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Larval and Acute Oral Toxicity of *Calotropis Procera* and *Ficus Umbellata*, Plants Traditionally Used to Treat Hemorrhoids in Benin

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Abstract In the modern days, investigation of the phytochemical compounds contained in the traditionally-acknowledged medicinal plants have established significant interest in drug research and developmental projects. In addition, phytochemical compounds in plants are compounds delivered by plants having pharmacological or toxicological properties in man and animals. *Calotropis procera* (Ait.) and *Ficus umbellata* (Vahl.) are two medicinal plants used in Benin. Their various preparations were used to treat a number of ailments and diseases, including hemorrhoids. Here, before checking their effects on hemorrhoids, toxicity studies were carried out on the decoction of *F. umbellata* leaves and those of *C. procera* with and without potash as used in the traditional treatment of hemorrhoids in Benin. Toxicity test results demonstrated tolerance of the leaves to *Artemia salina* larvae with all their LC_{50} > 0.10 mg/mL (0.147 for extract of *F. umbellata*, 0.224 for extract without potash of *C. procera* and 0.887 for extract with potash of *C. procera*) and albino rats of the wistar strain in acute oral toxicity at a single dose of 2000 mg/kg body weight. Studies of chronic and sub-chronic toxicity, as well as the anti-inflammatory activity of the extracts, are in prospect.

Keywords: *medicinal plants, extracts, phytochemistry, toxicity, cytotoxicity*

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1. Introduction

Different cultures use herbs, located in their geofigureic locations, in different ways to cure common diseases. The practice of herbal medicine history back to prehistoric times when people discovered through trial and error that certain plants had healing powers. Herbal remedies were coded and eventually compiled into books during the ancient civilizations of India, China, Egypt, Persia, and Greece. With the development of chemistry and the refinement of laboratory methods, herbal medicine gave way to the modern pharmaceutical industry, in which many drugs are manufactured in test tubes. Still, herbal ingredients are found in nearly half of all prescription and over-the-counter drugs used in mainstream medicine, including aspirin, digitalis, and atropine, and various anticancer drugs [1]. Additionally, phytochemical compounds in plants are compounds delivered by plants having pharmacological or toxicological properties in man and animals. These non-nutrient (e.g., vitamins and minerals) chemical compounds are often denoted as phytochemicals or plant secondary metabolites [2,3]. Infectious diseases represent a major health problem and considered as one of the main causes of morbidity and mortality in the world [4,5]. Hemorrhoids have always been a human ailment with a wide range of consequences. Like modern medicine, which offers preventive or sedative treatments, traditional medicine has its own system of values, units of measurement and ways of protecting the body, and can even offer effective treatment

for hemorrhoidal crises [6]. If combined by general practitioners and physicians with allopathic drugs for patients with chronic problems, it could be a positive step towards medical care. Because they are inexpensive, they are well within the budget of long-term care patients. The Beninese population has recourse to several plants and recipes to treat hemorrhoids, that two are the subject of our study. As these plants are within their reach, they are freely available. *Calotropis procera* and *Ficus umbellata* are two of the many plants whose leaves are traditionally used to treat hemorrhoids. These plants are widely used in traditional medicine and pharmacology, ... The objective of our study is to promote the consumption of these plants by the user population. For this, we are interested in studying their larval and acute toxicity in order to verify their harmlessness. The choice of plants is based on traditional usage and information provided by elders.

2. Materials and Methods

2.1. Plant Material

The plant material consisted of the leaves of Calotropis procera and Ficus umbellata. C. procera (Asclepiadaceae) is known in French as Arbre soie du Sénégal, Pomme de Sodome, in Fon as Kpɛntwe or Amon man, [7].

Ficus umbellata (Moraceae), is called in Fon Voma, Volima or Vo man Gblo gblo, in Bariba Ganu, Gangulu [7] en Gun Voun voun ti.

2.2. Animal Material

Brine shrimp larvae (*Artemia salina* Leach) were used for the larval toxicity study.

Female and male albino rats of Wistar strains, between 6 and 10 weeks old, and weighing between 149 and 217 g, were used for the acute oral toxicity study. The animals were stored in cages in a room maintained at 25°C with a relative humidity of 35-60%. They are kept under standard conditions with an alternating 12-hours light/12-hours dark cycle. They have free access to food and water.

