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Acute and Subacute Toxicity in Wistar Rats of the Methanolic Extract of the Roots of *Maytenus Senegalensis* (Lam.) Exell.

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Abstract

Background *: Maytenus senegalensis is a medicinal plant endemic to sub-Saharan Africa, widely used in traditional medicine. Genus Maytenus is an important source of bioactive secondary metabolites found in different parts of the plant. Phytochemical studies have reported the presence of several families of compounds such as flavonoids, pentacyclic triterpenes, sesquiterpenes, alkaloids, and tannins as well as maytansinoids which confer to these plants an important bioactivity and justify their use in traditional medicine. Pharmacologically it has been reported that the roots of the plant have antifalcemic activity in vitro on the red blood cells of sickle cell patients. This action gives hope for a future remedy against sickle cell disease. The objective of our study is in line with the general dynamics of improving knowledge on medicinal plants with a view to revalorising the African and Senegalese Pharmacopoeia for an efficient health care of the populations. The aim is to evaluate the safety of the extract of the roots of M. senegalensis by a study of toxicity in animals.*

Materials and Methods *: safety of the methanolic root extract was assessed by determining the acute and subacute toxicity after oral administration of the extract in Wistar rats in accordance with OECD guidelines 423 and 407 respectively.*

Results *: the results showed a high LD_{50} in the order of 5000 mg/kg, thus a low to none acute toxicity. The subacute toxicity allowed to determine a no observable adverse effect level (NOAEL) of 500 mg/kg*

Conclusion *: The methanolic extract of the roots of M. senegalensis was found to be practically non-toxic at the doses tested in rats by the oral route.*

Key word : *Toxicity, In vivo, rats, Maytenus senegalensis,*

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I. Introduction

Modern medicine has made enormous progress, particularly in terms of vaccines and the fight against chronic and endemic diseases such as malaria, AIDS and cancer. However, in Africa, the objectives of access to health care for all, set by the WHO in 2000,[1,2] are far from being achieved. The reasons for this include the uneven distribution of health infrastructure but above all the high cost of health services, even at primary level. As the majority of the population is made up of people with very limited incomes, the only alternative is to use medicinal plants. This form of medicine has always been practiced in the world and particularly in Africa which has great potential. In the 1990s, about 50,000 species of vascularized higher plants were counted in Africa out of 250,000 worldwide [3].With the crisis of the modern health system, Africa is faced with the need to rebuild an adapted health system and to valorize its ancient heritages in medicine, in accordance with the recommendations of the WHO [1,2]. However, according to the WHO, an optimal use of medicinal plants requires investigations on their effectiveness, quality and safety, thus allowing the establishment of regulations and the formalization of the traditional practice of medicine. Thus, plants used wisely can be a good alternative in the health care of populations.

In the present work, the safety level of *Maytenus senegalensis* (synonym *Gymnosporia senegalensis* (Lam.) was assessed by determining the in *vivo* toxicity of root extracts. M. senegalensis is a plant of the family *Celastraceae*, widely distributed in the world and widespread in tropical and subtropical areas of Africa, South America, Australia and Asia [4–7]. It is a small tree or shrub with a narrow, open crown about 6 m high, which can reach 15 m often occurring in bushes[8,9]. The tips of the twigs are lignified and spiny with straight, axillary spines 1-2 cm long or terminal and longer. The bark is scaly and grey with red slices and the leaves are alternate, elliptic, oblong or obovate, 3-13 x 2-10 cm with a finely toothed or more or less entire margin.

The genus *Maytenus* is an important source of bioactive secondary metabolites. Phytochemical studies have reported the presence of several families of compounds [8,10,11] which confer important bioactivity to these plants [10,12]. These plants are widely used worldwide as medicinal plants in the treatment of gastric disorders, such as gastric ulcer, but also as analgesic, anti-inflammatory, antiseptic, antiasthmatic, antitumor, diuretic, laxative [10,13–15]. In Senegal, *M. senegalensis* is one of the most widely used plants in traditional medicine, especially the roots often traded in markets and presented as a remedy for several diseases [16,17]. They are reputed to be febrifuge, tonic, sudorific, cholagogue and laxative [17,18] . Infusions of the roots are also used against gastrointestinal disorders such as stomach ache, dysentery and diarrhoea. The roots are associated with the treatment of leprosy by some healers [16]. Pharmacologically, the literature describes an *in vivo* effect of the roots against different species of *Plasmodium*, [19] anti-parasitic [17,20–22], antibacterial and anti-inflammatory activities [8,15,23,24].

