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Research Article
Assessment of *in vivo* and *in ovo* Toxicity of the *Maytenus senegalensis* Roots Hydroalcoholic Extract

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Abstract

Background and Objective: *Maytenus senegalensis*, is a plant traditionally used in Togo to treat several ailments including asthma. This study was undertaken to evaluate the toxicity of the root's hydroalcoholic extract *in vivo* and *in ovo*. **Materials and Methods:** *In vivo*, the oral acute assay was conducted on Sprague Dawley rats by an oral administration of a single dose of 5000 mg kg^{-1}. Subchronic toxicity was studied by a daily administration of the extract at 500 and 1000 mg kg^{-1} for 28 consecutive days. The micronucleus test was performed on ICR mice treated with the extract at 500, 1000 and 2000 mg kg^{-1} for 7 consecutive days to assess its genotoxicity. *In ovo*, the effect of the extract injection to SASSO hen eggs on chick embryo development was studied. **Results:** The acute toxicity test showed that the extract LD_{50} was over 5000 mg kg^{-1}. The subchronic toxicity study showed no significant variation in the rat's body weight increment and haematological and biochemical parameters. Similarly, organs' relative weight and histology were not affected by the *Maytenus senegalensis* extract administration. The micronucleus test revealed an increase ($p<0.05$) in the number of Micronuclei in Polychromatic Erythrocytes (MnPCE) in mice receiving the extract at 2000 mg kg^{-1} and a decrease ($p<0.05$) in the percentage of polychromatic erythrocyte in the 1000 and 2000 mg kg^{-1} groups. Administrated *in ovo*, the extract did not damage blood vessels or significantly affect the relative weights of the albumen and embryo. **Conclusion:** *Maytenus senegalensis* hydroalcoholic extract can be considered safe regarding the results of acute and subacute toxicity tests but further investigations are needed to state its genotoxicity and embryonic toxicity.

Key words: *Maytenus senegalensis*, acute toxicity, subchronic toxicity, genotoxicity, dulcitol, hydroethanolic extract, ephedrine

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Maytenus senegalensis (Lam.) Exell Celastraccae (synonym *Gymnosporia senegalensis*) is a shrub/tree widely distributed in Africa and Asia[1]. As a medicinal plant, it is traditionally used to treat various ailments including malaria, rheumatism, dysmenorrhea, infections, chronic wounds and chest pains[2-4] commonly called Confetti tree, the plant is recognized to have several pharmacological activities against various bacteria and helminths[5,6], inflammation[4,7], pain and malaria[1,2,8] and cough c[9]. *Maytenus senegalensis* contains alkaloids like ephedrine, 1-stachidrine and wilforine. Several phenolic compounds such as anthocyanins, epicatechin, epigallocatechin, glucopyranoside, tannins, prenyletin, quercetin derivatives, scopoletin, vanillic acid etc., as well as sugars (dulcitol, glucose, sucrose, xylose etc.) and triterpenes (maytenonic acid, β-sitosterol, pristimerin etc.), were also identified in the plant[10-12]. Some acute toxicity tests revealed that the extract is tolerated up to 5000 mg kg^{-1} when administered orally to rats but shows some toxicity at 1200 mg kg^{-1} when injected intraperitoneally into CD-6 mice[1,4,13]. In addition, the works of Ndako et *al.*[14] showed that the 21-day administration of the crude methanol extract (800 mg kg^{-1}) and the polar fraction (300 mg kg^{-1}) of the roots induced transitory changes in some serum biomarkers of hepato-renal integrity in male rats. In our previous work on allergic asthma models, hydroethanolic extract of *M. senegalensis* roots at 500 mg kg^{-1} significantly inhibited inflammatory cell infiltration, reduced MDA concentration and increased glutathione level in animal lungs[15,16].

To our knowledge, there are no reported studies on *in vivo* genotoxicity and embryonic toxicity of the plant. Thus, in the present study, the acute and subchronic toxicity, the genotoxicity, as well as the toxicity on chicken embryos was evaluated on *Maytenus senegalensis* root's hydroethanolic extract which was found to be a potential antiasthmatic.

