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Study of the zootoxicity of the leaf extracts from *Crotalaria bernieri baill*. (Fabaceae), a Malagasy medicinal plant

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Abstract

The aim of this study was to assess the toxicity of *Crotalaria bernieri*, a medicinal plant native to Madagascar, on animals. From leaf powder previously treated with hexane, a methanolic extract (LME) was obtained in 20.59% yield. LME contained alkaloids, flavonoids, amino acids, tannins and polyphenols. Total alkaloids (TAE) were extracted from LME with a yield of 30.25%. The toxicity of the extracts was tested on warm-blooded animals (*Mus musculus*, *Gallus gallus domesticus*) and cold-blooded animals (*Ptychadena mascareniensis, Cyprinus carpio* and *Xenopsylla cheopis*). LME was toxic to mice by the oral, subcutaneous and intraperitoneal (i.p.) routes, but the i.p. (LD_{50} = 43.57 mg/kg) route was far more toxic than the other two. Five min LME's injection by i.p. route, severe lesions were detected in the lungs, liver, and brain, characterized by vasodilatation and the presence of altered neutrophil polymorphonuclears. LME was also toxic to *Ptychadena mascareniensis* (LC_{50} = 52.59 µg/mL) and *Cyprinus carpio* (LC_{50} = 16.96 µg/mL). However, it was not toxic to *Gallus gallus domesticus* by oral and i.p. routes at a dose corresponding to the LD_{100} in mice. It was also not toxic to *Xenopsylla cheops*. TAE was far more toxic than LME, suggesting that the toxic principles could be alkaloidal in nature.

Keywords: *Crotalaria bernieri*; Fabaceae; Alkaloids; Zootoxicity; Warm-blooded animals; Cold-blooded animals

1. Introduction

The *Crotalaria* genus of the Fabaceae family comprises around 700 species, widely distributed in tropical and subtropical regions throughout the world [1, 2, 3, 4].

Different species are widely used as medicinal plants for the treatment of various diseases. They exhibit a broad range of biological activities, including antimicrobial [5], anti-inflammatory [6], antioxidant [7], anticancer [8, 9], cytotoxic [10], and anti-HIV [11]. These properties make them of great interest for the development of new treatments in both traditional and modern medicine.

However, despite their historical use in folk medicine, several *Crotalaria* species are known to contain toxic substances, mainly pyrrolizidine alkaloids and non-protein amino acids, which are associated with severe adverse effects [12, 13]. Poisoning is caused by accidental ingestion or medicinal use of the leaves and seeds where these toxins accumulate. For example, in Guadeloupe, severe poisonings were reported in children due to the consumption of "sonnettes tea" prepared from *C. retusa* [14]. In Jamaica, cases of severe hepatitis were observed following the consumption of herbal

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teas made from *C. fulva* [15]. The genus *Crotalaria* is also responsible for various animal poisonings, such as "crotalariosis" in livestock (*C. burkeana*) [16, 1] and "Kimberley horse disease" in horses (*C. crispata* and *C. retusa*) in Australia [17]. Several other species, such as *C. spectabilis*, *C. retusa*, and *C. aridicola*, are also toxic to livestock, poultry, and birds [18]. They can cause pulmonary and intrahepatic hemorrhages as well as liver necrosis in rats [19], mice [20], and pigs [21].

In Madagascar, 53 species of *Crotalaria* are present, of which 34 are endemic, 12 are introduced, and 7 are native [22]. Among of these, *Crotalaria bernieri* is a medicinal plant that is widely used in traditional medicine for its therapeutic properties, particularly for the treatment of diarrhea and stomach disorders using its leaves. This species, an annual herb, is found in open vegetation, grassy areas and roadsides throughout most of Madagascar. It flowers from July to October and from December to March [1, 23].

Our previous work has shown that the methanol extract of its leaves has significant antimicrobial activity against pathogenic bacteria and molds [5]. From this extract we characterised and isolated two guanidine alkaloids: Sphaerophysin and the novel Cyclocrotalarin, as well as a flavonoid: 2''-*O*-*α*-rhamnoside vitexin [24]. These results encouraged the deepening of the study.