Extracts from the roots are also reported to have an effect on metabolic diseases such as diabetes with hypoglycemic activity in rats [25] and *in vitro* antifalcemic activity on sickle cell red blood cells [26].

However, few studies on the toxicity of *M. senegalensis* roots are available. Some of them have been carried out on crude extracts of bark and roots and have focused on acute toxicity. In our previous study we performed an *in vitro* study and evaluated the cytotoxicity of the roots as well as their cell death mechanism by studying the fragmentation of ADN [27]. In this work, we propose to continue with an *in vivo* study by evaluating the acute toxicity of the root extracts and the subacute toxicity in Wistar rats.

II. Materials and Methods

This experimental study was carried out on Wistar rats raised at the Pharmacy Department of Cheikh Anta Diop University in Dakar. A total of 30 rats (males and females) between 8 and 12 weeks of age were used for this study.

Study Design: *In vivo* experimental study

Study Location: This was a study done in Department of Pharmacy Toxicology Laboratory, at Cheikh Anta Diop University, Dakar, Senegal.

Study Duration: 14 days / acute toxicity; 30 /sub-acute toxicity.

Sample size: 30 rats (12 rats/acute toxicity; 18 rats/sub-acute toxicity).

Sample size calculation: The sample size was estimated according to the OECD recommendations on chemical testing. We planned to include 42 rats (05 rats/each group for sub-acute toxicity) but this could not be achieved due to the unavailability of animals meeting the age criteria.

Reagents and chemicals

Biosystem test kits: aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and glucose were purchased from Biosystem S.A (Spain). Methanol, ethanol and all other chemicals and reagents were obatined in the purest form available from Scharlab S.L. (Spain). Urine reagent strips (Labistix, Siemens) were purchased from Diagnostic Laboratory Inc., France. Ketamine 10% and Xylazine 2% were obtained Alfasan laboratory (Holland). Buffered formaldehyde 10% was purchased from Ral Diagnostics, (France).

Procedure methodology

Herbal extractum preparation

The extract was prepared from the roots of *Maytenus senegalensis* which was harvested between May and June before the first rains in the commune of Sagalkam, 18 km from Dakar. The plant samples were identified and authenticated at the Pharmacognosy and Plant Biology Department of the Faculty of Medicine and Pharmacy of Dakar .The roots were washed, dried in a contamination-free environment and then ground. A quantity of the powder was mixed with methanol (100 g of powder for 1 L of methanol) for a double maceration at room temperature for 72h. The mixture was filtered through cotton wool and then on to Wattman paper and the extract was dried using a rotavapor and stored in a cool dry place. It was reconstituted in drinking water with 10% methanol at 100 mg/ L before use.

Animals

Male albino Wistar rats, 8-12 weeks old, obtained from the research laboratory, Toxicology and Hydrology Laboratory (LTH), Cheikh Anta Diop University in Dakar, Senegal, were used for the study. Throughout the study, all animals were fed standard rat food (cereal mixture) and received filtered drinking water twice. The research was conducted in accordance with the OECD guidelines on the use and care of laboratory animals [28]. The females were nulliparous and non-pregnant.

Evaluation of toxicity

Acute toxicity assessment: determination of the oral lethal dose 50 (LD_{50})

Acute toxicity was assessed in rats in accordance with OECD Test Guideline No. 423 for the testing of chemicals adopted on December 17, 2001 [29]. First set of femals rats received single dose of 500 mg/kg of the extract, then the 1000, 2000 and 5000 doses were tested on three groups of three femals rats. Dosing was spaced at 48 hours. The observation lasted 14 days for each set, and was carried out twice a day in order to determine possible deaths. It also focused on the appearance of clinical signs, changes in skin, fur, gait, breathing, the appearance of stereotyped behaviours (excessive or infrequent grooming, repetitive circling...) and bizarre behaviours such as self-mutilation, walking backwards etc.