MATERIALS AND METHODS

Study area: All the studies were carried out at the University of Lome for 3 months from 2019 to 2020. The *in vivo* toxicity tests were conducted at the Laboratory of Physiology-Pharmacology of the Animal physiology department (Faculty of Sciences, University of Lome). *In ovo* Test was done in the Laboratory of the Centre d'Excellence Regional sur les Sciences Aviaires (CERSA, University of Lomé).

Plant material: It is consisted of the roots of *Maytenus senegalensis* harvested at Tsévié-Boloumodji and authenticated at the Department of Ecology and Botany of the University of Lomé (number TG 15182). *Maytenus senegalensis* extract was prepared by macerating the roots powder in distilled water/ethanol 1:1 and evaporating the macerate as described earlier[15].

Animals: For this study, 24 Sprague Dawley rats of both sex (130-160 g) and 25 female ICR mice (25-35 g) provided by the animal house of the Department of Animal Physiology (University of Lome) were used. Rats and mice were housed under standard laboratory conditions with free access to food and water. The experiment was conducted according to OECD guidelines and approved by the national bioethics committee of the University of Lome, Togo.

Acute toxicity: The acute toxicity of the *Maytenus senegalensis* extract (MSE) was assessed by the limit test of the Organization for Economic Cooperation and Development (OECD) 423 guideline described by Dossou-Yovo *et al.*[17].

Three female rats received orally one by one within 24 hrs the MSE at 5000 mg kg^{-1}. After gavage, each animal was observed for 1 hr and then at least once daily for 14 days to detect any observable signs of toxicity. Other parameters were searched like signs of mortality, changes in skin and fur and changes in behaviour including lethargy, convulsions, salivation, diarrhoea and aggression.

Sub-chronic toxicity: Animals were exposed to MSE for 28-days to evaluate the sub-chronic toxicity of the extract following the OECD 407 protocol described by Kpemissi *et al.*[18]. Three groups of 8 rats (4 males and 4 females) were formed. During 28 consecutive days, animals received a daily oral gavage (10 mL kg^{-1} b.wt.) of distilled water (group 1) and MSE at respective doses of 500 mg kg^{-1} (group 2) and 1000 mg kg^{-1} (group 3).

Daily, rats were observed for any observable sign of disturbances or adverse effects and their body weight was measured. On day 28 after the last gavage, the rats were fasted for 14 hrs. They were then anaesthetized and blood was collected by retro-orbital puncture in heparinized and non-heparinized tubes. After blood collection, the rats were sacrificed and organs such as the liver, kidneys, heart, lung, spleen, testis and ovaries were removed.

Haematological and biochemical parameters: Blood collected in EDTA tubes was used to determine haematological parameters like White Blood Cell (WBC), red blood cell (RBC), Haemoglobin concentration (HB), hematocrit (HT), Mean Corpuscular Volume (MCV), mean haemoglobin

Concentration (MHC), Mean Corpuscular Haemoglobin Concentration (MCHC) and platelet count (PLT) with the automated hematologic analyzer AJ-2400 Auto.

The blood samples collected in non-heparinized tubes were centrifuged at 3000 rpm for 15 min and the serums were recuperated for biochemical analysis. Biochemical parameters such as Blood Glucose (Glu), Transaminases (AST, ALT), Gamma-GT (GGT), Creatine Phosphokinase (CPK), Urea, Creatinemia (CREA), Total Cholesterol (TC), Triglycerides (TG), HDL and LDL cholesterol were estimated. The analysis was performed with Rayto Chemray-120 automated analyzer.

Relative organ weight and histological study: After blood collection, the rats were sacrificed and organs such as the liver, kidneys, heart, lung, spleen, testis and ovaries were carefully removed. The organs were weighed and their relative weight was calculated. The liver, kidneys, lung and spleen were then preserved in 10% formalin for histopathological examination. The fixed organs were embedded in paraffin and sections were prepared. The sections were stained with hematoxylin and eosin. The slides were observed and microphotographed using a microscope coupled to camera[19].

