

**ASSESSMENT OF ACUTE TOXICITY AND CYTOTOXICITY OF AQUEOUS EXTRACT (EA), ETHYL ACETATE FRACTION (FA) AND METHANOL FRACTION (FM) OF *EUCALYPTUS CITRIODORA*.**

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**ABSTRACT**

The objective of this study was to investigate the toxicity of *E. citriodora*. In order to evaluate the toxicity of *E. citriodora*, the acute toxicity of the aqueous extract, ethyl acetate fraction and methanol fraction of this plant on female rats and cytotoxicity test on NIT-1 cell line were investigated. A fixed large dose of 2000 mg/kg b.w. of aqueous extract, ethyl acetate and methanol fractions was administrated by a single oral gavage according to the OECD procedure. In 2 weeks, aqueous extract, ethyl acetate and methanol fractions did not significantly affect the weight gain, hematological and biochemical parameters of rats treated groups compared with the control group. Cytotoxicity effect of aqueous extract, ethyl acetate fraction and methanol fraction was estimated through a MTT assay. Cytotoxicity tests on NIT-1 cell line disclosed that concentrations of 10, 100 1000 µg/mL inhibited the growth of cells. The concentration inducing cell growth inhibition by about 50% (IC<sub>50</sub>) were 100 µg/mL. The results of cytotoxicity show toxic effect of *E. citriodora* extract and fractions on NIT-1 cells. While this work did not showed acute toxicity effect on NIT-1 cells.

**KEYWORDS:** *Eucalyptus citriodora*; aqueous extract; ethyl acetate fraction, methanol fraction; cytotoxicity; oral acute toxicity; MTT assay.

**INTRODUCTION**

Nowadays, there has been increasing interest in the use of herbal medicines and natural products for the treatment of a variety of disorders.<sup>[1]</sup> Traditional medicine has maintained greater popularity all over the world and the use is rapidly on the increase.<sup>[2]</sup> One reason for the widespread use of medicinal species is the belief that these products from medicinal plants are risk free and considered by patients to be a safe alternative for the treatment of several disease.<sup>[3]</sup> Natural products are of great importance in the development of new pharmaceutical products or plant protection products.<sup>[4,5]</sup> But many studies have shown that these products contain potential toxic effects.<sup>[6,7]</sup> It is therefore essential, before the use as a therapeutic drug from plant extract to conduct its toxicological study to assess its safety for use without risk of poisoning.

*Eucalyptus citriodora* belongs to the family of Myrtaceae. Many studies have shown the biological effect of *E. citriodora* oil as antifungal, antibacterial, anticandidal,<sup>[8,9,10]</sup> anti-inflammatory, analgesic.<sup>[11]</sup> The

water extracts of dried leaves of *Eucalyptus citriodora* (EC) Hook are traditionally used as analgesic, anti-inflammatory, and antipyretic remedies for the symptoms of respiratory infections, such as cold, flu, and sinus congestion.<sup>[12]</sup> Reported the aqueous extract of *Eucalyptus citriodora* leaves have antidiabetic activity. The objectives of the present study are to determine the acute toxicity and cytotoxicity of aqueous extract, ethyl acetate fraction and methanol fraction.

**MATERIALS AND METHODS****Experimental animals**

Male albinos wistar rats (130-142 g) obtained from pharmacy department of University Felix Houphouët Boigny-Abidjan were used in this study. The animals were housed in polypropylene cages and in environmental conditions. They were had free access to water and food. The rats were fed on a standard pellet diet (IVOGRAIN Agro Industries Bingerville, Côte d'Ivoire).

### Plant collection and extract preparation

Leaves of *E. citriodora* were collected in the west region of Côte d'Ivoire during the dry season. Thereafter, the leaves were dried at room temperature and pulverized. One hundred fifty (150) g of powder was added in 3 L of water and boiled during 30 minutes. After decantation, the supernatant was taken and filtered with cotton and with Whatmann (N°1) filter paper. After filtration and lyophilization, the extracts were stored at a temperature of 4°C pending the time for chemical and biological investigations.