2.3. Methods

2.3.1. Harvesting and Preparation of Extracts

Harvested *C. procera* and *F. umbellata* leaves were washed in tap water and left to drip before study operations. A portion of the fresh leaves was used for extraction by decoction at 10% using the traditional method. 308 g fresh *C. procera* leaves + 1.5 L distilled water ; 308 g fresh C. procera leaves + 10 g traditional potash + 1.5 L distilled water and 308 g fresh *F. umbellata* leaves + 1.5 L distilled water.

2.3.2. Phytochemical Screening

The study is based on differential staining and precipitation reactions using the Houghton and Raman method [8]. Ferric chloride ($FeCl_3$) is used to detect tannins, the Shinoda reaction (cyanidine reaction) to characterize flavonoids, the Dragendorff reaction to detect alkaloids, and the Guignard reaction (paper soaked in 5% picric acid) to detect cyanogenic derivatives.

2.3.3. Larval Toxicity

This preliminary non-clinical toxicity test was proposed by Michael et al. and later developed by Vanhaeeke *et al*. [9], Solis *et al*. [10] and reported by Assogba [11].

The test is based on the survival of shrimp larvae in seawater in the presence of the extract or product to be tested. During the test, the larvae are not fed, as they can survive up to 48 hours without food, feeding on their yolk sac [12]. The study is carried out three times, and the average number of dead larvae is considered.

2.3.3.1. Incubation of Brine Shrimp Eggs and Production of Larvae

The eggs of *Artemia salina* (France, Northwest Atlantic) are incubated at laboratory temperature in a 1000 mL Erlenmeyer flask containing seawater from the Atlantic Ocean. The whole set is placed on a reciprocating shaker for forty-eight hours with gentle agitation to allow eggs to hatch and give birth to shrimp larvae. Young incubated larvae are collected using a pipette, in the presence of a light source.

2.3.3.2. Concentration Range of Extracts and Larval Inoculation

Stock solutions of aqueous extracts are prepared with seawater at a concentration of 100 mg/L. In 10 test tubes, each containing a range of decreasing concentrations of each extract, i.e. 25.0 mg/mL in the first tube and 0.0488 mg/mL in the tenth tube. Sixteen (16) larvae were inoculated into each extract in each tube, and the whole set was incubated for 24 h at laboratory temperature.

2.3.3.3. Reading and Counting Dead Larvae

After 24 hours, the test tubes are examined. The number of surviving larvae in each tube is counted and the number of dead larvae recorded. Larvae are considered dead if they show no internal or external movement for some five seconds of observation. Larvae are not fed. To ensure that the death observed was attributed solely to the extracts and not to starvation, we compared the tubes containing extracts with the control tube containing seawater and larvae only.

2.3.3.4. Determination of Lethal Concentration 50

The lethal half-concentration (LC_{50}) is determined from the figure showing the number of dead larvae as a function of different extract concentrations, together with the best-fit line for half the number of deaths on this figure, and then on the concentration axis [13]. The degree of toxicity of the solution is known by referring to the correspondence table drawn up by Mousseux [14].

2.3.4. Acute Oral Toxicity

The acute oral toxicity study on adult, nulliparous, non-pregnant male and female Wistar laboratory rats is performed using the acute toxicity class method described in OECD Test Guideline 425 for chemical testing by the dose adjustment method [15]. Animals are randomly selected, individually marked for identification purposes and kept in their cages for at least five days before

administration of the substance, to allow them to acclimatize to laboratory conditions.

Table. Chemical compounds of extracts obtained by phytochemical screening

	Extract of the leaves of *Calotropis procera*				Extract of the leaves of *Ficus umbellata*		
Chemical groups	**Aqueous without potash**	**Aqueous with potash (6,66 g/L)**	**Powder, aqueous extract**	**EtOH extract**	**Aqueous of fresh leaves**	**Powder, aqueous extract**	**Powder, EtOH extract**
Alkaloids	-	+	++	-	+	-	-
Tannins	+	+	+	+	++	++	+
Cathechic tannins	+	+	+	+	++	+	+
Gallic tannins	+	-	+	+	+	+	+
Flavonoids	+	+	+	-	++	++	-
Leuco-anthocyanins	++	+	+	-	+++	++	+++
Saponosides	++	+	+	-	+++	+++	-
Cardenolides	+	+	+	-	-	-	-
Cyanogenic derivatives	-	-	-	-	-	-	-
Mucilages	+	++	++	-	+++	+++	-
Coumarines	+	+	-	+	-	-	-
Reducing compounds	++	++	+	++	+++	+	++
Heterosides	-	-	+	-	-	+	-