Genotoxicity evaluation by the micronucleus test: The micronucleus test was conducted according to the OECD 474 guideline[20]. Mice were divided into five groups of five animals. During seven consecutive days, they received a daily gavage (5 mL kg^{-1} b.wt.) of distilled water (groups 1 and 2) and MSE at respective doses of 500 mg kg^{-1} (group 3), 1000 (group 4) and 2000 mg kg^{-1} (group 5). Mice were observed daily for any observable signs of toxicity. After the last gavage, cyclophosphamide (100 mg kg^{-1}) was injected by intraperitoneal route into mice of group 2. About 30 hrs later, all the animals were sacrificed and the micronucleus test was realized by the method described by Raymundo *et al.*[21] with slight modification. Briefly, mice femurs were removed, bone epiphyses were cut and the bone marrow was collected with NaCl 0.9%. After 7 min centrifugation, the pellets were fixed with May-Grunwald for 2 min and then stained with 5% Giemsa.

For the analysis, Micronucleus in Polychromatic Erythrocytes (MnPCE) were scored in a total of 5000 Polychromatic Erythrocytes (PCE) per mouse to determine the clastogenic properties of the MSE. The rapport PCE/total erythrocyte (500 erythrocytes/animal) was also calculated to detect its possible cytotoxic effects.

Evaluation of the toxicity of *Maytenus senegalensis* on chick embryo: The SASSO hen eggs were incubated for 10 days using PAS REFORM (temperature 37.8°C, humidity range of 55-60%). On the 11th day, fertile eggs were selected and then divided into 5 groups of 40. The eggs were weighed and treated as follows:

Batch 1 : Eggs have not received any treatment
Batch 2 : Eggs that received the HBSS buffer (100 µL)
Batches 3, 4 and 5 : Extract at 20, 40 and 80 µg/100 µL have respectively administrated these eggs

The extract and buffer were administered to the eggs by injection. For this purpose, the eggshell was carefully perforated in the marked area of the air bladder. The solutions were injected through the perforation, which was plugged immediately afterwards. Twenty four hours after the injections, 10 eggs from each batch were weighed and sacrificed. For each egg, the albumen and embryo were weighed, the presence of blood vessel damage and the vitality of the embryo were checked[22].

Statistical analysis: The statistical software GraphPad Prism Version 6.02 (USA) was used to analyze the results. All the values were expressed as Mean±SEM (n = 8, n = 6). Analysis of variance followed by Tukey's post-test was used for comparison, $p<0.05$ was considered significant.

RESULTS

Acute toxicity: At the dose of 5000 mg kg^{-1}, MSE did not produce mortality or any acute toxicity sign on the treated animals. Neither changes in skin and fur nor behavioural changes were observed during the first 24 hrs and 14 days after gavage. The result suggested that the LD_{50} of the MSE was higher than 5000 mg kg^{-1}.

Subchronic toxicity

Macroscopic observations: During the 28 days of exposure to the MSE, no mortality or behavioural disorders were recorded in the animals. Similarly, no diarrhoea, vomiting, or changes in skin and fur was observed.

Effect of MSE on body weight: Daily body weight was measured from days 0-28. At doses of 500 and 1000 mg kg^{-1}, the extract of *Maytenus senegalensis* did not cause significant changes in rat weights. Table 1 shows the variations in the body weight of the animals weekly.

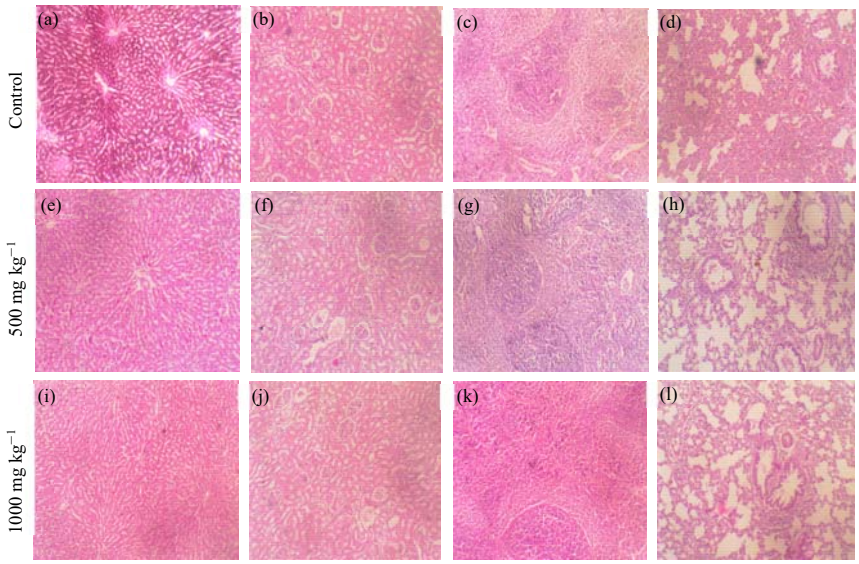

Fig. 1(a-l): Histopathological evaluation of subacute oral toxicity on liver, kidney, spleen and lung, (a-d) Study (from left to right) conducted on control rats, (e-h) MSE 500 mg kg^{-1} and (i-l) 1000 mg kg^{-1} rats

