample of the organ of interest (kidney, liver and spleen) In order to carry out a histological and histopathological study. Were removed from any adherent tissue and weighed in the fresh state immediately after dissection, in order to avoid desiccation. After weighing, these organs were preserved in 20% formalin for histopathological examinations.

Histopathological kidneys study

Histological procedures were performed according to Martoga and Martoga P.M. 1967 Histopathology: Organs of interest were stored in 20% formalin. The following procedure was then used for making the histological sections (Histopathology department at Mohammed V Hospital in Meknes):

1) "Trimming": thin and regular slices of each fixed organ were taken using a scalpel, then stored in cassettes.
2) Dehydration: the cassettes were then passed through ethanol baths of increasing concentration: 50 ° ethanol (1h) - 70 ° ethanol (1h) - 95 ° ethanol n ° 1 (1h) - 95 ° n ° ethanol 2 (1h30mn) - ethanol 100 ° n ° 1 (1h) - ethanol 100 ° n ° 2 (1h30mn) - ethanol 100 ° n ° 3 (2h); the cassettes were then passed through 2 xylene baths, respectively 1 hour and 2 hours; the cassettes were finally passed through 3 paraffin baths at 60 ° C respectively 1h, 1h30.
3) Inclusion: the tissues were then placed in molds filled with molten paraffin which was then put to solidify on a cold surface after proper orientation of tissue in the block.
4) Sections: 5 µm sections of the blocks were made using a microtome. Once cut, the sections were unfolded in a water bath (about 40 ° C) and then collected on slides. The slides were then left for 24 hours in an oven (45 ° C.) before staining.
5) Coloring: the sections were then passed through a dewaxing battery consisting of a series of baths of 5 to 10 minutes each according to the following sequence: xylene n ° 1 - xylene n ° 2 - xylene n ° 3 - ethanol at 100 ° n ° 1 - ethanol at 100 ° n ° 2 - ethanol at 100 ° n ° 3 - ethanol at 95 ° - ethanol at 70 ° - distilled water. The sections were then immersed for 10 minutes in a tank containing Mayer's

hematoxylin, then rinsed for 10 minutes in running tap water. The sections were then passed through a 95 ° ethanol bath for 5 minutes, then they were immersed for 5 minutes in 0.5% alcoholic eosin + 40 µl of acetic acid per 100 ml of solution. The sections were then dehydrated in 100 ° ethanol (3 × 5 min), thinned in xylene (3 × 5 min).

6) Assembly: once out of the xylene, a few drops of resin were deposited on the sections, then the latter were covered with a glass coverslip for observation under the microscope.

RESULTS

Curve of weight change

The figure shows the variation of the body weights of the male and female (animals until the end of the test and observation period). A significant difference was noted in the mean weight lost in treated males and females compared to their respective controls. (Figure 1)