+++, ++, + : presence (strong, medium and low) ; - : absence ; EtOH : ethanol

The 20 rats were divided into four batches of five (5) rats each, including two (2) females and three (3) males. The rats were fasted for 18h before the start of the experiment, but were given water ad libitum. Doses were administered at a volume of 1 mL/100g body weight. Lot 1 received the aqueous extract of *Calotropis procera* leaves without potash, lot 2 received the aqueous extract of *C. procera* leaves with potash and lot 3 received the aqueous extract of *Ficus umbellata* leaves. The dose limit was 2000 mg/kg body weight, respectively. Lot 4 served as a control, and took distilled water at 10 mL/kg bw. The animals were monitored individually for the first four hours and then daily for 14 days to record any signs of toxicity.

2.3.4.1. Observed Parameters

Observations included skin, hair and eye changes, respiration, locomotion, behavior, tremor, convulsion, salivation, diarrhea, lethargy, sleep and coma.

2.3.4.2. Body Weight (Bw)

The individual body weight of each animal was taken before administration of the extracts and monitored until the end of the experiment. The rate of increase expressed as a percentage was calculated using the formula :

$$\%\ Increase = \frac{(mass\ at\ the\ end - mass\ at\ the\ start) \times 100}{mass\ at\ the\ end}$$

2.3.4.3. Body Temperature

Each animal's individual temperature is taken at the start, before the extracts are administered, for the first 4 hours, and then daily for 14 days.

2.3.4.4. Organ Observation

Organs were removed on days 0, 7 and 14 for macroscopic observation of the heart, liver, kidneys and stomach. Images were taken with an INFINIX camera at a size of 3264X2448 and processed with Adobe Photoshop CC 2014 (Ps) at a resolution of 72 pixels/inch.

2.3.5. Statistical Analysis

Statistical data were processed with FigurePad Prism® software for Windows version 5.00, March 7, 2007 and Microsoft Excel® spreadsheet software version 2016. Results were expressed as mean ± standard error of the mean. Differences between means were determined by ANOVA, followed by a Bonferroni multiple comparison test for groups using FigurePad Prism5 software. A significant difference is represented by a $p < 0.05$.

3. Results

3.1. Phytochemical Screening

Chemical analysis of aqueous extracts of *C. procera* and *F. umbellata* leaves without and with potash showed the presence of flavonoids, tannins (hydrolyzable tannins or pyrogallic tannins and condensed non-hydrolyzable tannins or catechic tannins), flavonoid heterosides and alkaloids according to our previous study [16] (Table).

3.2. Evaluation of Larval Toxicity

Larval toxicity results are illustrated in figures 1, 2 and 3. Logarithmic fitting of the curves showing the number of dead larvae as a function of extract concentration was used to determine the correlation coefficients (R^2) and the equations of the fitting curves to find the lethal LC_{50} concentrations.

C. procera with potash (LC_{50} = 0.887 mg/mL) is the least toxic extract, followed by *C. procera* without potash (0.224 mg/mL). *F. umbellata* is the most toxic of the three extracts (0.147 mg/mL).

3.3. Acute Oral Toxicity

3.3.1. Physical Parameters

Clinical observations made during the acute oral toxicity test revealed no major signs of toxicity. During the first 30 minutes after gavage, some rats showed signs of fatigue. However, these signs disappeared before the end of the first hour post-gavage. We observed only a few

isolated cases (1 case/15rats) of diarrhoea one hour after gavage for the aqueous extract of *Calotropis procera*, and 3 hours later for the aqueous extract of *Ficus umbellata*. No signs of irritation, corrosion, sleep, salivation, convulsion, tremor, hetargy, coma or rat death were recorded during the experiment.

