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The Cytotoxic Effect of Aqueous Extract of Moringa Oleifera (Moringaceae) on Wistar Rats

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Abstract Introduction: Moringa oleifera was a medicinal plant generally used by populations in the food as well as therapeutic field. Its use to treat anemia was observed in the Djougou area in northern Benin in traditional medicine. Although this plant underwent a lot of scientific study, there was still to our knowledge, a lack of scientific data on its effectiveness in the treatment of hemolytic type anemia. The aim of our study was to test its safety in vivo on Wistar rats. Methods: Wistar rats received by gavage a single dose of 2000 mg/kg of aqueous extract of Moringa oleifera leaves for the acute oral toxicity test (TOA). For the subchronic oral toxicity test (TSC), the Wistar rats received the aqueous extract by gavage at a daily dose of 200 mg/Kg of body weight per day for 28 days. The rats were weighed and blood samples were taken on day 0, then on day 14 for TOA and on day 28 for TSC, respectively. The renal assessment was carried out by the dosage of creatinine, the liver assessment by the transaminases AST and ALT and the immune assessment by the hemogram. The liver, kidneys and spleen were removed for histological analysis. The results were analyzed using the Mann Whitney test, the significance level being set at 5%. Results: The weight of the rats did not change significantly in the various acute or subchronic oral toxicity tests, suggesting the absence of physical disturbance in the rats. Serum creatinine did not change significantly, indicating preservation of renal function. It was the same for AST and ALT transaminases, indicating an absence of hepatic cytolysis. Hepatic, renal and splenic parenchyma showed no atypia. Conclusion: The aqueous extract of Moringa oleifera leaves did not reveal any toxic effect in the acute or subchronic state and offered prospects for its use in the treatment of anemia.

Keywords: *Moringa oleifera, oral toxicity, subchronic liver, kidney and spleen toxicity*

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1. Introduction

Anemia was defined by the fall in the level of hemoglobin in the circulating blood compared to normal values [1]. It was a syndrome observed in various pathologies such as genetic anomalies, infections [2]. The World Health Organization (WHO) estimated that 1.62 billion people suffer from anemia, i.e. a global prevalence of 24.8% with a predominance in Africa and South-East Asia, i.e. 67 respectively. 7% and 65.5% [3]. In Benin, 72% of children aged 6-59 months suffered from anemia, of which 3% in its severe form and 41% in its moderate form, then 58% of women aged 15 to 49 suffered from anemia: 26% in its mild form, 30% in its moderate form and 2% in its severe form [4]. Treatment varied depending on the type. This could be iron, vitamin B12 or B9 [5-6]. Given the high cost of treatment, the inaccessibility and adverse effects of conventional synthetic drugs used in developing countries, the search for alternative and/or complementary strategies was necessary for the use of synthetic antianaemics and other conventional drugs. WHO estimated that 80% of rural populations in developing

countries depend on traditional medicine for primary health care [7], including anemia and its complications. In Benin, out of the 30,700 species of plants inventoried in forest ecosystems [8] which were used by local populations as food plants and others as medicinal plants, including Moringa oleifera and Jatropha Gossypiifolia [8]. Moringa oleifera leaf powder contained most of the essential nutrients needed for good health [9-12]. The leaf powder was rich in multiple minerals and vitamins, including iron, vitamin A (carotenoid) and vitamin C, which were important for iron metabolism. Additionally, Moringa had an added benefit for solving multiple malnutrition problems because it was rich in all essential amino acids, which were building blocks of proteins necessary for cell growth [2,13] [15,16]. Studies conducted in different countries like Senegal and India, the use of Moringa has been reported to reduce malnutrition in children as well as vitamin A and protein deficiencies [13,17] [18,19,20] without any risk of toxicity [21,22,23] and no adverse side effects [18]. The nutritional potential of Moringa leaf powder maked it an important ingredient for improving nutrient diversification in complementary foods for children [13]. In the rich and diversified flora of Benin, Moringa oleifera and many other plants were used in traditional medicine to treat anemia. These plants, although having already been the subject of several scientific studies on their therapeutic virtue; have not yet been proven to our knowledge in Benin, their effectiveness on hemolytic anemia. It is for this reason that this study was undertaken with the aim of evaluating the cytotoxic effect of the aqueous extract of Moringa oleifera (Moringaceae) on Wistar rats. Such an evaluation would allow not only their rational use but also their possible transformation into improved traditional medicines (MTA) to be used without toxic risk by poor populations.

