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Toxicological study of Manihot esculenta Crantz (Euphorbiaceae) leaf extracts

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ABSTRACT

Manihot esculenta, also named cassava, is a plant widely used as food but also in traditional medicine to treat many diseases such as anemia. However, consumption of *M. esculenta* is limited by its content in cyanogenic glycosides. Few studies have evaluated the toxicity of *M. esculenta* leaves. This study aims to evaluate the *in vitro* cytotoxicity of *Artemia salina* and the 28 days subchronic toxicity of *M. esculenta* leaves by oral administration of the extract to male Wistar rats. The *A. salina* assay showed that the alcoholic extract of *M. esculenta* presented some toxicity. The results of 28 days subchronic toxicity showed a significant increase in the relative weight of the liver. No changes in hematological parameters were observed apart from a significant increase in platelet number. Biochemical parameters such as SGOT, SGPT, glycemia, and ALP were not significantly changed. The histological sections showed no organ damage.

Keywords: *Manihot esculenta*, cytotoxicity, *Artemia salina*, sub-chronic toxicity, Wistar rats.

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INTRODUCTION

Manihot esculenta is a worldwide plant belonging to Euphorbiaceae family's, extensively cultivated in tropical and subtropical regions [1]. Though, the main part of the plant used is its starchy tuberous root, its leaves are also consumed as vegetables [2, 3] and used for various medicinal purposes by local communities. Interestingly, cassava leaves are reported to have bioactivities such as anti-oxidant, anti-tyrosinase, anti-inflammatory, and hepatoprotective [2, 4, 5, 6]. It is a source of calories in the human diet in the tropics [7]. The common use of these leaves is for disorders such as rheumatism, fever, headache, and loss of appetite.[8] In Nigeria, they are also utilized in the treatment of ringworm, tumor, conjunctivitis, sores, and abscesses. Apart from these, various literature have mentioned the use of plant leaves in hypertension, irritable bowel syndrome, aches, cancerous affections, affective excrescences of the eyes and tumors, antiseptic, cyanogenetic, demulcent, diuretic, dysentery, flu, marasmus, snake bites, prostatitis, and spasms, etc.[9, 10, 8]

M. esculenta leaves are a good source of acids [11]. Additionally, it contains a cyanogenic glucoside, linamarin and lotaustralin [12]. Generaly, there is a detoxification mechanism which can avoid death when cyanide release is slow [13]. The consumption of large amounts of the bitter variety (containing higher cyanogenic glucosides) can cause severe toxicity resulting in Konzo, a paralytic disorder or even death. Routine ingestion of low levels of cyanide leads to chronic toxicity possibly developing into goiter (enlargement of the thyroid gland) or tropical ataxic neuropathy (a disorder involving the nervous system). However, scientific research is still needed to support these findings [9, 8]. This study aims to evaluate the *in vitro* cytotoxicity of *M. esculenta* leaves on *Artemia salina* and its 28 days subchronic toxicity by oral administration of the extract on male Wistar rats.

MATERIALS AND METHOD

Collection and extraction of plant material

Manihot esculenta leaves were collected from the Agronomic farmstead of University of Lomé (TOGO) after identification by the Botany and Ecology Department of the same University. A voucher specimen was registered in the herbarium under the number TG 15184. Leaves were cleaned and dried under climatization (20°C). Then 500 g of dried and pulverized leaves were soaked in 7.5 L of ethanol-water (70-30) for 72 hours with occasional agitation. After double filtration with cotton and Whatman paper, the filtrate was evaporated using a rotating evaporator Heidolph 2 (Germany). The extract was dissolved in distilled water with 2% of Tween 80. The yield of this extraction was calculated according to the formula: R = (weight of extract / dry weight) x 100.

Animals

Male Wistar rats (130-200 g) were used for the experimentations. They were provided by the Animal Physiology's Department and were acclimated at least one week prior the beginning of the manipulations. The animals were fed with rodent standard diets and water *ad libitum*. Animal's care and handling were conformed to accepted guidelines [10, 8]. Ethical approval was obtained from the institutional Ethical Committee for Teaching and Research under the number (ref no. CNCB- CEER 2801/2010).

Phytochemical screening

The screening was performed to assess the presence of some chemical groups such as tannins and alkaloids, saponins, and flavonoids. Flavonoids, saponins, and tannins were checked as described by Karumi *et al.* [14]. For alkaloids, Bourchadat, Dragendorff, and Mayer reagents were used to determine their presence as described by Evans [15].