The main aim of this work was therefore to determine the toxicological effects of methanol extracts from *C. bernieri* leaves on different animals in order to better assess the risks associated with its use and to highlight the need for caution in its application as a natural remedy.

2. Experimental

2.1. Plant Materials

C. bernieri (Figure 1), known in Madagascar by the vernacular name “Ranomanja”, was harvested in Ibity, in the Vakinankaratra region, located at 200 km South of Antananarivo (Madagascar). The plant was collected in April and identified by R.M. Polhill, botanist at the Royal Botanic Garden of Kew (England). Voucher specimens (No. Herizo R.010) were deposited at the herbarium of Plant Biology and Ecology Department of the Faculty of Sciences of the University of Antananarivo.

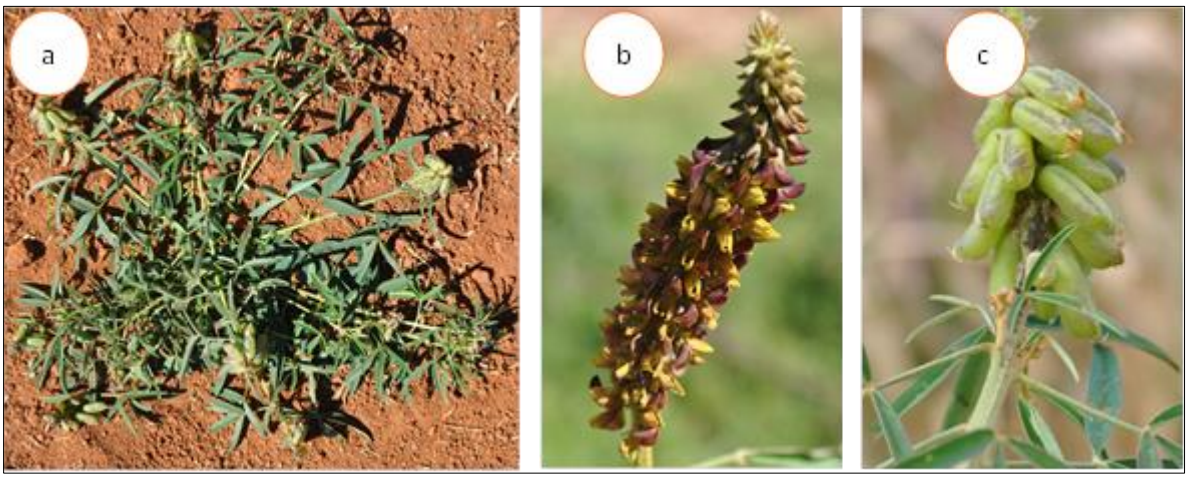


Figure 1 *Crotalaria bernieri* (a) the whole plant; (b) flowers; (c) fruits (Source: the authors)

After drying in the shade, the leaves were reduced to a fine powder, which was sieved to obtain the starting material. This was then stored at room temperature in a sealed container.

2.2. Animals

OF-1 strain albino mice (*Mus musculus*), weighing 25 ± 2 g, came from the Pasteur Institute of Madagascar (IPM) breeding farm.

One day old chicks (*Gallus gallus domesticus*), Hubbard classic strain, were provided by poultry farmer.

Carp alvins (*Cyprinus carpio*), Royal strain, 2-4 cm size, were provided by an approved fish farmer.

Legless frog tadpoles (*Ptychadena mascareniensis*) were harvested from the ponds in the vicinity of the Antananarivo University site. Fishes and tadpoles were allowed to acclimatize to the aquarium conditions for three days after their arrival in laboratory.

The plague vector fleas (*Xenopsylla cheopis*), collected from captured rats, came from the IPM Entomology Unit insectarium. They are reared at a fixed temperature of 25°C and a humidity level of 85%, optimum conditions that are strictly favourable to their growth and survival.

2.3. Extracts Preparations

2.3.1. Leaf Methanolic Extract (LME) Preparation

The fine leaf fine powder was extracted with hexane (3 x 1000 mL) until complete discoloration. The colorless powder was extracted with 3 x 1000 mL of methanol for 24 h under stirring. The suspension obtained was filtered. The filtrate was then evaporated under reduced pressure to yield dry extract powder. The aqueous solution of this extract was named LME.