Sub-acute toxicity assessment

The subacute toxicity of the methanolic extract of the plant was assessed in rats according to the OECD guideline 407 for chemical testing adopted on October 3, 2008 [30]. Rats were divided into three groups of six animals / three per sex. Each group of animals (three males and three females) received either 10% methanol in drinking water or a preparation of the extract daily. The control group received drinking water with 10% methanol.Two groups of test animals were dosed with 500 mg/kg and 1000 mg/kg respectively.The choice of doses was based on the results reported in the literature, the acute toxicity and cytotoxicity tests that were carried out [27]. All animals received the treatment for 30 days. All animals were weighed the day before day 0 and fasted for 12 hours. Then, they were weighed weekly until the end of the treatment. The observation conducted twice daily and were made at the same times of day to record changes in skin, coat, gait, breathing, stereotypic behaviour and any other bizarre behaviour that was not usual. For the study of blood parameters, blood samples in each treatment group were collected from the heart after anaesthesia with a mixture of ketamine and xylazine in dry tubes and tubes containing sodium fluoride and EDTA for biochemical and haematological analysis respectively. For histopathological studies the liver and kidneys of all animals were removed and preserved in 10% buffered formalin after macroscopic examination of all organs.

Biochemical analyses

Blood samples for biochemical analyses were centrifuged at 3000×g for 5 min and the serum and plasma collected and stored in Eppendorf tubes at +4◦C and analyzed 24 hours later. Plasma glucose, serum AST, ALT and creatinine were determined by UV- spectrophotometric assays according to the Biosystem kit instruction manual.

Haematological analyses

Haematological analyses were done on day of blood collection. Red blood cell (RBC), Haematocrits, white blood cell (WBC), platelets counts and Hemoglobin (HGB) levels of EDTA blood samples were determined using a Mindray MIN-BC-3000 Plus auto hematology analyzer, which provides a complete blood count (CBC).

Urinalysis

Urine was collected from rats in each group at days 7, 15 and 30 and urinalysis for glucose, pH, proteins, in the urine were determined using urine reagent strips (Labistix, Siemens, Diagnostic Laboratory Inc., France).

Histology:

All organs stored in buffered formalin 10 % were dehydrated in ethanol. The tissues were then cleared with chloroform and impregnated into paraffin wax. Five µm thick sections were mounted on slides and stained with haematoxylin and eosin for light microscopic examinations.

Statistical analyses

Statistical analyses were performed using Graph Pad prism 6.0 software. Data analysis was performed using one-way analysis of variance (ANOVA) followed by multiple comparison tests (Dunnett's multiple comparisons test). Differences were considered statistically significant at P value < 0.05.

III. Results

Acute toxicity

The acute toxicity test for the determination of the LD_{50} of the methanolic extract of the roots of *M. senegalensis* was carried out with four dose levels, the highest of which was 5000 mg/kg. During the test, no disturbances and no suspicious clinical signs were observed, nor were any cases of death noted.

Sub-acute toxicity

The results of the sub-acute toxicity study on body weight changes are shown in *Figures 1* and 2 for males and females respectively, and the biochemical and haematological parameters in *Tables 1* and *2*. Daily administration of the methanolic extract of the roots of *M. senegalensis* to male and female Wistar rats for 30 days at doses of 500 and 1000 mg/kg did not induce mortality in either sex and no apparent signs of toxicity were observed.

Body and organs weights

Figures 1 and *2* show a graphical representation of the evolution of the average body weight of the animals after treatment. No significant differences in body weight were observed in the treated rats compared to the control rats. The weight changes are physiological. There was also no significant difference in liver and kidney weights, expressed as a percentage of body weight, between the control and extract treated groups.