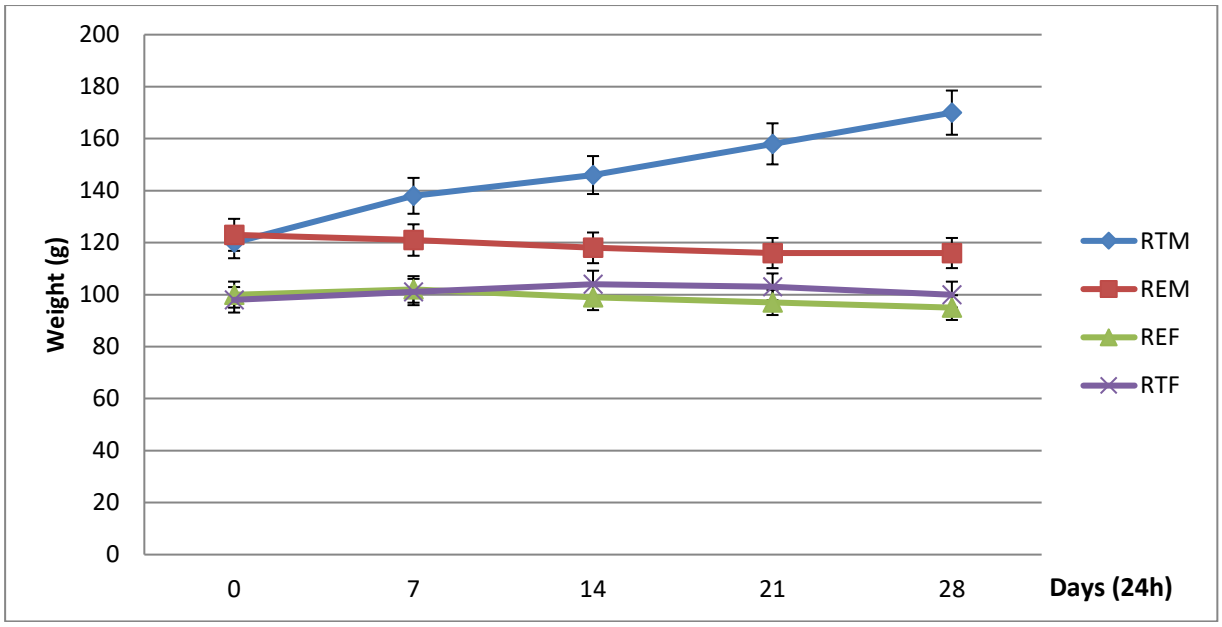

Figure 1. variation of male and female body weights in subchronic testing. (The dots represent average weights and n=5)

Effect of the extract on the relative weight of the organs

The table shows the effect of the aqueous extract of *Ruta Chalepensis L* on the relative weight (%) of some organs. (Table 1)

- In treated males, mean relative weights of kidney and spleen at 10g/kg were reduced ($p<0.05$) compared to controls.
- In females, mean relative weights of spleen at 10g/kg were significantly reduced ($p < 0.001$) and for kidney ($p < 0.05$) relative to controls. Inducing atrophy of internal organs except the liver.

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Table 1. Effects of the extract on the relative weight of some organs (% of body weight) [(5)]n=5 ; Values are averages; ESM ;*p<0.05 ; ***p<0.001 : significant differences from the control.

Male	Control	The experimental dose 10 g/kg
Liver	3.00 ± 0,07[(5)]	3 ± 0,20[(5)]
Kidney	0,30 ± 0,01[(5)]	0,27 ± 0,01*[(5)]
Spleen	0,20 ± 0,01[(5)]	0,18 ± 0,02[(5)]
Female	**Control**	**The experimental dose 10 g/kg**
Liver	3.00 ± 0,20[(5)]	3.00 ± 0,10[(5)]
Kidney	0,30 ± 0,01[(5)]	0,28 ± 0,01* [(5)]
Spleen	0,40 ± 0,03[(5)]	0,30 ± 0,01***[(5)]

Urinary volume monitoring

The monitoring of the urinary volume shows an increase in the males with quite important and impressive values. Thus, a chronic polyuria was observed during the whole period of the treatment with *Ruta Chalepensis L* aqueous extract. The peak of the urinary volume 41.64±1.63 was recorded on the 9th day of the treatment in all treated males. (Figure 2)

Monitoring of urinary volume also shows an increase in urinary volume in females that is less significant than in males (Figure 2). Polyuria was observed throughout the period of treatment with *Ruta Chalepensis L*. The peak recorded urine volume23±07 on day 17 of treatment in all treated females. (Figure 3)

Figure 2: Monitoring of urine volume in treated and control male rats.