### Fractionation procedures

The aqueous extract (20 g) was extracted successively with cyclohexane, ethyl acetate, a mixture of water and methanol (20/80; v/v). Briefly, 20 g of water dried extract was dissolved in 100 mL of distilled water and added in a separation funnel. Thereafter, 4x100 mL of cyclohexane were added in the funnel and shaken vigorously. After 15 minutes, two phases were observed and separated. The upper cyclohexane phase was collected and evaporated to dryness in a rotary evaporator. The lower aqueous phase was submitted to a following separation with 4x100 mL of ethyl acetate. As the previous operation, after the addition of ethyl acetate, the funnel was shaken and two phases were separated after a period of 30 minutes. The upper ethyl acetate phases were assembled and evaporated to dryness. The aqueous phases were also collected and evaporated to dryness in the rotary evaporator. The aqueous extract was submitted to a subsequent extraction with 4x100 mL of a mixture of water and methanol (20/80; v/v). For this operation, the extract was mixed with the solvent during 2 hours on a magnetic stirring. After this period; the mixture was left to cooled and the layer was separated from the supernatant by filtration. The supernatant was then dried with a rotary evaporator and the layer phase was also dried at 50 °C in a stove.

$$\text{Relative organ weight} = \frac{\text{absolute organ weight (g)}}{\text{body weight of rat on sacrifice day(g)}} \times 100$$

### Biochemical analysis

For biochemical analysis, blood was centrifuged at 3500 rpm for 5 min and serum was also obtained and stored at -20°C. The serum was analyzed for various parameters such as Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), LDH, serum urea, serum creatinine, serum glucose, Creatine kinase (CK), Total protein (TP), Total cholesterol (Chol-T), total bilirubine and triglycerides. Dosages were made using Cobas integras 400 plus. The protocol for each assay was preset and then incorporated into the device during the assays.

### Hematological study

The EDTA tube was used and white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular

### Acute toxicity study

**Assessment of acute toxicity:** The acute toxicity was investigated using the OECD Guideline 423.<sup>[13]</sup> for testing of chemicals. This method is a sequential process using three animals of one sex by step. Depending on the mortality and/or moribund state of animals, two to four steps are needed to assess the average acute toxicity of the test substance. Female rats aged 2 to 3 months and weighing between 130 and 142 grams on average were used for experimentation. We did not have information on the toxicity of *Eucalyptus citriodora* fraction so we started the test with a dose of 300 mg/kg bw. Three rats deprived of food during the night but not water, are weighed and then were given orally 1 mL of fraction at a dose of 300 mg/kg bw as a single dose using a cannula intubation. After administration of fractions, the three rats are again fasted for three hours before giving them food. They are observed individually the first thirty minutes, periodically during the first 24 hours after treatment. They were then observed daily for up to 14 days. Twenty four (24) hours after administration of the fractions at a dose of 300 mg/kg bw, in case of no mortality and no moribund observed, the superior dose of 2000 mg/kg bw was tested. After this dose, rats were observed for mortality and morbidity. At the end of the study (on day 14), rats were then anesthetized with ether and blood samples were obtained into sterile tubes with anticoagulant EDTA (ethylene diamine tetra acetic acid) for hematological tests and without anticoagulant tubes for biochemical tests. Immediately after blood collection, the animals were sacrificed. The organs (liver, kidney and heart) of rats in the various groups were weighed. The organ weight ratio was calculated and relative organ weights was also calculated).

hemoglobin concentration (MCHC), platelet count and percentage of total lymphocytes (LYMP) were assessed with an automatic hematological analyzer (Sysmex KX-21).

### CYTOTOXICITY ASSAY

#### Cell viability assay

Cells were seeded onto 96-well plates at a cell concentration of  $1 \times 10^5$  cells per well and were pre-incubated overnight. After pre-incubation, NIT-1 cells were exposed to extract and fractions at different doses. The cells were incubated for 24 h with or without different concentrations (10, 100, 1000 µg/ mL). The cytotoxicity effect on the NIT-1 cells was determined using MTT reduction assay.