Table 1: Effect of MSE on body weight of rats

		Maytenus senegalensis extract	
Days	Distilled water	500 mg kg^{-1}	1000 mg kg^{-1}
0	141.50±4.14	142.5±4.49	141.00±3.26
7	154.37±5.70	152.75±5.03	148.12±3.88
14	163.50±5.70	158.37±4.59	158.62±4.31
21	172.37±5.94	164.12±7,69	168.87±4.61
28	178.25±5.82	174.50±6.02	177.00±5.22

Values are expressed as Mean±SEM, n = 8, one-way ANOVA followed by Tukey's Multiple Comparison Test and p>0.05

Table 2: Effect of MSE on relative organ weights of rats after 28 days of treatment

		Maytenus senegalensis extract	
Organs	Distilled water	500 mg kg^{-1}	1000 mg kg^{-1}
Liver	3.052±0.141	3.034±0.088	3.199±0.107
Kidneys	0.670±0,041	0.605±0.010	0.585±0.023
Spleen	0.278±0.020	0.229±0.011	0.243±0.017
Lungs	0.784±0.038	0.710±0.036	0.793±0.051
Heart	0.389±0.015	0.387±0.013	0.386±0.018
Brain	0.966±0.033	0.999±0.070	0.992±0.022
Testis	1.188±0.056	0.969±0.113	0.985±0.109
Ovaries	0.096±0.17	0.086± 0.006	0.072±0.012

Each data represent the Mean±SEM of 8 rats, n = 4 for testis and ovaries, one-way ANOVA was followed by Tukey's post-test and p>0.05

Relative organ weight: Exposure of the rats to MSE for 28 consecutive days did not induce significant changes in relative organ weights when compared to the control at either 500 or 1000 mg kg^{-1} (Table 2).

Effect of MSE on haematological and biochemical parameters: Treatment with MSE at 500 and 1000 mg kg^{-1} doses did not significantly alter the haematological parameters of the animals. Similarly, biochemical parameters did not change significantly after the 28 days of oral administration of the MSE. The results are summarized in Table 3 and 4.

Histopathological study: Histopathological examination of the liver, lung, kidney and spleen was performed in rats (male and female) treated with distilled water and the two doses of MSE. The results showed normal histology and did not reveal any significant alterations in the tissues (Fig. 1a-i).

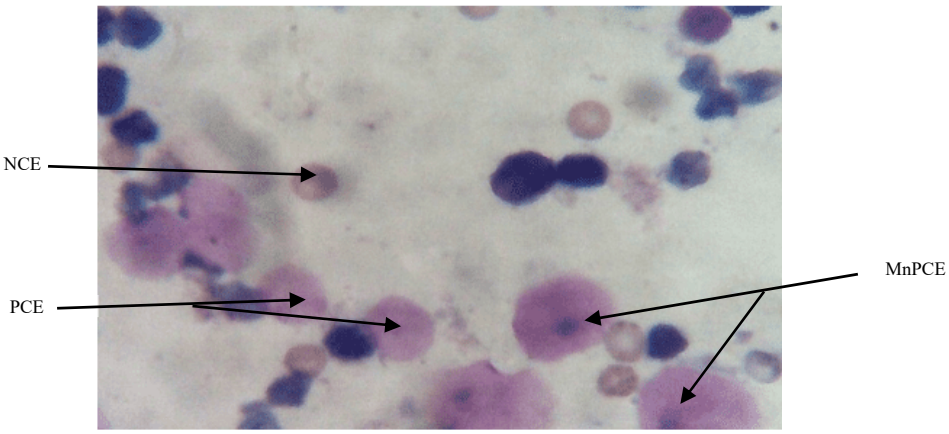

Fig. 2: Images of normochromic erythrocyte (NCE), Micronuclei in a Polychromatic Erythrocyte (MnPCE) and polychromatic erythrocyte (PCE)
May-Grünwald Giemsa staining and 100x enlargement