2.3.2. Extraction of Total Alkaloids

To extract the total alkaloids, 2 g of LME were extracted with a mixture of methanol (MeOH 15 mL) and 5 mL of aqueous hydrochloric acid (10%) under stirring at room temperature for 24 h. After filtration using a Buchner funnel, the filtrate was evaporated to dryness under reduced pressure at 40°C. The residue was dissolved in distilled water and extracted with dichloromethane (CH_2Cl_2 3 x 10 mL). The organic and aqueous phases were separately alkalinized with NH_4OH (20%) up to pH 9. The CH_2Cl_2 part was then evaporated to dryness, while the aqueous solution was extracted with CH_2Cl_2 (3 x 10 mL) to yield water and organic solutions. The water solution was mixed with n-butanol (v/v) in a separating funnel. The operation was repeated three times. These phases were collected separately and evaporated to dryness. The residue from the butanol phase, solubilised in distilled water, constituted the total alkaloid extract (TAE).

2.4. Phytochemical Screening

The reactions of chemical group detection were those developed by Fong *et al.* [25] and Marini-Bettolo *et al.* [26].

2.5. Methods Used to Study Effects in Animals

2.5.1. Acute Toxicity Test

Toxic effects in mice and chicks were evaluated by intraperitoneal (i.p.) and oral routes. By i.p. route, LME was injected at a volume of 0.3 mL per 25 g of body weight while by oral route, it was given by gavage by means of a curved distal end needle at the rate of 0.25 mL per 25 g of body weight.

The toxicity of LME was also assessed in mice by the subcutaneous route using a dose of 0.25 mL per 25 g of body weight.

2.5.2. LD_{50} (24 h) Assessment in Mice

The dose that killed 50% of mice in 24 h or LD_{50} of LME was determined by calculation and graphical methods [27]. Seven different doses were injected by i.p. route on seven groups of five male mice. Another group receiving physiological serum served as control. Results were interpreted using the Hodge and Sterner scale [28].

2.5.3. LC_{50} Assessment on Cold-Blooded Animals

Experiments on cold-blooded aquatic animals involved testing their resistance to environmental intoxication. The animals were placed in batches of 5 in crystallizers containing spring water. The extract was added so that the final concentrations followed a geometric progression of the determined reason, from LC_0 (maximum concentration at which all animals survived) to LC_{100} (minimum concentration killing 100% of animals). An untreated batch served as a control. The tests were carried out in triplicate.

2.5.4. Tests on Plague Vector Fleas

The method used to study the effects of LME in fleas (*Xenopsylla cheopis*) was that of [29].

2.5.5. Histopathological Examination

The aim was to examine the organ damage caused by acute and sub-chronic intoxication. Three groups of 10 mice were used. Animals of group 1 were injected with LD_{50} (43.57 mg/kg), then sacrificed 3, 5, 10, 15 min and 24 h after treatment. Animals of group 2 were treated orally with a single daily dose of 28.56 mg/kg (LD_0 dose) for 10 and 30 days (Sub-chronic exposure to LME). Animals of group 3 served as control. At the end of experiences, the animals were sacrificed.

Brain, lungs, heart, stomach, liver, kidneys, and intestines were harvested afterwards and preserved in formol 10%. The preparation of the organ sections for histopathological examinations was carried out as described by [30].

2.5.6. Evaluation of the Effects of LME on Renal and Hepatic Functions

The effect of LME on hepatic and renal function was assessed utilizing the method described in previous paper [31]. It was evaluated in healthy mice after daily administration of a sub-acute dose (28.56 mg/kg) during 30 days.

At the end of experiment, 1 mL of the blood was collected and immediately centrifuged at 4 °C for 10 min at 13,000 rpm. Mean plasma levels of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), creatinine and urea from the extract treated animals were statistically compared with those of control animals.

2.6. Statistical Analysis

The determination of the LC_{50} (24 h) was performed using the method of statistical analysis ANOVA with Graphpad Prism 7 software. Those of the effects on renal and hepatic functions were expressed as mean ± standard error mean (SEM). Significant differences were determined using a Student's t-test and the differences were considered significant if $p < 0.05$ [32].