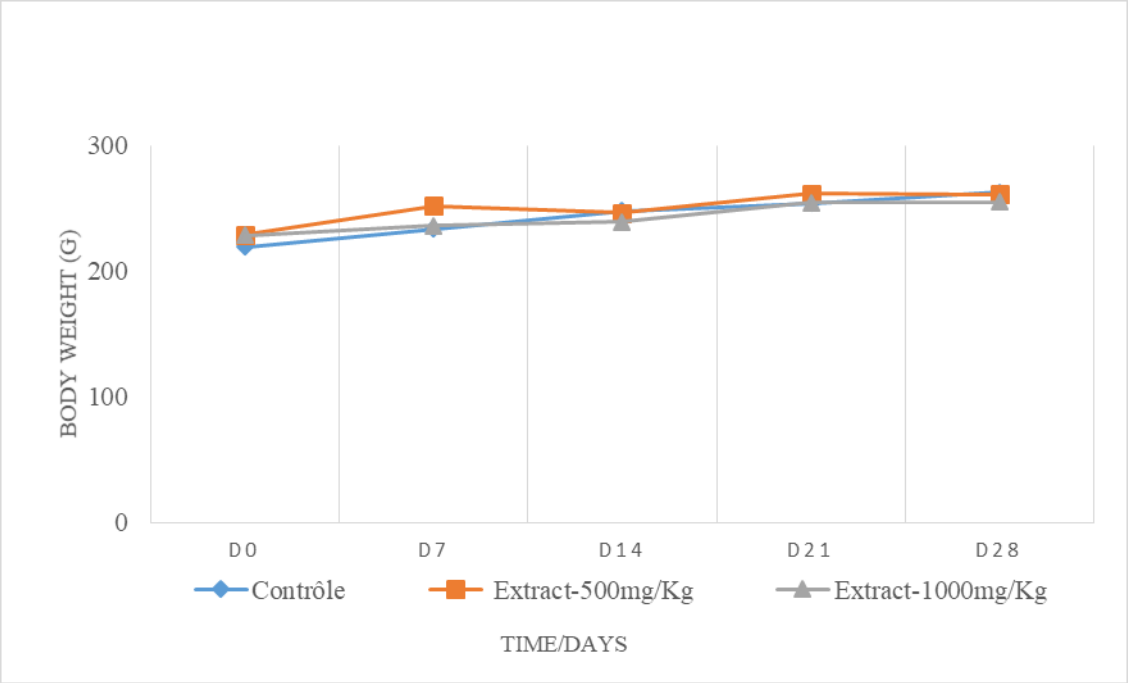

Figure no 1: Changes in males rats's body weight with duration of methanolic extrac of *M. senegalensis* treatment (30 days). Each point represents mean±S.E.M. of *N*=3. Treated animals are compared with a group of control animals (ANOVA).