Table 3: Effect of MSE on haematological parameters of rats

		Maytenus senegalensis extract	
Parameters	Distilled water	500 mg kg^{-1}	1000 mg kg^{-1}
WBC ($\times 10^3/\mu L$)	4.000±0.207	3.688±0.383	3.800±0.462
RBC ($\times 10^6/\mu L$)	6.435±0.116	6.023±0.174	5.931±0.085
HB (g dL^{-1})	13.813±0.445	12.450±0.199	12.250±0.210
HT (%)	31.663±0.745	29.350±0.536	30.525±0.939
MCV (fl)	49.159±0.422	49.815±2.189	51.469±1.412
MCH (pg)	21.431±0.383	20.864±0.864	20.656±0.185
MCHC (g dL^{-1})	43.585±0.600	42.464±0.379	41.335±1.617
PLT ($\times 10^3/\mu L$)	735.000±42.012	786.094±47.785	750.812±42.546

Each value represents the Mean±SEM, n = 8, one-way ANOVA followed by Tukey's Multiple Comparison Test and no significant difference was observed

Table 4: Effect of MSE on biochemical parameters

		Maytenus senegalensis extract	
Parameters	Distilled water	500 mg kg^{-1}	1000 mg kg^{-1}
UREA (g L^{-1})	0.507±0.052	0.495±0.050	0.559±0.044
GLU (mg dL^{-1})	68.750±2.782	63.875±2.216	60.625±2.283
CREA (mg L^{-1})	7.937±0.271	7.062±0.248	7.537±0.423
ASAT (U L^{-1})	217.250±10.785	190.125±10.286	204.375±16.947
ALAT (U L^{-1})	76.500±10.328	55.375±9.658	58.500±2.061
GGT (U L^{-1})	6.375±0.844	6.750±0.796	6.000±0.732
CPK (U L^{-1})	619.356±73.338	651.393±90.512	653.361±119.314
TC (g L^{-1})	0.775±0.040	0.755±0.040	0.766±0.040
TG (g L^{-1})	0.665±0.077	0.706±0.105	0.816±0.180
HDL (g L^{-1})	0.536±0.046	0.530±0.023	0.540±0.033
LDL (g L^{-1})	0.132±0.056	0.112±0.050	0.095±0.072

Data are expressed as Mean±SEM, n = 8, one-way ANOVA followed by Tukey's Multiple Comparison Test and $p>0.05$ (no significant difference)

Micronucleus test: During the 7 days of gavage with MSE, no toxicity signs or mortality were observed in animals. In mice treated with cyclophosphamide (CP), the number of Micronuclei in Polychromatic Erythrocytes (MnPCE) significantly increased ($p<0.001$) when compared to the control group. There is no significant difference between the MnPCE of the control and the MnPCE of the MSE 500 and 1000 mg kg^{-1} extract groups. However, a significant increase ($p<0.05$) in MnPCE was observed in mice receiving the extract at 2000 mg kg^{-1}. Compared to the control, a significant decrease ($p<0.05$) in the percentage of polychromatic erythrocytes was observed in the 1000 and 2000 mg kg^{-1} extract groups. The results are summarized in Table 5. Normochromic erythrocyte (NCE), PCE and MnPCE are illustrated in Fig. 2.