Assessment of larval toxicity of the substance

Bioactivity of the extract was monitored by the brine shrimp lethality test [16]. Different concentrations of extract from 25 mg/mL to 0.049 mg/mL were prepared. Using a cone micropipette, a colony of 16 live larvae was brought into contact with a series of solutions of progressive concentrations of plant drug extracts. These media and controls were allowed to stir and live larvae were counted 24 hours after incubation. The mean percentage mortality was plotted against the logarithm of concentrations. The concentration (LC50), at which 50% of the larvae were killed, was determined from the graph [1, 17, 18].

Acute toxicity test

The limit dose of 5000 mg/kg was carried on three female Wistar rats according to the Organization for Economic Cooperation and Development (OECD) guidelines [19]. Each rat was sequentially dosed at the interval of 48 h. The animals were observed individually to check acute toxicity signs or behavioral changes, 30 minutes post-dosing during the first 24 h. The rats were observed at least once daily for 14 days.

Sub-chronic toxicity

Repeated-dose oral toxicity was achieved according to OECD guidelines 407 [20]. The animals were divided into three groups of 6 each. The first group (group 1) received distilled water containing 2% of Tween 80 and served as control group. The second (group 2) and the third (group 3) groups received, respectively, the extract at 500 mg/kg and 1000 mg/kg body weight. The extract was administrated daily for 28 days at the same time and the animals were observed at least twice daily for morbidity and mortality. All groups were administrated 1 mL/100 g body weight of solution. The body weights were recorded every day. The rats were observed for aggressiveness, mobility, diarrhea, appetite, and answer to sound stimulation. On the 29th day, after 12 h of fast, the rats were anesthetized first by ether. Blood samples were collected from

the retroorbital sinus in dry tubes for biochemical analyses and in EDTA tubes for haematological analyses. Samples for biochemical analyses were centrifugated at 2500 rpm for 15minutes and the serums were collected. Biochemical parameters such as Serum Glutamic Pyruvic Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), alkaline phosphatase (ALP), creatinine and glucose were performed. Haematological parameters achieved were white blood cell count (WBC), red blood cell count (RBC), haematocrit (HCT), haemoglobin (HB), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), platelet count (PLT) and mean corpuscular volume (MCV). Necroscopy of all rats was carried out and some organ weights (kidney, liver, spleen, heart, and testis) were recorded.

Statistical analysis

Results were expressed in mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance (ANOVA) with Tukey test to evaluate the difference between two groups. Values of $p<0.05$ were considered significant. The Instat statistical package (GraphPad Prism 6.02) was used to carry out all statistical analyses.

RESULTS AND DISCUSSION

Phytochemical screening and extraction of plant materials

The evaporation allowed us to obtain a yield of 8.15%. The results of the phytochemical screening have shown the presence of flavonoids, saponins, tannins, and the lack of alkaloids. (data was not shown).