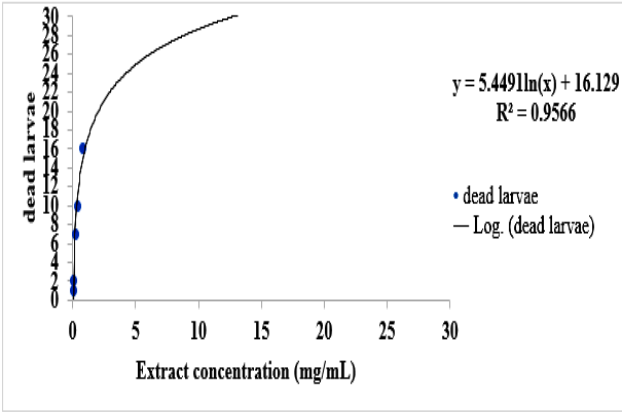

figure 1. Evaluation of larval toxicity of without potash aqueous extract of *C. procera*

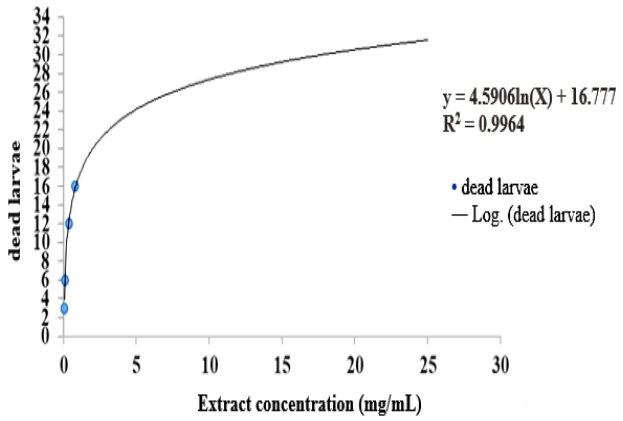

Figure 2. Evaluation of larval toxicity of *C. procera* aqueous extract with potash

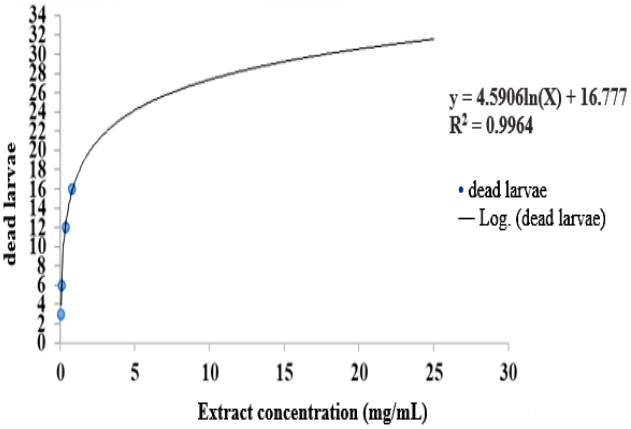

Figure 3. Evaluation of larval toxicity of *F. umbellata* aqueous extract

3.3.2. Body Weight

The rats, aged 6-8 weeks and still growing, continued to grow during the experiment. The average body weight of rats treated with the potassium-free aqueous extract of *C. procera* ranged from 201 to 210.33 g, with an increase of 4.437% (Figure 4).

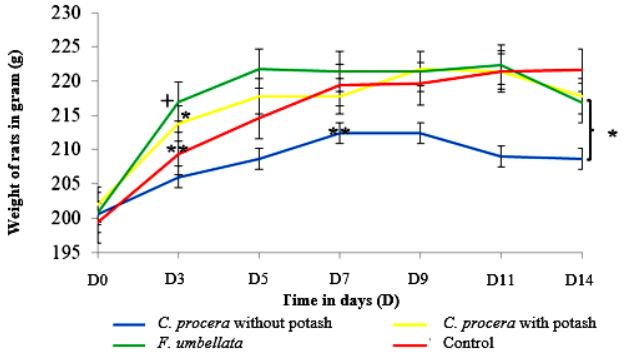

Figure 4. Extract- and time-dependent variation in rat body weight during acute oral toxicity (N = 5)