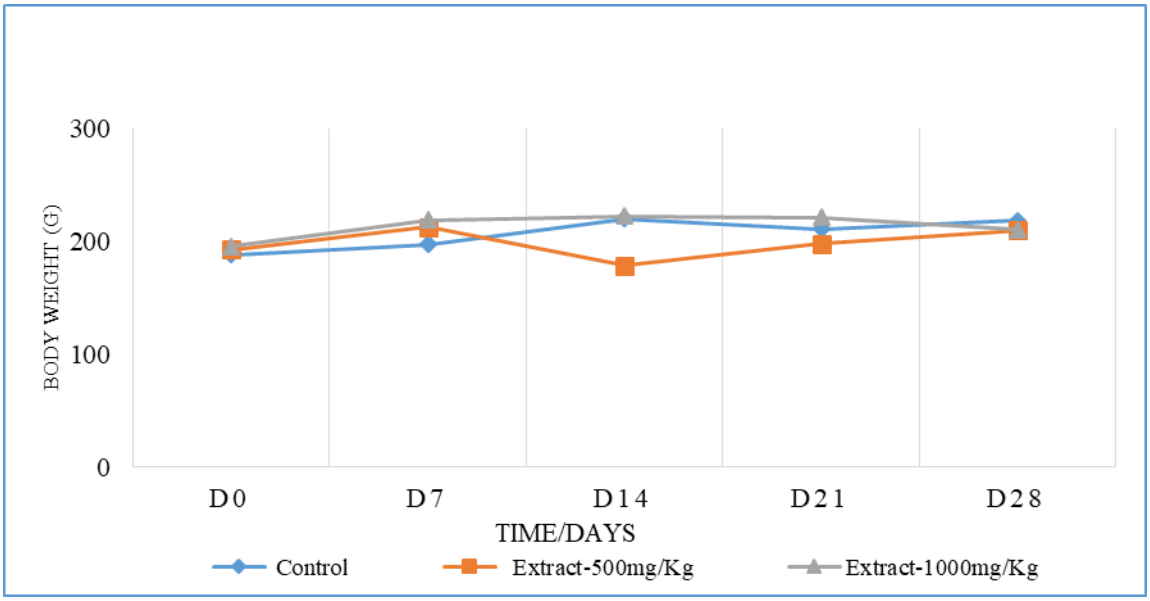

Figure no 2: Changes in femal rats's body weight with duration of methanolic extrac of *M. senegalensis* treatment (30 days). Each point represents mean±S.E.M. of *N*=3. Treated animals are compared with a group of control animals (ANOVA).

Blood biochemistry and urinalysis

Serum, Plasma biochemical data at termination of the study are presented in *Tables 1*. No significant changes were observed in the clinical chemistry parameters (glucose, creatinine, AST and ALT,) measured between the extract treatment groups and controls. There were also no differences observed between extract treatment groups and controls in respect of urinary parameters (glucose, ketones, pH, leucocytes, nitrite, and proteins).

Table 1: Effects of methanolic extract of *M. senegalensis* roots on certain plasma biochemical parameters at termination of treatment (30 days)

	Control	Extract-500mg/Kg	Extract-1000 mg/kg
		Males	
Blood glucose (g/L)	3.15±0.12	3.02±0.17	2.92±0.53
Creatinine (mg/L)	7.27±0.46	6.17±0.29	7.90±1.31
ALT (UI/L)	51.33±0.88	45.90±0.58	53.58±0.96
AST (UI/L)	271.60±10.23	279.83±9.50	248.00±12.24
		Femals	
Blood glucose (g/L)	3.35±0.02	3.12±0.02	3.32±0.73
Creatinine (mg/L)	7.27±0.46	6.17±0.29	7.90±1.31
ALT (UI/L)	41.33±0.58	46.33±1.15	47.00±10.00
AST (UI/L)	241.33±20.03	261.00±29.50	298.00±17.04

Values are means±S.E.M. for $N = 3$

Haematological studies

Results of the haematological studies are presented in *Table 2*. The data show that WBC, RBC, platelets, hemoglobin, hematocrit and mean corpuscular volume, levels, for control rats were not significantly different from those treated with extract during the period of study *(Table 2).*

Table no 2: Effects of methanolic extract of *M. senegalensis* roots on Hematological parameters assessed at termination of treatment (30 days)

	Control	Extract-500mg/Kg	Extract-1000mg/Kg
		Males	
WBC (10^3/µL)	9.95±0.78	9.33±0.42	9.20±0.99
RBC (10^6/µL)	7.80±0.26	7.90±0.21	7.59±0.27
HGB (g/dL)	14.35±0.52	14.70±0.48	14.55±0.35
HCT (%)	45.00±1.44	47.45±0.22	46.90±0.57
MCH (fL)	52.00±1.13	50.00±1.13	50.60±0.14
PLT(10^3/µL)	924.00 ±107.5	888.00±133.45	954.00±43.13
		Females	
WBC (10^3/µL)	8.38±0.14	7.97±0.12	8.27±2.62
RBC (10^6/µL)	7.59±0.55	8.05±0.12	7.12±0.95
HGB (g/dL)	13.10±1.15	14.43±0.12	13±1.51
HCT (%)	37.67±2.71	35.63±0.12	38.90±4.78
MCH (fL)	53.43±0.29	51.67±0.58	54.67±0.81
PLT (10^3/µL)	895.67±210.16	853.33±174.36	985±166.33