**MTT assay**

Cells were seeded at  $1 \times 10^5$  per well in a 96-well plate for viability assay. The media cultured with the cells were changed and 20  $\mu$ L of MTT solution (5 mg/mL in PBS) was added to each well and the plates were further incubated for another 6 h. Supernatants were then discarded and 150 $\mu$ L of DMSO was added to each incubation well and mixed thoroughly to dissolve the dark blue crystal formazan. The absorbance at 570 nm (formation of formazan) was recorded with a microplate spectrophotometer.<sup>[14]</sup>

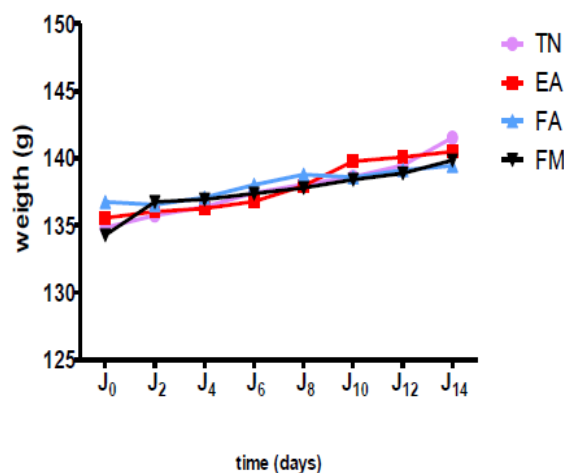
**Statistical analysis**

The graphical representation of the data was performed using the Graph Pad Prism 5.0 software (Microsoft, USA). Results are expressed as mean  $\pm$  SEM. The difference between two values is considered significant when  $P < 0.05$ .

**RESULTS**

The results showed that, all the animals which were given acute dose of 2000 mg/kg bw, orally were remained active and healthy throughout the period of study. The animals did not show any changes in general behavior or other physiological activities and no symptoms of adverse effects were recorded during the study. Also no manifestations of tremors, convulsions, salivation, diarrhea, coma or abnormal behaviors such as

self-mutilation or walking backwards were observed. The **figure 1** shows the evolution of body weight of rats. **Table 1** shows the effect of extract and fractions on the relative weight of the heart, liver and kidneys on the total weight of the animal. Analysis of the average of the relative weight of the organs studied shows no statistical difference ( $P > 0.05$ ) between the control group and the treated groups.



**Figure 1: Effect of aqueous extract (2000 mg/kg) and fractions (2000 mg/kg) on rats body weight**

**Table 1: Body and organ weights of rats in acute toxicity study in control and groups treated with aqueous extracts and ethyl acetate and methanol fractions of *E. citriodora*.**

	TN	EA (2000 mg/kg)	FA(2000 mg/kg)	FM (2000 mg/kg)
Body weight (g)				
Day 14	141.52 $\pm$ 0.81	140.48 $\pm$ 0.81	139.43 $\pm$ 0.72	139.85 $\pm$ 0.42
Absolute weight (g)				
Heart	0.58 $\pm$ 0.02	0.63 $\pm$ 0.01 <sup>ns</sup>	0.59 $\pm$ 0.02 <sup>ns</sup>	0.58 $\pm$ 0.03 <sup>ns</sup>
Liver	4.13 $\pm$ 0.24	4.35 $\pm$ 0.37 <sup>ns</sup>	4.46 $\pm$ 0.30 <sup>ns</sup>	4.50 $\pm$ 0.25 <sup>ns</sup>
Kidney	0.70 $\pm$ 0.03	0.71 $\pm$ 0.06 <sup>ns</sup>	0.70 $\pm$ 0.05 <sup>ns</sup>	0.70 $\pm$ 0.00 <sup>ns</sup>
Relative weight (%)				
Heart	0.41 $\pm$ 0.02	0.45 $\pm$ 0.04	0.42 $\pm$ 0.02	0.41 $\pm$ 0.02
Liver	2.92 $\pm$ 0.17	3.10 $\pm$ 0.26	3.20 $\pm$ 0.21	3.22 $\pm$ 0.18
Kidney	0.49 $\pm$ 0.03	0.47 $\pm$ 0.04	0.50 $\pm$ 0.04	0.50 $\pm$ 0.01

Data are expressed as mean  $\pm$  SEM,  $n=3$  for each group. No statistical difference was found between the control and treated groups ( $P > 0.05$ ).

**Hematological**

Hematological parameters of rats were measured after 14 days. The effect of hematological parameters in rats treated is presented in **Table 2**. The results show that all hematologic parameters have not evolved significantly compared to the control group. At dose used in this experiment, the extract and fractions caused no significant change in blood parameters that are the White blood cells (WBC), Red blood cells (RBC), Hemoglobin (Hb), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin concentration (MCHC), Platelets (PLT) and Lymphocytes (Lym) compared to the control group.