Brine shrimp toxicity screening

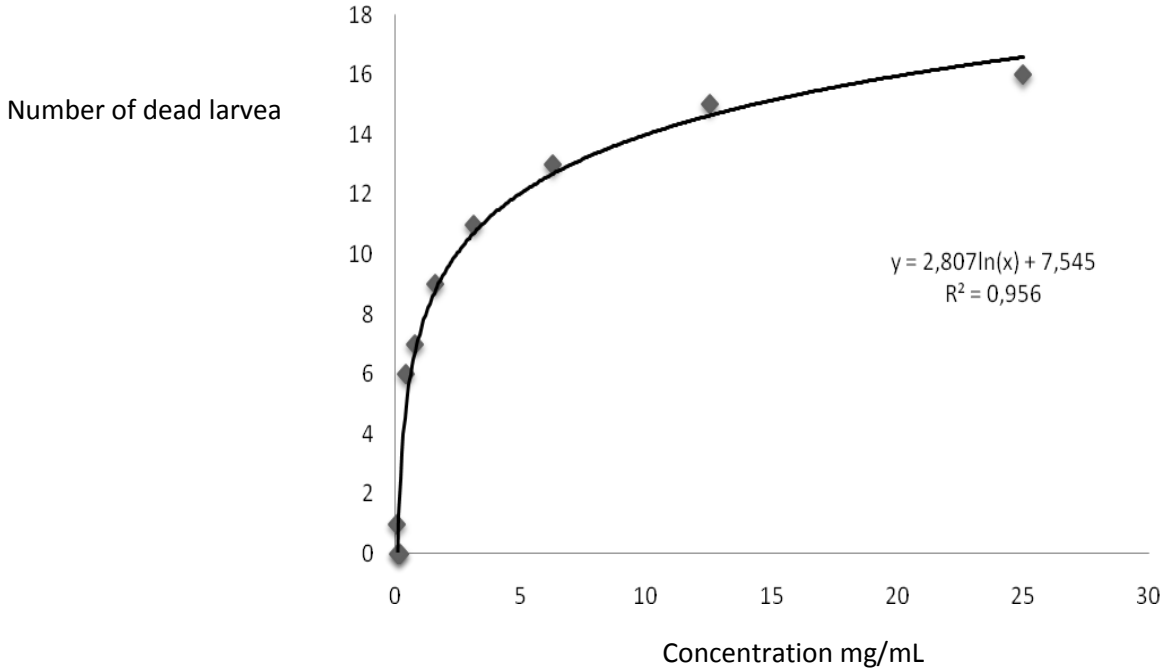

Figure 1: *In vitro* cytotoxicity of *M. esculenta* leaves on *Artemia salina* larvae.

Figure 1 shows the results of the cytotoxicity of *A. salina*. The LC50 values of *M. esculenta* were determined and were found to be 1.031 ± 0.11 mg/mL.

Acute toxicity

For a short (48 h) or long period of observation, the limit dose 5000 mg/kg of *M. esculenta* didn't lead to mortality or acute toxic effects of the three rats dosed. The LD_{50} was then over 5000 mg/kg.

Sub-chronic toxicity

In the clinical evaluation, no behavioural changes and death were observed at the end of treatment. Similarly, no significant differences in body weight were observed between the control and treated groups during this period (Table 1).

Table 1: Effect of hydroethanolic extract of *M. esculenta* on rat weight after 28 days of experiment.

Week	Control	*M. esculenta* extract dose	
		500 mg/kg	1000 mg/kg
0	153.0±11.6	154.0±6.3	156.0±7.8
1	158.0±10.1	164.0±7.0	161.0± 8.4
2	165.0±10.4	182.0±8.5	169.0±7.4
3	168.0±11.1	177.0±8.0	173.0±8.3
4	177.0±10.9	189.0±8.1	184.0±6.6

Each valor is a mean ±S.E.M with n (number of rats per group) =6.

The extract was administrated daily for 28 days. The body weights were recorded every day.

M. esculenta at 1000 mg/kg increased significantly ($p < 0.001$) the relative weight of the liver as compared to the control group (Table 2).

Table 2: Effect of hydroethanolic extract of *M. esculenta* on rat's organ ratio weight after 28 days of experiment.

Parameters	Control	*M. esculenta* extract dose	
		500 mg/kg	1000 mg/kg
WBC ($\times 10^3$/µl)	7.40±0.82	7.50±0.62	6.60±0.49
RBC ($\times 10^6$/µl)	8.20±0.12	7.80±0.18	7.88±0.16
HB (g/dl)	14.20±0.21	13.20±0.11	13.70±0.27

Each valor is a mean ±S.E.M with n (number of rats per group) =6.

The extract was administrated daily for 28 days. The organ ratios were recorded every day.

** $p < 0.001$, significant difference as compared to the control.

Tables 3 and 4 show the haematological and biochemical parameters, respectively. No changes in parameters were observed apart from a significant increase in the relative weight of the liver and in platelet number when compared with controls. Biochemical parameters such SGOT, SGPT, glycemia, and PAL were not significantly changed when compared with control. The histological sections showed no organ damage.

Table 3: Effect of hydroethanolic extract of *M. esculenta* on haematological parameters after 28 days of experiment.

Parameters	Control	*M. esculenta* extract dose	
		500 mg/kg	1000 mg/kg
WBC ($\times 10^3$/µl)	7.40±0.82	7.50±0.62	6.60±0.49
RBC ($\times 10^6$/µl)	8.20±0.12	7.80±0.18	7.88±0.16
HB (g/dl)	14.20±0.21	13.20±0.11	13.70±0.27
HCT (%)	43.80±0.61	40.50±0.43	42.20±0.87
MVC (fl)	53.30±0.97	53.00±0.63	53.90±0.96
MCH (pg)	17.20±0.32	17.20±0.14	17.50±0.33
MCHC (g/dl)	32.30±0.15	32.50±0.16	32.50±0.20
PLT ($\times 10^5$/µl)	768.10±25.3	799.50±32.70	744.80±24.6***

Each valor is a mean ±S.E.M with n (number of rats per group) =6.

The extract was administrated daily for 28 days. The haematological parameters were performed at the end of the administration. *** p <0.0001 significant difference as compared to the control

Table 4: Effect of hydroethanolic extract of *M. esculenta* on biochemical parameters after 28 days of experiment.