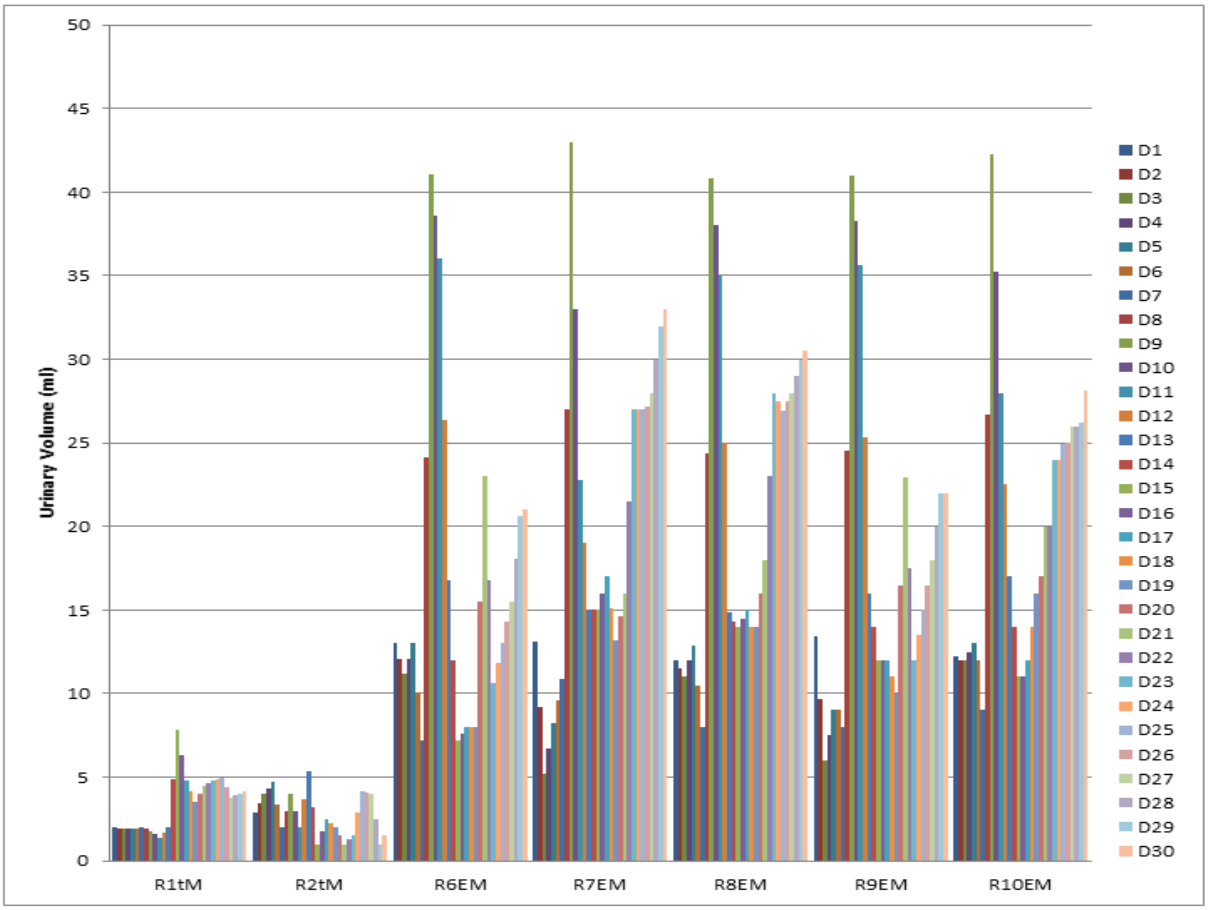

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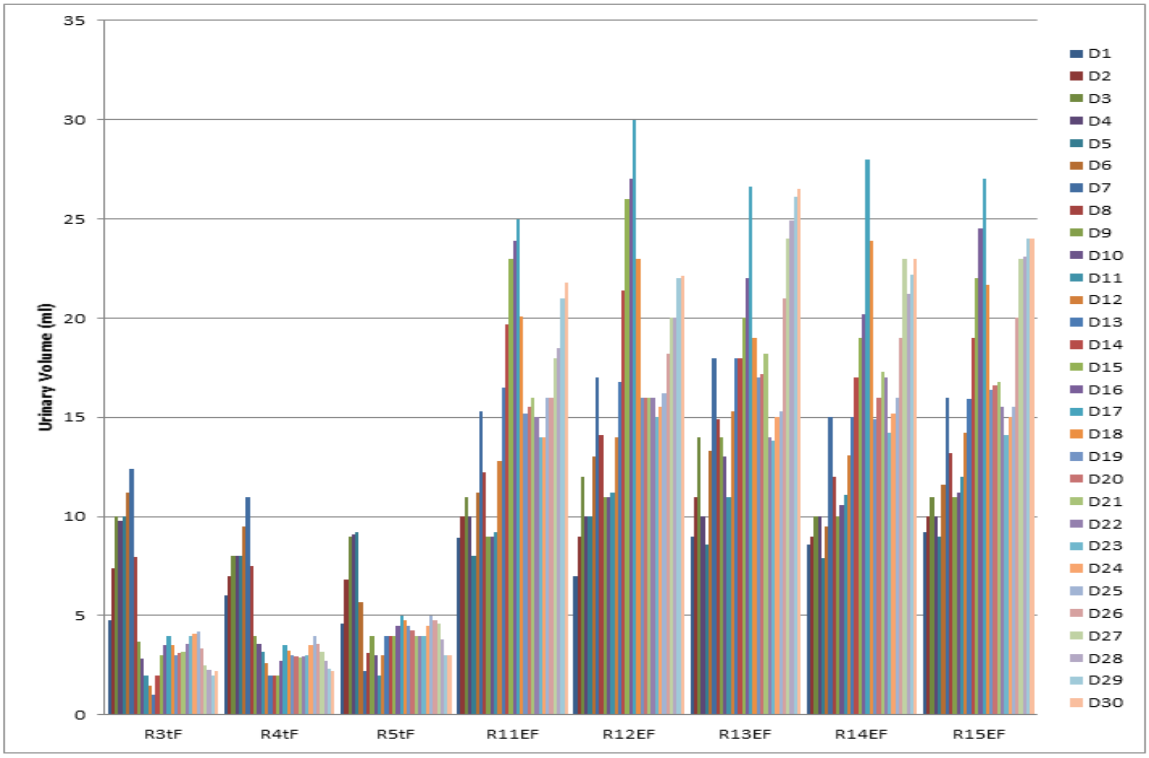
Figure 3. Monitoring of urine volume in treated and control female rats.



Observations of clinical signs

Table 2: Observations in subchronic testing.

		week 1		week 2		week 3		week 4	
		Number of animals showing signs of toxicity and nature of these signs	Morbidity or Mortality	Number of animals showing signs of toxicity and nature of these signs	Morbidity or Mortality	Number of animals showing signs of toxicity and nature of these signs	Morbidity or Mortality	Number of animals showing signs of toxicity and nature of these signs	Morbidity or Mortality
Males	Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	10 g/kg *Ruta Chalepensis L*	1/5 : lethargy, thinness, tachypnea, bristly coat	0/5	1/5 : insensitivity of the left side legs, torticollis pushed, turns round and round repeatedly	0/5	0/5	0/5	0/5	0/5
Females	Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	10 g/kg *Ruta Chalepensis L*	1/5 : thinness	0/5	2/5 :lethargy; thinness; tachypnea; bristly coat; insensitivity of front legs (1/5)	0/5	1/5:slight lethargy	0/5	1/5: lethargy, tachypnea, thinness, bristly coat	0/5

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Observations of histopathological sections