**Biochemical parameter**

In this study, transaminases (AST, ALT), blood glucose, total cholesterol, and total bilirubin, creatinine, urea, total protein, creatinin kinase, tryglicerides and Lactate dehydrogenase were measured. The results are shown in **table 3**. The statistical analysis showed that the parameters of rats treated with dose of 2000 mg/kg bw are identical ( $P > 0.05$ ) when compared to the control group.

**Cytotoxicity effect of extract and fractions on NIT-1 cells**

The **figure 2** shows the effect of extract and fractions on NIT-1 cells. The results show that the aqueous extract and fractions exhibited NIT-1 cell lines death at all

concentrations (10, 100 and 1000 µg/mL). The percentage of cell viability for each concentration was for aqueous extract 59.80; 49.28; 5.81%, ethyl acetate fraction 66.65, 50.27, 38.66% and methanol fraction

60.02, 49.12, 17.69% respectively. At 100µg/mL of extract and fractions, nearly 50% of the cells were necrotic.

**Table2: Hematological values of rats in acute toxicity study in control and groups treated with aqueous extracts and ethyl acetate and methanol fractions of *E. citriodora* leaves.**

Parameters	TN	EA (2000 mg/kg)	FA (2000 mg/kg)	FM (2000 mg/kg)
WBC (10 <sup>3</sup> /µL)	6.8±0.12	6.10±0.15 <sup>ns</sup>	6.04±0.11 <sup>ns</sup>	7.04±0.604 <sup>ns</sup>
RBC (10 <sup>6</sup> / µL)	7.03±0.26	7.11±0.13 <sup>ns</sup>	7.37±0.46 <sup>ns</sup>	6.90±0.67 <sup>ns</sup>
HGB (g/ dL)	13.20±0.31	13.75±0.14 <sup>ns</sup>	13.72±0.43 <sup>ns</sup>	13.90±0.46 <sup>ns</sup>
HCT(%)	41.48±0.42	41.10±0.55 <sup>ns</sup>	41.67±0.83 <sup>ns</sup>	41.57±0.91 <sup>ns</sup>
VGM (fl)	54.70±1.44	55.81±0.80 <sup>ns</sup>	54.74±2.04 <sup>ns</sup>	53.80±0.94 <sup>ns</sup>
CCMH (g/dl)	33.37±0.64	32.80±0.51 <sup>ns</sup>	34.17±0.41 <sup>ns</sup>	33.37±1.35 <sup>ns</sup>
CMH (pg)	18.77±0.23	18.37±0.15 <sup>ns</sup>	18.97±0.15 <sup>ns</sup>	18.70±0.4 <sup>ns</sup>
PLT (10 <sup>3</sup> / µL)	436.67±40.96	477.67±40.50 <sup>ns</sup>	401.33±44.03 <sup>ns</sup>	484.33±33.83 <sup>ns</sup>
LYM(%)	70.66±2.08	75.33±3.84 <sup>ns</sup>	74.00±2.08 <sup>ns</sup>	75.00±2.89 <sup>ns</sup>

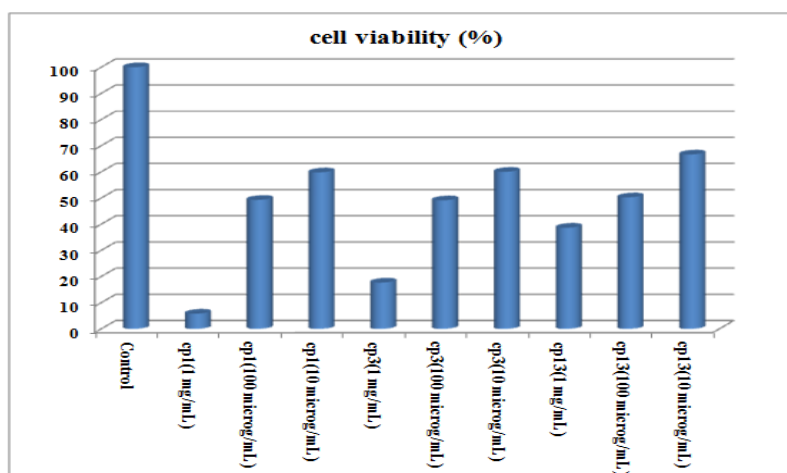
Data are expressed as mean ± SEM; (n=3); ns (not significant, p<0.05); White blood cells (x10<sup>3</sup>/uL); Red blood cells (x10<sup>6</sup>/uL), Hemoglobin (g/dL); Mean corpuscular hemoglobin concentration (g/dL); Platelets (x10<sup>3</sup>/uL); Lymphocyte (%); Hematocrit (%); Mean corpuscular volume (FL/cell).