Values are means±S.E.M. for $N = 3$

Histopathological studies

Macroscopic examination of tissues shows that *M.senegalensis* extract did not affect liver and kidney morphology. However, in the liver, minor histopathological lesions such as cytoplasmic granulation with mild vacuolation of hepatocytes were observed as shown in *Figure 3 (B).*

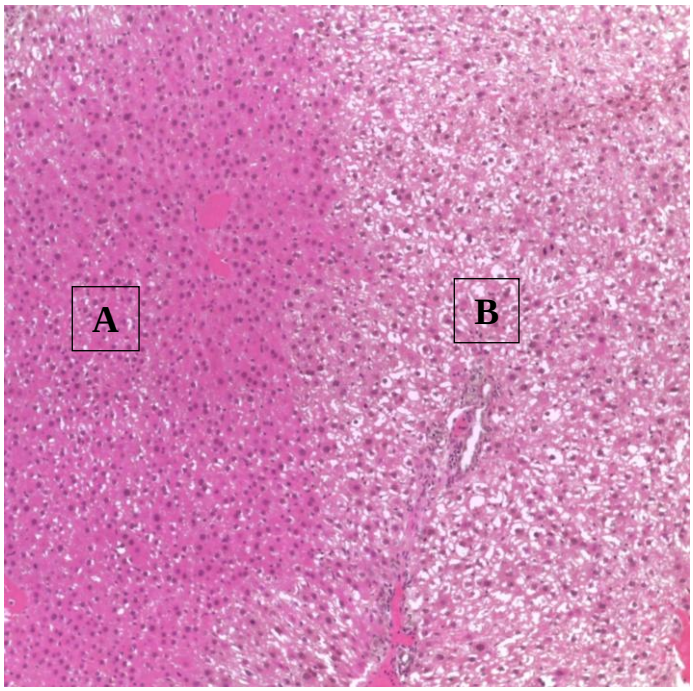

Figure no 3 : Rat liver treated with 1000 mg/kg of the methanolic extract of *M. senegalensis*'roots. Normal appearing (A) and granular and vacuolated (B) hepatocyte territories (H&E, 10x).

IV. Discussion

In a previous study we assessed the cytotoxicity of *Maytenus senegalensis* roots and their effect on DNA to determine a possible mechanism of cell death by apoptosis [27]. The results obtained encouraged us to continue the investigation in animals. In order to complement these data with *in vivo* studies, we investigated the toxic effects of single and repeated 30-day administration of the methanolic extract of *M. senegalensis* roots in the Wistar rat. These studies are important and mandatory for the marketing authorisation of pharmaceutical products for human use, whether they are chemical or herbal products [31].

The results presented in these studies did not reveal any overt toxic reactions of root's estract. Acute toxicity results gave an LD_{50} of over 5000 mg/kg for the methanolic extract of *M. senegalensis* roots. At this dose, no signs of toxicity were observed, and no deaths were noted. In the GHS and according to the OECD, the methanolic extract would be classified as practically non-toxic because the LD_{50} is very high [29,32]. Single oral administration in Wistar rats of *M. senegalensis* root extract up to 5000 mg /kg would therefore be safe. In mice the root extract would also be weakly toxic with an LD_{50} higher than 1600 mg/kg orally [19], the highest dose tested in this study. Regarding the other parts of the plant such as leaves and trunk bark, it would appear that these parts are more toxic with an LD_{50} equal to 1200 mg/kg intraperitoneally in mice for leaves [8] . These results are consistent with those of [13] who had reported an LD_{50} equal to 1264.91 mg/kg intraperitoneally in both mice and rats. However, it should be noted that these doses were sometimes ten times higher than the pharmacological dose found [8,13]. These results obtained from an internationally approved methodology show that the plant and especially the roots are practically safe for single use in humans and animals even at high doses.