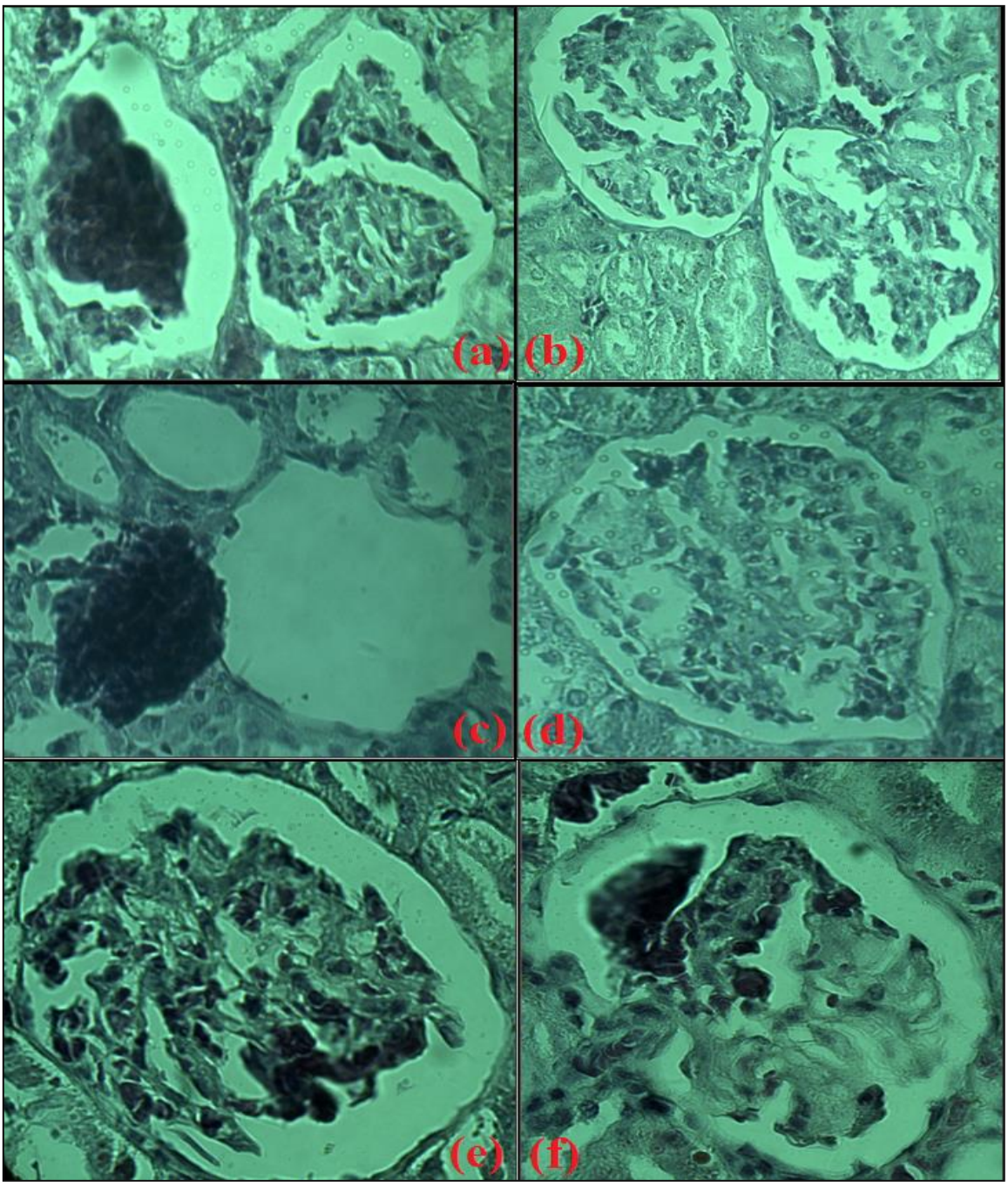

Figure 4: Microscopic findings of the renal biopsy. (a) the glomerulus in the right showing global sclerosis : sclerotic glomerulus ≪ sealing bread ≫ the glomerulus on the left showing well-defined peripheral segmental sclerosis.(b)and(d) glomerulus showing no lesions in this section (normal glomerulus). (c) global sclerosis : sclerotic glomerulus ≪ sealing bread ≫ glomerulus totally destroyed and expelled from the bawman capsule. (f)In this biopsy, the segmental sclerotic lesion on the right with FSGS is composed of obliteration of capillary lumens and increased mesangial matrix. The remaining portion of the glomerular tuft appears unremarkable, without basement membrane changes or any apparent deposits. (e)The visceral epithelial cells overlying this early sclerotic lesion appear activated. (microscope optique, X400)