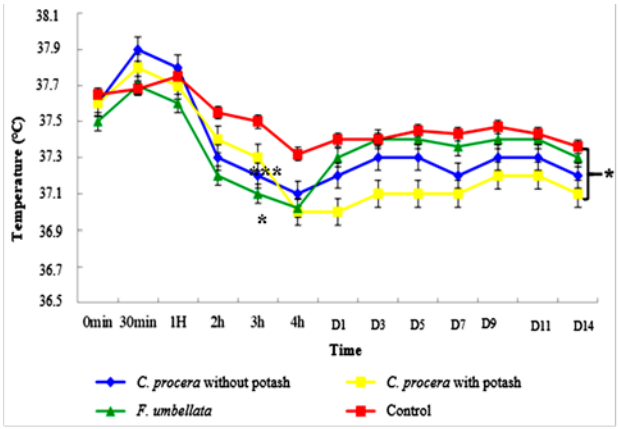

Figure 5. Extract- and time-dependent variation in rat body temperature during acute oral toxicity (N = 5)

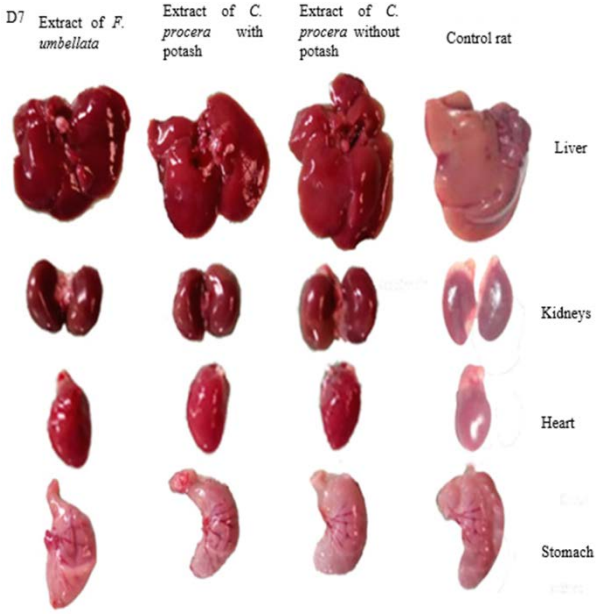

Figure 6. Liver, kidney, heart and stomach of treated rats on day 7, as a function of extract.

In rats treated with aqueous extract of *C. procera* with potash, the average weight varied from 200 to 216.66 g, with an increase of 7.692% (Figure 4). For rats treated with *F. umbellata* aqueous extract, mean body weight ranged from 197.33 to 210 g, with an increase of 6.031%

(Figure 4). The difference in weight over the course of the experiment between the groups of treated rats was not significant (P = 0.33).

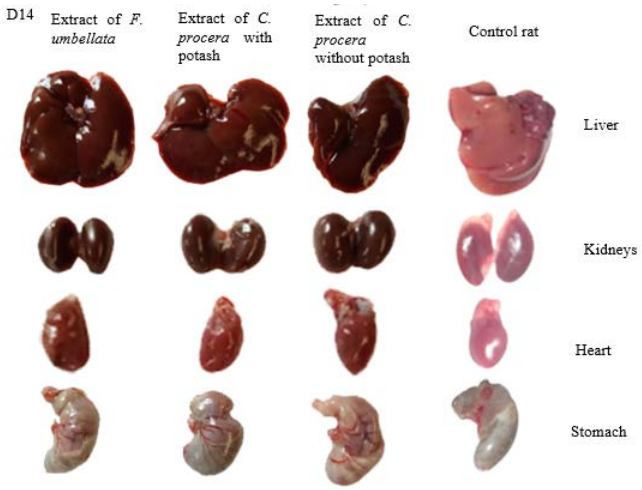

Figure 7. Liver, kidney, heart and stomach of rats treated on day D14 according to extract