3. Results

3.1. LME and TAE Yields

Extraction of the dried leaves of *C. bernieri* gave a brown colored extract with a yield of 20.59%. Meanwhile, the extraction of alkaloids from 2 g of LME gave 605 mg of TAE, corresponding to a yield of 30.25%.

3.2. Qualitative phytochemical analysis

Phytochemical analysis was carried out on the leaf powder and LME. Results revealed the presence of alkaloids, flavonoids, tannins, polyphenols and amino acids (Table 1).

Table 1 Phytochemical screening of leaf preparations of *C. bernieri*

Chemical groups	Tests	Leaf powder	LME
Alkaloids	Mayer	+	+
	Wagner	+	+
	Dragendorff	+	+
	Confirmatory test (solubility in ethanol)	+	+
Saponins	Foam test	-	-
	Confirmatory test (hemolytic test)	-	-
Flavonoids	Willstätter	+	+
Amino acids	Ninhydrine 0,1%	+	+
Leucoanthocyanins	Bate-Smith	-	-
Tannins and Polyphenols	Gelatin 1%	+	+
	Gelatin-salt 10%	+	+
	$FeCl_3$	+	+

Quinones	Borntrager	-	-
Steroids	Liebermann-Burchard	-	-
Iridoids	Hot HCl	-	-
Triterpenes	Liebermann-Burchard	-	-
Unsaturated sterols	Salkowsky	-	-

+: positive test; -: negative test

3.3. Effects of LME on animals

3.3.1. In Mice

Developed Symptoms and LD_{50} value

The influence of the administration route on LME effect was shown in Table 2.

Table 2 Effect of LME on mice according to the administration route

DOSE (mg/kg)	Mortality rate (%)		
	Intraperitoneal	Subcutaneous	Oral
81	100	0	0
1200	100	0	0
3300	100	0	0
3600	100	100	0
6000	100	100	100

At the lethal dose of 81 mg/kg, an agitated state occurred immediately after injection, followed by abdominal contortion and an increase in respiratory rate. Motor activity gradually decreased until the animal was completely immobilised. Death of the mice, preceded by a decrease in respiratory rate and severe clonic convulsions, occurred 10 min after administration of the extract.

At a sublethal dose of 28.56 mg/kg, symptoms were similar to those induced by the lethal dose, without being followed by death and with a longer time to onset. Progressive remission was observed 2 h after administration of the extract. No mortality was observed 24 h after the test.

Subcutaneous injection of LME at the doses of 81 mg/kg (LD_{100} i.p.) and 28.56 mg/kg (LD_0 i.p.) produced practically the same symptoms of intoxication. However, the difference lay in the time taken for these symptoms to appear. No mortality was observed 24 h after the test.

By the oral route, administration of i.p. lethal dose (81 mg/kg) did not cause mortality. Immediately after administration of the extract, there was abdominal contortion, followed by exophthalmos, muzzle pruritus and hiccups. The mice returned to normal after 1 h. In addition, after ingestion of LME at a dose 70 times greater than the LD_{100} by i.p. route, mortality was observed after 1 h, meaning that the oral LD_{100} was 6000 mg/kg. LD_{50} (24 h) of LME was assessed at 43.57 mg/kg body weight by i.p. route.