2. Materials and Methods

Animal material

The animal material used in this study consisted of albino Wistar strain rats aged 4 to 5 months and with an average body weight of approximately 210 g. These rats were acclimatized to ambient conditions at a constant temperature of 25 ± 1°C in the animal facility of the Experimental and Clinical Biology Unit (UBEC), of the National School of Applied Biosciences and Biotechnologies (ENSBBA) of Dassa, National University of Sciences, Technologies, Engineering and Mathematics (UNSTIM) in Benin. The rearing took place in a well-ventilated room with a cycle of 12 hours of light and 12 hours of darkness. The rats were kept in wire mesh cages with feeders and drinkers, and had free access to drinking water and food. They were supplied with drinking tap water and their daily diet consisted of a mixture of granulated feed presented in the form of kibble and marketed by Véto Services (Benin). The breeding enclosure was regularly cleaned to guarantee optimal development of the animals protected from any infection.

Identification and preparation of plants

The samples studied were fresh leaves collected in the northern Benin region, specifically in Djougou in the department of Donga and brought back to the laboratory for drying. The samples were spread out in a cold drying room (22°C) for about 14 days, after which time the samples were brittle and were practically anhydrous. The identification was carried out at the National Herbarium of Benin. The extraction of the total chemical principles was done using the method of maceration in accordance with the traditional use of plants. Based on the extraction techniques cited in the literature [24], 50 g of powder were dissolved in 500 mL of distilled water. The mixture was left stirring continuously for 48 hours. After cooling, the mixture obtained was filtered (3 times in a row) on absorbent cotton and the filtrate was transferred to a 1000 mL flask then subjected to evaporation until dryness at 40° C. using a rotavapor (Heidolph Laborota 4000 efficient) coupled to a water chiller (Julabo FL 300). The dry residue obtained represented the maceration.

Larval toxicity test

Larval cytotoxicity was commonly performed using shrimp larvae in seawater (Artemia salina) in the presence of the test solution. Its interest lied in the knowledge of the safety of use of the plants whose extracts were tested. Indeed, there was a correlation between toxicity on shrimp larvae and cytotoxicity on 9PS and 9KB cells (human nasopharygeal carcinoma) on the one hand, lung carcinoma A-549 cells and carcinoma HT-29 cells. colon on the other hand. The test, used successfully in 2010 by Fatondji et al., in 2012 by Sakirigui et al., and in 2014 by Houngbeme et al., was carried out according to the method of Michael et al., taken over by Vanhaecke et al., then by Sleet & Brendel. Artemia salina eggs were incubated in seawater until young larvae hatch (48 hours). A stock solution of the extract was prepared by dissolving 200 mg of extract in 4 mL of distilled water; i.e. a concentration by weight of 50 mg/mL. Ten [11] successive half (1/2) dilutions of the stock solution with seawater were carried out. A defined number of larvae [17] was introduced into each dilution.

All the solutions as well as control solutions containing no active substance were left under stirring for 24 hours. Macroscopic counting of the number of surviving larvae in each solution maked it possible to assess toxicity. In the case where deaths were observed in the control medium, the data were corrected by Abbott's formula: %death = [(test − control) / control)] x 100. The dose-response data were transformed by logarithm and the LC50 is thus determined by linear regression.

To assess the degree of toxicity from the LC50 values, we used the correspondence table (Table 1) drawn up by Mousseux in 1995 and widely used in the literature [24].

Table 1. Correspondence between LC50 and toxicity

LC_{50}	Toxicity
$LC_{50} \geq 100$ µg/mL	**- (nontoxic)**
100 µg/mL > $LC_{50} \geq 50$µg/mL	**+ (weak)**
50 µg/mL > $LC_{50} \geq 10$ µg/mL	**+ + (moderate)**
$LC_{50} < 10$ µg/Ml	**+ + + (strong)**

Assessment of acute and subchronic oral toxicity

The Toxicity Test was performed according to the recommendations of the modified Organization for Economic Cooperation and Development (OECD) Guideline 423 at doses of 200, 300, 1200, 2000 and 5000 for testing chemicals [25]. Three (03) animals were used for the test. Their weights were determined before and after the test. Hepatic and renal functions were assessed at the beginning and at the end of the experiment on blood samples (assay of transaminases and serum creatinine) and histological sections of the kidney, liver and spleen (search for cell lesions).