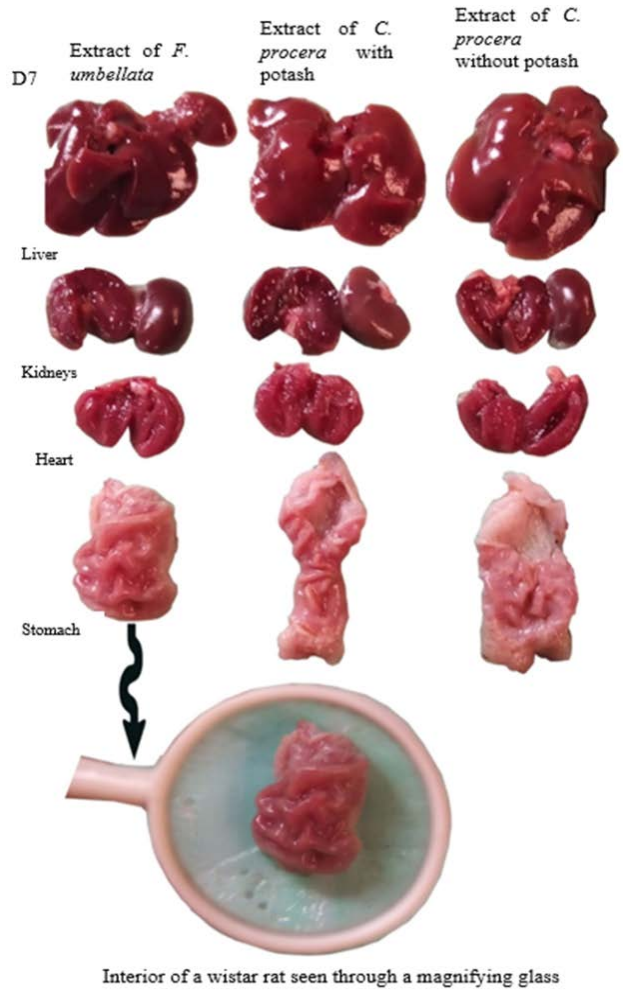

Figure 8. Longitudinal cross-section of organs: liver, kidney, heart and stomach of rats treated on day 7, according to extract

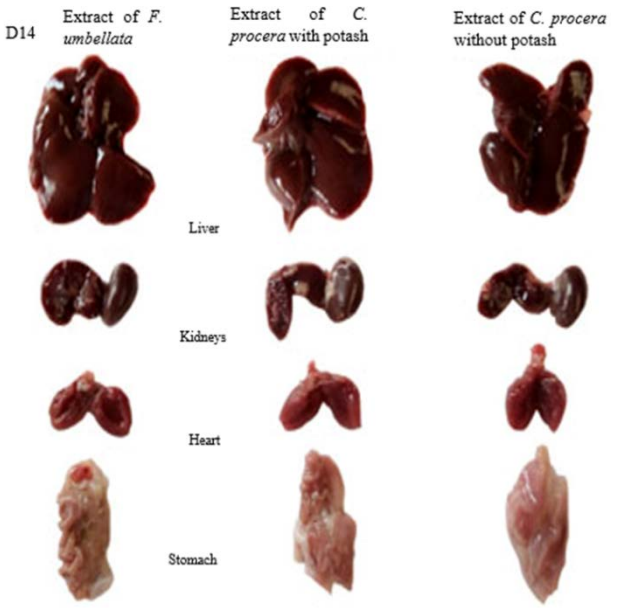

Figure 9. Longitudinal cross-section of liver, kidney, heart and stomach organs of treated rats on day 14 as a function of extract.

3.3.3. Body Temperature

Rats' body temperature did not vary significantly during the experiment (Figure 5).

3.3.4. Macroscopic Observation of Selected Organs

The kidneys, livers, hearts and stomachs of the rats were analyzed macroscopically following sampling on days 7 and 14. These organs showed no changes under magnifying glass. There were no external or internal abnormalities (Figure 6 and 7).

Longitudinal sections of the liver, kidneys, heart and stomach of extract-fed rats necropsied on days D7 and D14 showed no internal abnormalities (Figure 8 and 9).

4. Discuss

We chose to perform extractions based on the traditional decoction principle to carry out our studies, while trying out maceration. The aqueous extracts without potash obtained from the fresh leaves of *Calotropis procera* and *Ficus umbellata* do not contain cyanogenic derivatives, chemical groups that are potentially harmful to the organism. The two aqueous extracts of fresh *C. procera* leaves do not contain quinone and cyanogen derivatives, steroids and triterpenoids, free anthracenes and heterosides. These phytochemical results confirm the probable non-toxicity of our extracts. While the extract with potash showed alkaloids, this presence in fresh leaf extracts of *C. procera* would be harmless according to our results on acute oral toxicity.