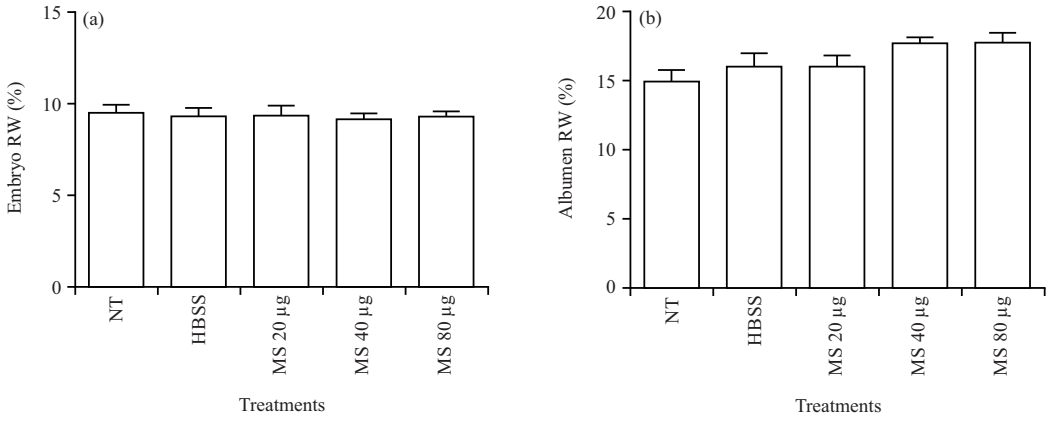

Fig. 3(a-b): Effect of hydroalcoholic extract of *Maytenus senegalensis* on relative weights of chick (a) Embryo and (b) Albumen
Values are expressed as mean±SEM, n = 10, no significant variation was observed (Tukey's follow-up one-way ANOVA), RW: Relative weight, NT: Not treated and MS: *Maytenus senegalensis*

Table 5: Number of MnPCE and PCE (%) observed in the bone marrow of mice treated with distilled water and MSE

	MnPCE number per mouse					Mean±SEM	
Treatments	M_1	M_2	M_3	M_4	M_5	MnPCE	PCE (%)
Distilled water	3.97	6.48	4.75	6.42	5.26	5.46±0.53	45.86±2.85
CP 100	20.91	19.87	15.54	12.31	17.04	17.13±1.54$^{\#\#\#}$	41.84±1.49
MSE 500	4.5	6	4	4.2	5.5	4.84±0.88***	42.1±0.98
MSE 1000	11	8	13	4,5	3.5	8±1.82***	37.02±0.81*
MSE 2000	11	7.5	13	14	11.5	11.4±1.11$^{\#}$**	38.37±1.91*

MnPCE: Micronuclei in Polychromatic Erythrocytes, $^{\#}$p<0.05 and$^{\#\#\#}$p<0.001 (PC/MS vs distilled water), *p<0.05, ***p< 0.001 (MS vs PC), n = 5, PCE (%): Percentage of polychromatic erythrocyte, PCE (%): (PCE/TE)×100, TE: Total erythrocyte (TE = PCE+NCE (normochromic erythrocyte)) and *p<0.05 (MS vs distilled water)

Table 6: MSE effect on eggs hatchability and mortality

			Doses *Maytenus senegalensis* (µg)		
	NT	HBSS	20	40	80
Hatching rate (%)	100	90	86.67	78.13	81.87
Mortality rate (%)	0	5.88	7.69	12	11.53

Eggs that received the extract and buffer did not hatch 100%, also, some mortalities were recorded and NT: Not treated

Effect of *Maytenus senegalensis* on chick embryo development

Macroscopic observations: On the day of sacrifice, the embryos of the batches treated with the different concentrations of the extract were all alive and no visible blood vessel lesions were observed.

Effect on relative weights of albumen and embryo: The *Maytenus senegalensis* extract injected into the eggs did not induce any significant variation in the relative weights of their albumen and embryo compared to eggs that received no treatment or received the buffer (Fig. 3a-b).

Effect on hatchability and chick shape: The extract of *Maytenus senegalensis* did not prevent the chicks from hatching. Macroscopic observation of the hatched chicks showed that they had the normal shape and size of those of the NT and HBSS groups. The hatching and mortality rates of each batch are summarized in Table 6.

DISCUSSION

The current study was carried out to evaluate the toxicity of *M. senegalensis* hydroalcoholic extract through oral acute and subacute toxicity, the micronucleus test and *in ovo* injection of the extract.

Animals treated with MSE at 5000 mg kg^{-1} did not show symptoms of toxicity or mortality. Thus, MSE is practically not toxic through acute exposure in rats as its LD_{50} is higher than 5000 mg kg^{-1}. This result is following previous acute toxicity studies conducted on plants[1,13] except da Silva *et al.*[4], who observed toxicity signs and mortality at the dose of 1200 mg kg^{-1} intraperitoneally administrated to mice.