Acute toxicity

It consisted of testing Moringa oleifera extract at a single dose of 2000 mg/kg on rats. The test was carried out on 6 Wistar rats. The behavior of the rats was observed as well as the number of deaths over a period of 14 days. After 15 h of fasting, they were distributed as follows: control group consisting of 3 rats receiving distilled water, at a rate of 10 ml/kg; experimental batch consisting of 3 rats, receiving the extract of Moringa oleifera, at a rate of 2000 mg/kg. A behavioral observation was carried out 3 hours after administration of the extracts. Then hydration and feeding were performed daily for 14 days. During this period, signs of toxicity including coat change, motility, tremors, stool appearance, mobility and death were monitored. At the end of the treatment, the rats were fasted for 24 h, then a blood sample was taken, followed by a dissection after general anesthesia with ether. The organs removed were the liver, kidneys and spleen. The latter were rinsed with a 0.9% saline solution, then placed in 10% diluted formalin for the histological study.

Subchronic toxicity

It was carried out on 6 Wistar rats divided into 3 equal groups of 3 rats each as follows: group 1, receiving distilled water at a rate of 1 ml/100 mg of body weight (control group); batch 2 (experimental batch) receiving a solution of Moringa oleifera extract at 200 mg/kg body weight every day for 28 days. The rats were fed and hydrated ad libitum. At the end of the treatment, the rats were fasted for 24 h, then a blood sample was taken, followed by a dissection after general anesthesia with ether. The organs removed were the liver, kidneys and spleen. The latter were rinsed with a 0.9% saline solution, then placed in 10% diluted formalin for the histological study.

Blood tests

Serum creatinine was measured to explore renal function. AST and ALT transaminases were measured for liver function. The number of white blood cells was measured as hematological parameters.

Histological studies

A standard historical study was carried out for the acute and subchronic oral toxicity test on the organs removed after sacrifice of the different groups of animals. Kidneys, liver and spleen were removed, fixed in a 10% buffered formalin solution and embedded in paraffin. Sample sections (5 µm) were mounted on glass slides, deparaffinized and hydrated. For histological analysis, the sections were stained with hematoxylin and eosin (H&E), according to a standard protocol [18]. Pictures were taken at 400X magnification.

Statistical analyzes

Parameter means were compared using the Mann Whitney test. The significance level was set at 5%.

3. Results

In vitro Cytotoxicity of the extract

The sensitivity curve of the Artemia salina larvae to the extract was plotted from the numbers of dead larvae recorded after reading the test, as a function of the concentration of the extract. The curve was represented by the figure below.

The analysis of the LC50 values of the extracts tested by the correspondence table (Table 1), allowed to say that the tested extract did not present any toxicity in the range of concentrations analyzed. In fact, the LC50 values obtained were above the toxicity limit set (0.1 mg/mL) Figure 1.

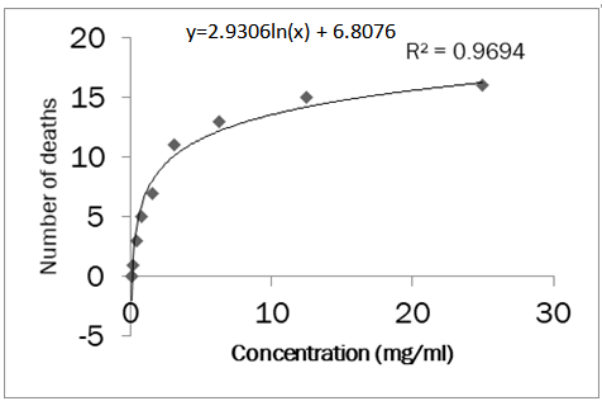

Figure 1. Sensitivity curve

The aqueous extract of Moringa oleifera leaves showed no acute oral toxicity. The results of the acute oral toxicity test of the extract were presented in (Table 2).

On D0, the weight of the rats was 147 ± 9 g, the serum creatinine was 9 ± 1 mg/L, the AST and ALT transaminases were respectively 101 ± 12 U/L and 73 ± 11 U/L, the mean number of blood leukocytes was 10.1 ± 1.4 G/L. These different parameters did not change significantly on D14, indicating an absence of physical disturbance, an absence of alteration of the renal, hepatic and immune functions of the rats. This result suggested an absence of toxicity of the extract in the acute state (Table 2).