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DISCUSSION

Our research on the possible nephrotoxic effects of the aqueous extract of *Ruta Chalepensis L* areal part in Wistar rats, aims to study her harmful effects on the physiology and function of the renal system. In our experimental conditions, the injection of the aqueous extract of *Ruta Chalepensis L* induced a nephrotoxic effect, common glomerular pathologies: the Focal segmental glomerulosclerosis (FSGS) dose and time dependent, evidenced by a histopathological study of the kidneys (Figure 4).

Surprisingly, this nephrotoxicity is appeared for the dose of 10g/kg/vi, the dose responsible for the acute diuretic effect .In previous work in our laboratory (Bellahmar et *al*, 2020) The aqueous extract was administered intraperitoneally produced a significant increment on diuresis from the first hour to the 24 hour. Furosemide at a dose of 20 mg/kg, adminestred intraperitoneally had a similar effect when compared to *Ruta Chalepensis L* administration suggesting a similar mechanism of action. The mechanism of action of furosemide is by inducing a loss of water through the inhibition of NaCl reabsorption. The results suggest that this receptor-mediated mechanism may account for the diuretic effect of *Ruta chalepensis L* and explains the polyuria (Figure 2 and 3) and the loss of weight (Figure 1) observed in rats treated under subchronic test acting as a loop diuretic.

Renal biopsy of experimental rats reveals a glomerular pathology: the Focal segmental glomerulosclerosis (FSGS). Focal segmental glomerulosclerosis (FSGS) is one of the most common glomerular pathologies. It was first described in 1925 by Fahr.

1. The diagnosis of FSGS relies on histopathologic findings characterized by the presence of adhesions between the glomerular tuft and Bowman's capsule (BC), focal and segmental lesions with mesangial sclerosis, and obliteration of glomerular capillaries with hyalinosis.

2. FSGS is considered primary or idiopathic when no etiology can be identified. Secondary FSGS is associated with infections, obesity, chronic hypertension, immunologic processes (e.g., IgA nephropathy and immune complex nephritis), and drug abuse.

Kidney damage mainly affects the glomerulus by reducing filtration, but also the proximal tubules which concentrate the toxic substances because of their high absorption and secretion activity. On the other hand, their high content of cytochrome P450 enables them to detoxify or activate the toxins. The main nephrotoxics are heavy metals, antibiotics, analgesics and certain halogenated hydrocarbons (chlorine derivatives) (Lu, 1992).

Plants can absorb and accumulate heavy metals from the environment. The most common are lead, cadmium and mercury. These metals are absorbed by the roots, some can pass into the aerial parts (stems, leaves), especially if their concentration increases in the soil. For example, lead remains in the roots while cadmium passes more easily into the aerial parts. The quantities absorbed remain low, and vary according to the plants, the concentration of metals in the environment and the characteristics of the environment. The latest edition of the European Pharmacopoeia does not mention a method for controlling heavy metals present in plant drugs (Kabelitz L et *al*, 1999).

Chronic lead exposure is associated with alterations in nephrosclerosis and cortical atrophy. The question of the possible impact of lead at lower levels of exposure remains much more controversial. The difficulty in making a precise judgement of risk at low doses is probably partly due to the inhomogeneity of the markers used so far, both to assess the intensity of lead exposure (blood lead levels, protoporphyrin-zinc or PPZ, bone lead, etc.) and to measure the renal effect (creatinine, blood urea, N-acetyl-B-D-glucosaminidase (NAG) or urinary prostaglandins, etc). In addition, the pathological significance of several of the effect markers used (NAG, prostaglandins, fibronectin, etc.) is still very incompletely specified (Lin et *al*, 1993; Chia et *al*, 1994a and b, 1995a and b; Pergande et *al*, 1994).