The results obtained from the subchronic study showed that there were no significant changes in body weight increment of treated animals when compared to control. This implied that MSE did not significantly influence the appetite or interfere with the normal metabolism of treated animals. Haematological parameters of treated animals were not significantly different from those of the control group suggesting that MSE did not alter the hematopoietic and the immune system of the rats[23]. MSE administration during 28 days result in no significant variation of biochemical parameters in treated rats compared to the controls. No significant changes in AST, ALT and GGT indicate that the extract did not alter the rat's hepatocytes. Similarly, the absence of significant variations in creatinine and urea of treated animals implies the nontoxic effect of MSE on kidneys. Blood glucose and lipid parameters were not altered by MSE treatment and CPK values indicate that the extract did not injure animals' heart[24]. The non-adverse effect of MSE on these organs was confirmed by the relative organ weights and the histopathological results. The 28 days subchronic toxicity study conducted on MSE showed that the extract is almost nontoxic.

Micronuclei are small nuclei formed when a chromosome or chromosome fragment is not incorporated into one of the daughter nuclei during cell division due to DNA damage or genomic instability[25,26]. The *in vivo* micronucleus test serves to determine the adverse effect of a test chemical on the genetic material of erythroblasts. Indeed, an increase in the frequency of micronucleated immature erythrocytes in treated animals is indicative of a chromosomal lesion[17]. The micronucleus test results revealed that MSE induces a dose-dependent increase of MnPCE in treated mice compared to those of the control group. Yet, this rise is not statistically significant except in the group treated with MSE 2000 mg kg^{-1} ($p<0.05$). Thus MSE would have a clastogenic effect while its dose is enhancing. The results also showed a significant ($p<0.05$) but not dose-depending decrease of PCE percentage in mice treated with MSE 1000 and 2000 mg kg^{-1} suggesting a possible cytotoxic effect of MSE at these doses. In the normal bone marrow, the PCE:NCE (normochromic erythrocyte) ratio is generally about 1:1. The decrease in the percentage of PCE observed implies that the normal proliferation of bone marrow cells is affected by MSE at the doses of 1000 and 2000 mg kg^{-1}[27]. However, a study conducted by Ahmed *et al.*[28] showed that *Maytenus senegalensis* leaves extract is noncytotoxic to Vero cell lines. At the dose of 500 mg kg^{-1} which is our highest therapeutic dose, neither genotoxic nor cytotoxic effects were observed in treated mice. A study on another plant of the genus *Maytenus robusta* showed that the plant is non-genotoxic at the same dose[21].

Chicken embryos are widely used in developmental biology to access early embryonic stages and to study embryo development ex utero or test developmental toxicity[29,30]. *In ovo*, the MSE did not damage a blood vessel or significantly affect the relative weights of the albumen and embryo. This suggested that the extract did not alter the normal growth of chicken embryos. Hatching rates were approximately 80% and mortalities were observed. This may be related to a variety of factors, including handling conditions. The non-variability of relative embryo weight, shape and size of chicks in batches treated with the extract compared to the control batch suggests that the extract would be non-teratogenic *in ovo*. This characteristic of the extract remains to be deepened and determined *in vivo* in other animal models.

CONCLUSION

From this study, it appears that *Maytenus senegalensis* root extract is nontoxic to rats through acute and subchronic studies. Subsequent investigations should be done to affirm the clastogenic and cytotoxic effects observed with high doses in the micronucleus test. Similarly, *in vivo,* embryonic toxicity tests need to be performed to confirm the results obtained *in ovo*. In sum, it can be stated that *Maytenus senegalensis* root extract is not or slightly toxic but further toxicological studies are necessary to conclude the plant's safety.

SIGNIFICANCE STATEMENT

This work was carried out to study the toxicity of the roots of *Maytenus senegalensis* used in traditional Togolese medicine and which would have an anti-asthmatic effect according to our previous work. *Maytenus senegalensis* roots is almost nontoxic when administrated orally to rat for 28 days at 500 and 1000 mg kg^{-1}. From 1000 mg kg^{-1}, it has a clastogenic and cytotoxic effect on mice bone marrow. These results provide data on some of the implications of the continued use of the plant as well as its dosage and pave the way for further research on the toxicological profile of the plant.

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