**Table3: Blood chemistry values of rats in acute toxicity study in control and groups treated with aqueous extracts, ethyl acetate fraction and methanol fraction of *E. citriodor*.**

Parameters	TN	EA (2000 mg/kg)	FA(2000 mg/kg)	FM (2000 mg/kg)
Glyc (g/L)	0.92±0.01	0.90±0.02 <sup>ns</sup>	0.91±0.02 <sup>ns</sup>	0.93±0.04 <sup>ns</sup>
Urea(g/L)	0.31±0.03	0.30±0.02 <sup>ns</sup>	0.29±0.00 <sup>ns</sup>	0.29±0.01 <sup>ns</sup>
Creat (mg/L)	4.33±0.33	5.03±0.33 <sup>ns</sup>	4.30±0.02 <sup>ns</sup>	5.00±0.6 <sup>ns</sup>
Total Chol (g/L)	0.72±0.02	0.77±0.01 <sup>ns</sup>	0.81±0.03 <sup>ns</sup>	0.78±0.06 <sup>ns</sup>
Trigly (g/L)	0.52±0.01	0.51±0.03 <sup>ns</sup>	0.50±0.03 <sup>ns</sup>	0.51±0.02 <sup>ns</sup>
AST (U/L)	128±8.98	119±6.0 <sup>ns</sup>	119±3.30 <sup>ns</sup>	114±3.52 <sup>ns</sup>
ALT (U/L)	52±2.31	54±2.33 <sup>ns</sup>	55±1.20 <sup>ns</sup>	55±2.90 <sup>ns</sup>
Total Prot (g/L)	67.00±4.73	71.33±2.03 <sup>ns</sup>	72.67±1.76 <sup>ns</sup>	70.00±1.53 <sup>ns</sup>
Total Bili (mg/L)	5.25±0.63	5.19±0.55 <sup>ns</sup>	5.17±0.52 <sup>ns</sup>	4.27±0.75 <sup>ns</sup>
CK (UI/L)	1341±57.58	1209±102.94 <sup>ns</sup>	1332±51.11 <sup>ns</sup>	1314±73.50 <sup>ns</sup>
LDH (UI/L)	1605±41.53	1533±37.02 <sup>ns</sup>	1576±62.55 <sup>ns</sup>	1621±25.52 <sup>ns</sup>

Data are expressed as mean ± SEM (n=3).; ns (not significant, p<0.05); AST: Aspartate aminotransferase (U/L); ALT: Alanine aminotransferase (U/L); Urea (mg/L); Creat: Creatinine (mg/L), LDH: Lactate dehydrogenase (U/L); CK: Creatine kinase (U/L); Total proteins (g/L); Total Chol: Total Cholesterol (g/L), Trigly: Triglycerides (g/L); Total Bili: Total Bilirubine (g/L). Glyc: Glycemia(g/L).

EA=aqueous extract of *E. citriodora* (cp1), FM=methanol fraction *E. citriodora* (cp3) and FA= ethyl acetate fraction (cp13).