The morphological adaptations and physiological regulations of plants are essential to tolerate changes in the external environment. These adaptations tend to amplify the root absorption of these plants in the soil, thus making it possible to tolerate and accumulate metals up to possibly toxic levels.

Generally the concentration of metal in plants is not proportional to the concentration of metal in the soil. It is even higher. To explain this phenomenon, several hypotheses are considered, among which metal tolerance, drought resistance, competition with neighboring plants and the defense mechanism against herbivores and pathogens.

The evaluation of the total metal content by treatment and extraction of the different organs of *Ruta Chalepensis L* with HCL, HNO3, and aqua regia, respectively, shows that the concentration of heavy metals varies from one organ to another of the plant. The heavy metals Co, Cu, Fe, and Pb are distributed throughout the plant but are more concentrated in the leaves (part used in our experiment) and roots. In addition, Cd is found throughout the plant while Zn concentrates more mainly in the leaves and fruits. Indeed, the contents in Pb, Co and Fe exceed very largely the threshold of phytotoxicity (Meriah .S, 2007).

Considering the supposedly low concentrations in the soil, both in total and available forms, and the very high levels of the metals observed, it is clear that *Ruta Chalepensis L* is a plant accumulator of heavy metals, especially Pb, Cd, Cu, Co and Fe (Meriah .S, 2007).

The hyperaccumulation of heavy metals in plants, is the capacity to accumulate certain metals up to abnormally high concentrations in leaves (T.Jaffré et *al*, 1976) (R.R Brooks et *al*, 1977).This character remains still misunderstood from an ecological and evolutionary point of view. However, several hypotheses have been proposed to explain the adaptive advantage of hyperaccumulation. Hyperaccumulation would notably influence resistance to drought, interactions with adjacent plants and defence against herbivores and pathogenic bacteria (A.G.I.Assuncao et *al*, 2001)(Y.S.M.Ghaderian et *al*, 2000).

Regarding atrophy of internal organs (Table 1). According to several previous studies, cadmium damages vital organs such as the kidneys and spleen (Kara et *al*, 2005) which may explain their decrease.

As for the kidneys, a significant amount of blood mass passes through them, making them extremely vulnerable to the cytotoxic effect of cadmium (Boujelbene et *al*, 2002).therefore, the kidneys remain the main targets after cadmium ingestion (Wang et *al*, 2014) where 50% of the metal accumulates, creating kidney damage (Siddiqui, 2010; Poontawee et *al*, 2016). Through this action, cadmium damages the kidneys, which could explain the decrease in the average weight of this organ (Kowalczyk et *al*, 2002).

For the spleen (lymphoid organ), the mechanism of splenic atrophy is unknown, but in inflammatory bowel disease splenic atrophy may also occur (Trewby et *al*, 1981).

CONCLUSION

The objective of this study was to assess the association between regular intake of *Ruta Chalepensis L* and nephrotoxic risk through the study of the sub-chronic toxicity.

At the end of this work, it appears that subchronic toxicity study of the aqueous extract aerial part of *Ruta Chalepensis L* in single daily doses of 10g/kg administered intraperitoneally during 2 months in wistar rats induces a nephrotoxic effect, a common glomerular pathologies: The Focal segmental glomerulosclerosis (FSGS) in wistar rats under the conditions of our experiment.

Intoxication by plants and products of the traditional Moroccan pharmacopoeia is a real public health problem in Morocco. Medicinal plants contain many active ingredients that can induce harmful effects on the kidney that are often underestimated (Figueredo MS et *al*, 2018). Thus, it is imperative to reinforce the knowledge of health professionals on the toxicity of the medicinal plants used.

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