Repeated administration of the methanolic extract of the roots of *M. senegalensis for* 30 days for the study of subacute toxicity did not result in any deaths and revealed no signs of toxicity. In terms of body weight, no significant difference was observed between the rats that received the extract and the control rats, and the weight changes noted remained physiological. Appetite was not affected either. The extract of the roots of *M. senegalensis* would thus have no effect on the evolution of the body weight of the rats and therefore would not act on the growth of the rats.

Medicinal plants contain secondary metabolites that give them their therapeutic effect. These same metabolites individually or by cocktail effect may have toxic actions on the haematopoietic system. In our study no changes in blood cells were observed at the doses tested.

Determination of transaminase activity, blood sugar and creatinine levels were performed for biological investigation of liver and kidney functions. The liver and kidney are important organs that play a vital role in the body's metabolic processes. The liver detoxifies substances that are harmful to the body, the kidney helps to maintain the homeostasis of the body by reabsorption of vital substances and excretion of waste products [33,34]. The liver is the main site of metabolism of drugs and other substances. Absorbed at the intestinal level, they are

processed within the hepatocytes before reaching the general circulation [33]. One of the ways to verify liver damage is the determination of transaminase activity (ALT, AST) in serum or plasma which is a sensitive indicator of hepatocellular damage. It is very high in case of disruption of hepatocellular membrane integrity during hepatocellular damage thus indicating cytolysis[35]. However ALT is the parameter that best indicates such damage, AST is not specific and could be related to a cardiac problem or hemolysis. The causes of hepatic cytolysis are very frequent and varied and among them there are drug-induced hepatitis that can appear following the use of phytomedicines [33]. Regarding the kidney, it is known that it is responsible for plasma filtration, reabsorption of almost all blood proteins and elimination of waste products such as creatinine which is a degradation product of creatine localized in the muscle [34–36] . Several factors explain the vulnerability of the kidney to toxins in medicinal plants namely: urinary pH, renal blood flow, renal endothelial surface area, high metabolic activity of the kidney, active absorption of toxins by renal tubular cells and their accumulation at the medullary interstitium [37]. On the other hand, the pharmacokinetics and pharmacodynamics of active ingredients in medicinal plants are not well known, let alone their interaction with the kidney [37]. The nephrotoxicity induced by chemicals or any other metabolite may be reversible or permanent and almost always involves the glomerulus, tubule and interstitium. The mechanism of toxicity is often direct and dose dependent [34,36]. Increased creatinine levels indicate a decrease in glomerular filtration rate (GFR), and by definition renal failure. Despite the fact that this parameter is not perfect, as it is influenced by several extrarenal factors such as age, gender, ethnicity, body mass, muscle metabolism and some medications, it remains to date the best marker of renal function [34,36].

We did not observe any abnormalities in clinical chemistry parameters and urinalysis that would suggest that extract treatment had any adverse effects on either the liver or kidney.

These biological results were confirmed by histopathological examination. Compared to the organs of control rats, the organs of rats given the extract showed no difference in texture, colour and size on gross examination. Microscopic examination did not reveal any alterations consistent with a toxic effect. Some cytoplasmic granulations with vacuolation of hepatocytes were observed. Plants are often considered harmless because they are natural. The results we obtained confirm this hypothesis for the roots of *M. senegalensis*. This study provides additional data on the safety of repeated and medium-term use of the roots.

V. Conclusion

This study provides additional data on the safety of repeated and medium-term use of the roots *M. senegalensis.* In developing countries, traditional medicine is still widely used to meet some primary health care needs and there is a growing awareness of the need to improve knowledge about these plants. While the pharmacological efficacy of several plants has been proven, more work needs to be done on their safety and use. The results of the study carried out on the methanolic crude extract of the roots of *M. senegalensis* in order to evaluate their toxicity showed that they can be used at pharmacological doses without major danger, as any toxicity was revealed at high doses well above the supposedly therapeutic doses. These data encourage further study of the pharmacological activities of M. senegalensis roots.

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