In order to prove the safety of our three aqueous extracts, we carried out a toxicity study on shrimp larvae and an acute oral toxicity study on Wistar rats.

All three extracts showed lethality on larvae, indicating that our extracts are biologically active. We obtained LC_{50} values in mg/mL including 0.224 for the aqueous extract without potash of *C. procera*, 0.887 for the aqueous extract containing potash of *C. procera*, and 0.147 for the

aqueous extract of *F. umbellata*. These values compared with those of the toxicity correspondence table established by Mousseux revealed that none of the three extracts is toxic to shrimp larvae, since the LC_{50} > 0.10 mg/mL [14]. The extract with traditional potash from *C. procera* appears less toxic to shrimp larvae than the extract without potash, since its LC_{50} is higher.

The acute oral toxicity test carried out at a single dose of 2000 mg/kg bw on aqueous leaf extracts of *C. procera* without and with potash, and of *F. umbellata*, produced no mortality. At this dose, the extracts did not induce any signs of toxicity in treated rats. The body mass of the rats normally increased, as they were still growing. The extracts would therefore not be slimming at this single dose. There was no significant difference between the relative weights of treated and control rats.

The rats' body temperature varied normally. Compared with controls, rats reacted normally. The three aqueous extracts did not create a large thermal variation in the rats. Rats maintained their body temperature almost constant throughout the 14-day experiment, except those rats treated with the potash extract showed a slight drop in body temperature compared with other rats. The extract did not induce hypothermia, as body temperature remained around 37°C and above 36°C.

Naked eye observation of the organs on days 7 and 14 showed no abnormalities. Our plant extracts had no effect on the morphological appearance of the organs of treated rats compared with control rats given a single oral dose of 2000 mg/kg bw. These results for the *C. procera* extract are in line with those obtained by Traore [17], in his studies on the antioxidant activities and toxicity of four plants including *C. procera*.

In its study, Traore showed that the alcoholic extract of the aerial parts of *C. procera* did not cause death or symptoms of intoxication up to a dose of 3g/kg orally. He also reported that the aqueous macerate of *Calotropis procera* powder assessed by determination of the LD_{50} in male mice is 973 mg/kg body weight, classifying the drug as low toxicity [17]. Dolo showed that aqueous extracts of *C. procera* leaf twigs are lethal to mice at a dose of 0.5g [18]. The difference between results of our work and their results could be explained by the difference in route of administration, that of the intra parenteral IP route used. The oral route used in our study is that used for treatment in traditional medicine.

The rats' organs showed no internal changes after cutting in the sagittal plane. Our three extracts at a dose of 2000 mg/kg body weight had no apparent toxic effect on the liver, kidneys, heart or stomach.

Finally, no rats died during the study, and only one developed diarrhoea during the first hours of the experiment. The aqueous extracts of *C. procera* and *F. umbellata*, in accordance with the toxicity scale of the Globally Harmonized System of Classification (GHS) of OECD 425 guidelines [15], enable us to classify our three extracts as category 5 plant drugs and to say that they are bio-tolerant. This result confirms our phytochemical results [16] and our hypothesis of the oral non-toxicity of our extracts for the treatment of hemorrhoidal attacks.

5. Conclusion

The toxicity test carried out on *Artemia salina* shrimp larvae and wistar rats showed that the aqueous extracts of *Calotropis procera* leaves without/with potash and *Ficus umbellata* are lethal, with low toxicity on *A. salina* shrimp larvae, and are classified in GHS category 5 at a dose of 2000 mg/kg body weight. They are therefore non-toxic substances that had no effect on rodent organs under macroscopic observation.

This work has enabled us to begin justifying the traditional use of *C. procera* and *F. umbellata* leaves in the treatment of hemorrhoidal attacks. With a view to enhancing the value of the recipes derived from these two plants, further studies are currently underway, with the aim of :

- in vivo subacute, chronic and subchronic toxicity tests on the three extracts;
- ipreventive and curative tests on the anti-inflammatory activities of the three extracts (edema, pain and hyperthermia);
- ithe efficacy of the extracts on induced hemorrhoids;
- isolate, characterize and test the active ingredient(s) of *F. umbellata* and *C. procera* extracts for effective treatment of hemorrhoidal diseases.

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