Parameters	Control	*M. esculenta* extract dose	
		500 mg/kg	1000 mg/kg
Urea (mg/dL)	0.20±0.01	0.10±0.01	0.20±0.10
Creatinine (mg/dL)	7.80±0.30	7.10±0.16	7.60±0.21
Glucose (mmol/L)	4.30±0.67	4.20±0.42	3.90±0.23
SGOT (UI/L)	160.30±15.14	153.30±13.43	145.80± 9.75
SGPT (UI/L)	42.10±4.40	44.80±4.07	40.80±2.66
ALP (UI/L)	168.10±19.62	185.30±24.20	196.60±10.38

Each valor is a mean ±S.E.M with n (number of rats per group) =6.

The extract was administrated daily for 28 days. The biochemical parameters were performed at the end of the administration

DISCUSSION

Plants (medicinal or otherwise) are a source of new molecules that can be used in medicine [21] and take a major part in the diet of people in developing countries. The use of these plants can bring direct answers to some health problems. It is therefore important to check the toxicity of the plants used. In this work, we have evaluated the toxicity of repeated doses of 28 days according to the protocol OECD 407 [20].

The phytochemical screening was first performed; it showed the presence of flavonoids and tannins and reducing compounds. These results confirm those of several authors [22, 23]. The screening has also identified a lack of alkaloids in our extract. Some authors [22, 24] found during their work in the hydroalcoholic extract of *M. esculenta* leaves, traces of alkaloids. Anbuselvi and Balamurugan [23], noted a strong presence of alkaloids during work on the alcoholic extract of the leaves of *M. esculenta*.

The LC_{50} value of *M. esculenta* was 1.031 ± 0.11 mg/mL. Therefore, the hydroalcoholic extract of *M. esculenta*. leaves is not toxic according to Meyer *et al.* [1], who classified crude extracts and pure substances into toxic (LC_{50} value < 0.1 mg/ml) and nontoxic (LC_{50} value > 0.1 mg/ml).

Regarding body weight, *M. esculenta* hydroethanolic extract had not induce significant changes in Wistar rat body weight. In our study, *M. esculenta* increased significantly ($p < 0.05$) the liver relative weight. The assessment of the weight of organs such as the liver, kidney, spleen, testis, heart, pancreas, brain, and language is very essential in toxicological studies. The weight of a body or more, the relative weight is an important index used in physiology and toxicology [25]. The increase in liver relative weight could be due to protein synthesis induction [26] or peroxisome proliferation or inflammation [25, 27, 28]

No significant effect was observed on haematological and biochemical parameters. The dosage of transaminases (SGPT, SGOT), alkaline phosphatase, and glycemia is essential in the evaluation of liver toxicity [29, 30]. As for creatinine and urea, they are used to assess renal toxicity [31]. The increase in platelets with the dose of 1000 mg/kg could be due to the action of a substance isolated from *M. esculenta*. The increase in the liver relative weight is not associated with changes in biochemical parameters (SGOT, SGPT, and ALP), we can say that after 28 days *M. esculenta* does not cause severe liver damage. Contrary to our results, Awe and Kolawole [32] after their biochemical, hematological, and histopathological evaluation of an aqueous extract of *M. esculenta* leaves in Wistar rats concluded that the extract appeared to be toxic at doses of 200 and 400 mg / kg body weight respectively for 28 days.

A similar study conducted by Soto-Blanco and Gorniak [33], on goats fed directly with leaves whose cyanide hydroxide content was evaluated at 6 mg/kg and then decreased to 4.5 mg/kg, revealed hepatotoxicity. The difference with our study could be explained by the concentration of cyanogenic glycosides contained in the leaves, the animal material used and the duration of exposure.

Similarly, hepatic damage was found in cassava-treated dogs and hepatopathy was characterized by periportal hepato-laryngeal vacuolation [34]. Similar degenerative changes have been described in the livers of rabbits [35] and HCN-treated rats [36], but in goats only minimal changes in the liver are observed [33]. Thus, it is possible that the liver is a target tissue for cyanide toxicity [37]. These results could explain the increase in the relative weight of the liver in our study.

CONCLUSION

In this study, we have evaluated the *in vitro* cytotoxicity of *M. esculenta* leaves on *Artemia salina* and its 28 days subchronic toxicity by oral administration of the extract on male Wistar

rats. No changes in parameters were observed apart from a significant increase in the relative weight of the liver and in platelet number at the dose of 1000 mg/kg. Therefore, some caution should be taken when administering *M. esculenta* leaves for long periods.

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