Table 2. Acute oral toxicity of Moringa oleifera extract

Parameters	D0	D14	P value
Weight (g)	147 ± 9	168 ± 11	0.1
Serum creatinine (mg/L)	9 ± 1	8 ± 2	0.4
Transaminase AST (U/L)	101 ± 12	107 ± 12	0.7
Transaminase ALT (U/L)	73 ± 11	81 ± 12	0.4
Number of blood leukocytes (G/L)	10,1 ± 1.4	12,3 ± 1.6	0.2

The aqueous extract of Moringa oleifera leaves showed no subchronic toxicity. The results of subchronic oral toxicity test of the extract were presented in (Table 3).

On D0, the weight of the rats was 185 ± 29 g, the serum creatinine was 9 ± 2 mg/L, the AST and ALT transaminases were respectively 91 ± 13 U/L and 53 ± 12 U/L, the mean number of blood leukocytes was 13.3 ± 2.1 G/L. These different parameters did not change significantly on D28, indicating an absence of physical disturbance, an absence of alteration of the renal, hepatic and immune functions of the rats. This result suggested an absence of toxicity of the extract in the subchronic state (Table 3).

Table 3. Subchronic oral toxicity of Moringa oleifera extracts

Parameters	D0	D28	P value
Weight (g)	185 ± 29	205 ± 30	0.4
Serum creatinine (mg/L)	9 ± 2	8 ± 2	0.7
Transaminase AST (U/L)	91 ± 13	87 ± 10	0.7
Transaminase ALT (U/L)	53 ± 12	46 ± 10	0.4
Number of blood leukocytes (G/L)	13.3 ± 2.1	14.7 ± 2.0	0.9

Moringa oleifera leaf aqueous extract did not change liver parenchyma

Figure 2 showed the renal paremchyma. In acute (AOT) and subchronic (SCT) oral toxicity tests, the liver of rats did not show atypia with a normal parenchyma like that of control rats. The hepatocytes (arrows) were organized into laminae separated by conspicuous venous sinusoids (VS) which communicate with the centrilobular vein (CV) Figure 2.

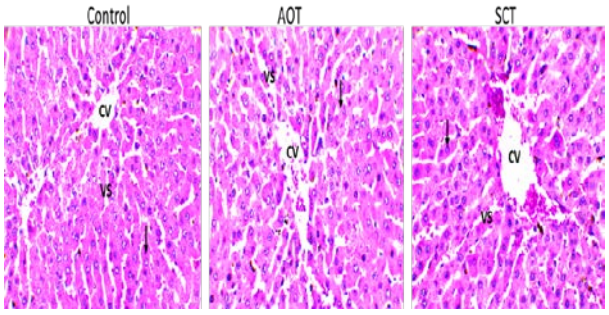

Figure 2. Liver histology in acute and subchronic oral toxicity tests of aqueous Moringa oleifera extract (400X magnification)

Moringa oleifera leaf aqueous extract did not change renal parenchyma

Figure 3 showed the renal parenchyma. In the acute (AOT) and subchronic (SCT) oral toxicity tests, the renal parenchyma did not show any atypia and was like that of the control rats. The glomeruli (G), the proximal tubes (PT) and the distal tubes (DT) were clearly visible Figure 3.

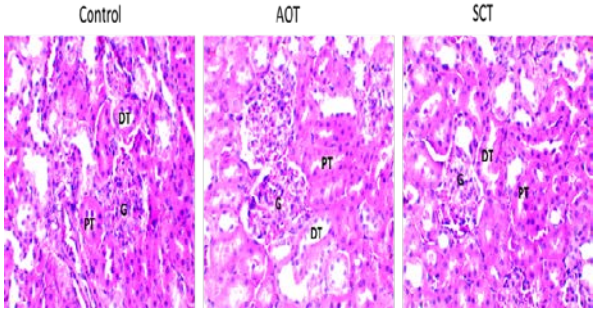

Figure 3. Renal histology in acute and subchronic oral toxicity tests of aqueous Moringa oleifera extract (400X magnification)

Moringa oleifera leaf aqueous extract did not change splenic parenchyma

Figure 4 showed the splenic parenchyma. In acute (AOT) and subchronic (SCT) oral toxicity tests, the splenic parenchyma was typical as in control rats. The central arteries (CA) with their periarteriolar sheaths (PS) and germinal centers (GC) were clearly visible in the white pulp. The venous sinusoids (VS) were well separated by the Billroth cords (BC) in the red pulp Figure 4.