**Figure 2. Effect of aqueous extract, ethyl acetate fraction and methanol fraction on NIT-1 cells viability.**

## DISCUSSION

After administration of extract and fractions at doses ranging of 300 and 2000 mg/kg bw to rats, no signs of toxicity were observed during the 14 days of observation. According to OECD Guideline 423 line for testing chemicals, these extract and fractions which have their lethal dose 50 (LD<sub>50</sub>) between 2000 and 5000 mg/kg bw. In principle, this process is not intended to determine a precise value of the LD<sub>50</sub>, but serves as a suggestion for the classification of the crude extract based on the prediction of the dose at which animals have to survive.<sup>[15]</sup> The extract and fractions can be classified in the hazard category 5. Acute toxicity is relatively low but may, under certain conditions, be dangerous for vulnerable populations. The vast majority of exogenous composites administered to the body are done gastrointestinal tract. These composites will therefore be subsequently distributed to the liver to be metabolized before being eliminated by the kidneys.<sup>[16]</sup> The heart which is also a vital organ undergoes the toxic effect of substances. That is why, although having no rats died following treatment, we focused on these three organs. The analysis of the relative organ weights and body weight showed that they were all statistically identical to those of control rats. The toxicity effect of extract and fractions were studied by measurement of some biochemical parameters of the kidney, heart and liver. Our results showed that the activities of enzymes such as the ALT and AST were not disturbed by extract and fractions. These enzymes are liver markers whose activities increase in liver toxicity.<sup>[17,18]</sup> Their concentration in serum informs about a hepatocyte injury.<sup>[19]</sup> ALT is an enzyme specific to the liver in dogs, rats, rabbits, cats and primates.<sup>[20]</sup> It can provide a quantitative assessment of the degree of damage to the liver.<sup>[21]</sup> Our results showed that extract and fractions caused no damage to the liver as shown in view of ALT and AST values.<sup>[22]</sup> Urea and creatinine are markers of renal function.<sup>[18]</sup> There was no variation of their rates after treatment. No significant change in the CK and LDH activities was observed. These enzymes are markers of cardiac function.<sup>[23]</sup> Our results showed that extract and fractions, at the used doses, did not provoked significantly change ( $P < 0.05$ ) of serum biochemical values of these parameters compared with rats in the control group, suggesting that the aqueous extract, ethyl acetate fraction and methanol fraction did not affect kidney heart, or the liver. Blood sugar, serum values of total cholesterol, triglycerides and total protein have not changed significantly ( $P < 0.05$ ), during treatment, suggesting that extract and fractions did not affect their control system. Assessment of the hematological indices showed that, the treatment of the extracts and fractions of *E. citriodora* did not cause any significant effect on RBC and indices relating to it (Hb, MCV, MCH and MCHC) throughout the experimental period. This result is an indication that there was no destruction of matured RBC's and no change in the rate of RBCs.<sup>[24]</sup> RBC and Hb are very important in the transfer respiratory gases; and the non-significant effect of extract and fractions on

the RBC and Hb indicates that there have been no changes in the oxygen-carrying capacity and amount of oxygen delivered to the tissues. In addition, the normal levels of MCV and MCHC indicates that the morphology and osmotic fragility of the red blood cells were not affected.<sup>[25]</sup> The determination of blood indices MCV, MCH and MCHC have a particular importance in anaemia diagnosis in most animals.<sup>[26]</sup> The non-significant effects on these indices relating to RBC suggest that there was no effect on the average size of RBC (microcytes) and also in the haemoglobin weight per RBC. This implies that extracts does not possess any potential of inducing anaemia throughout the 14 days period of study. However, cytotoxicity study on NIT-1 cells shows cytotoxic effect. NIT-1 cells are a mouse beta cell adenoma cell line derived non obese diabetic mouse.<sup>[27]</sup> The NIT-1 cells line used in this study is a normal line. In our study, the extract and fractions exert cytotoxic effect on NIT-1 cells. All the extract and fractions inhibited cell growth in a dose-dependent manner and the concentrations inducing cell growth inhibition by about 50% (IC<sub>50</sub>) were 100 µg/mL. Our results are similar to those of some authors with the methanolic extract of *Originum siriicum* on normal diploid human embryonic fibroblasts MRC-5 and human peripheral blood mononuclear cells (PBMCs) respectively.<sup>[28,29]</sup> These authors showed that the methanolic extract of *O siriicum* exerts a cytotoxic effect on these normal cells. These cytotoxic effects observed could be due to its cytotoxic compounds.

## CONCLUSIONS

The data of this study suggest that the oral administration of aqueous extract and ethyl acetate and methanol fractions of *E. citriodora* does not induce any toxic effects on the rats, no significant variation of body and organs weight and the biochemical parameters studied. However, they cause the death of NIT-1 cells at concentrations of 10, 100 and 1000 µg/mL. The extract and fractions inhibited cell growth in a dose-dependent manner and the concentrations inducing cell growth inhibition by about 50% (IC<sub>50</sub>) were 100 µg/mL. Others studies are needed to identify the chemical compound present in this extract and fractions.

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