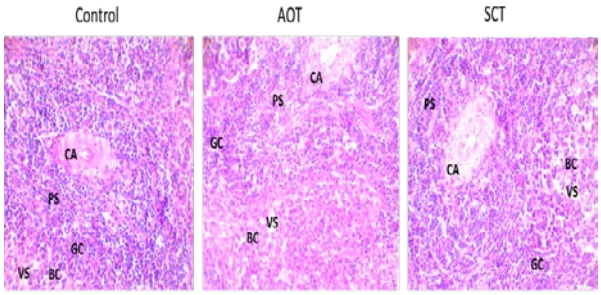

Figure 4. Histology of the spleen in acute and subchronic oral toxicity tests of aqueous Moringa oleifera extract (400X magnification)

4. Discussion

This study focused on the evaluation of the cytotoxic effect of the aqueous extract of Moringa oleifera (Moringaceae) on rats Wistar. That was a plant with anti-anemic property whose leaves could be used for the prevention and correction of malnutrition in because of its exceptional nutritional qualities [26-32]. The in vitro toxicity study using the larval toxicity test did not show any toxic effect. This result is comparable to that obtained with the aqueous extract of Cocos nucifera L. (Arecaceae) root, also used to treat anemia [41]. In the acute or subchronic in vivo toxicity study, the behavior and weight of the rats were not modified. Similar results were obtained by Fikriansyah et al [32] during their study on the cardioprotective effect of Moringa oleifera but by injecting a dose of 4.67 mg/kg. However, these results were contrary to those obtained by Sudha P. et al [33] who reported increased mortality of rats tested at a dose of 2000 mg/kg of body weight, prompting them to opt for doses of 750 mg/ kg and 250 mg/kg in their evaluation tests. In addition, Lemita N. et al [34] reported an increase in the weight of rats after administration of Moringa Oleifera leaf extract to rats at a dose of 150 mg/kg by the intraperitoneal route. In the acute or subchronic oral toxicity tests of the extract, the ALAT and ASAT transaminases were not modified. These observations suggested that the aqueous extract of Moringa Oleifera would have hepatoprotective effects. These results were similar to those reported by Senou et al [35], who noted a non-significant decrease in ALT enzyme levels after subchronic administration of an aqueous extract of Senna alata at a dose of 200 mg/kg in the treated rats. In a similar study, Luka et al [36] explained that a decrease in ASAT and ALAT hepatic enzymes could indicate a hepatoprotective effect of the crude ethanolic extract of the roots of Diospyros mespiliformis hochst (Ebenaceae). In a study carried out in diabetic rats, Lemita N. et al [34]

observed this hepatoprotective effect when they noted a very significant increase in ALT and AST transaminases in untreated diabetic rats compared to controls, and compared to diabetic rats treated with Moringa Oleifera. This result was confirmed by a liver histology that remained normal, an observation also noted by [34] and [35] in their respective study. Evaluation of acute and subchronic toxicity of Moringa oleifera extracts in rats showed that creatinine did not experience a significant increase, suggesting that the aqueous extract of Moringa oleifera leaves was not nephrotoxic. This observation was confirmed by histology which showed that the renal parenchyma with glomeruli, proximal and distal tubules and collecting tubules were normal. These results were similar to those reported by Lemita N. et al [34] in diabetic rats treated with Moringa oleifera compared to untreated rats, noting a total absence of lesions in all the organs studied, including the kidneys in the diabetic rats treated with Moringa Oleifera. These results were also observed by other authors [37] on the bark of Psorospermum febrifugum, an anti-anemic plant. An absence of subchronic oral toxicity of the flowers of Senna alata, an antiamemic plant, was also observed by authors [35] and [38], on the liver, kidneys and heart of Rat Wistar. In acute and subchronic oral toxicity tests, immune function as assessed by white blood cell count was not affected. The histology of the spleen, a peripheral immune organ, was not altered, confirming the non-toxic nature of the plant. Similarly, similar observations have been made with the aqueous extract of the root bark of Cocos nucifera, an anti-anaemic plant [40].

5. Conclusion

The different results obtained on the biochemical, hematological and histological level during in vitro and in vivo toxicity tests, reassure on the safe use of Moringa oleifera leaves in the perspective of treatment of anemia. Nevertheless, it would be important to continue the evaluation of the toxicity of the leaves of this plant in the long term with chronic toxicity tests. This would make it possible to envisage the formulation of these extracts in improved traditional medicine and accessible to the rural populations who were the most numerous and the most exposed.

Statement of Competing Interests

The authors declared no conflicts of interest.

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