Histopathological Lesions

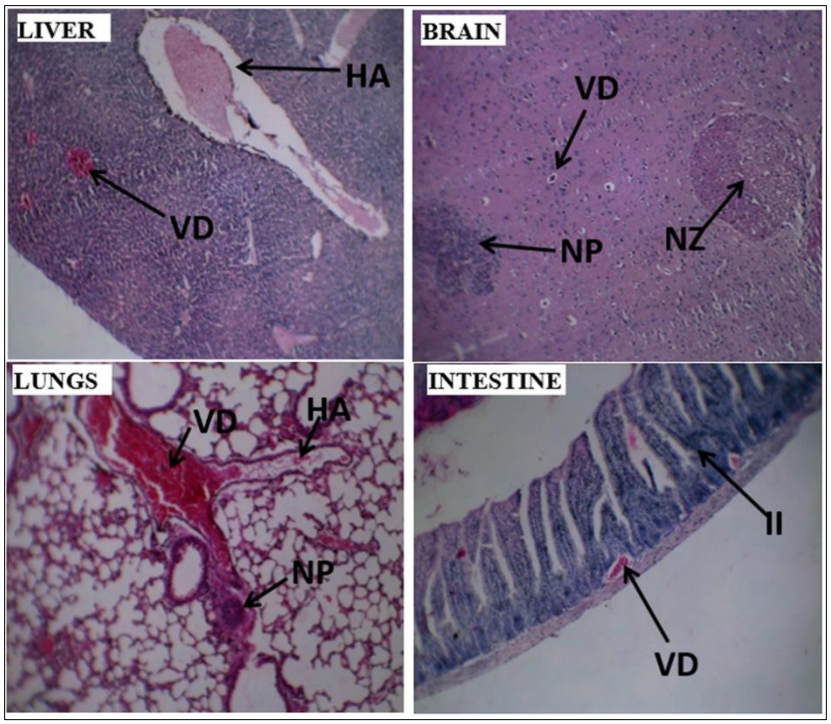
Macroscopic analysis of the organs showed the presence of whitish spots on the liver of the mice intoxicated via the i.p. route. The other organs, namely the brain, heart, lungs, liver, kidneys, stomach, and intestines, showed no significant changes in appearance, size, color, or texture compared to the control group.

Organ sections examination showed that the lesions varied by organ and route of administration. In the i.p. route, severe lesions were detected 5 min after LME injection in lungs, liver, and brain, characterized by vasodilation, the presence of altered neutrophil polymorphonuclears, necrotic (or necrosis) and hemorrhagic areas, as well as edematous infiltration

foci. The presence of some neutrophil polymorphonuclears and slight dilation of blood vessels were also observed in heart, kidneys, stomach, and intestines after 15 min of intoxication.

By oral route, lungs, liver, brain, and intestines were the most affected organs. The lesions were characterized by vasodilation, the presence of altered neutrophil polymorphonuclears, edematous foci, and large necrotic areas. Additionally, areas of destruction were noted in lungs and intestines after 30 days of exposure.

Some photos displaying the most remarkable lesions caused by i.p. administration of LME at a dose of 43.57 mg/kg body weight and by oral route of LME at a dose of 28.56 mg/kg body weight are shown in Figure 2.



HA: hemorrhagic area; VD: vasodilatation; NP: neutrophil polymorphonuclears; NZ: necrotic zone; II: inflammatory infiltrate

Figure 2 Main histological lesions in liver, brain, lungs and intestines caused by i.p and oral administration of LME

Effects on Renal and Hepatic Functions

No significant changes of serum concentration of ASAT, ALAT, creatinine and urea were noted in mice after 30 days of treatment at a dose of 28.56 mg/kg (Table 3).

Table 3 Effect of subchronic dose of LME on renal and hepatic functions in mice after 30 days of continuous treatment by oral route

Mice	Hepatic function		Renal function	
	ALAT (UI/L)	ASAT (UI/L)	Creatinine (mg/L)	Urea (mg/L)
Control	369.5 ± 223.5	470 ± 129.9	6.3 ± 0.4	5.6 ± 0.2
LME effect	139.5 ± 39.8	311.2 ± 49.6	5.03 ± 0.6	3.27 ± 0.7

3.3.2. Effects on Chicks and Cold-Blooded Animals

As shown in Table 4, LME had no effect on chicks by i.p. and oral routes at 81 mg/kg, a dose corresponding to LD_{100} on mice.

Table 4 Toxicity of LME on different animal species

Animal class	Species	LD_{50} or LC_{50}	Observations
Bird	*Gallus gallus domesticus*	-	A dose corresponding to LD100 on mice had no effect by intraperitoneal and oral routes
Amphibian	*Ptychadena mascareniensis*	52.59 µg/mL	
Fish	*Cyprinus carpio*	16.96 µg/mL	
Insect	*Xenopsylla cheopis*	-	No effect at the concentrations of 1 mg/mL, 2 mg/mL and 5 mg/mL

At the concentrations of 1 mg/mL, 2 mg/mL and 5 mg/mL, no mortality of the plague vector fleas (*Xenopsylla cheopis*) was observed after 24 h of exposure. In other words, LME was not toxic at the doses tested.

LME had toxic effects on *Ptychadena mascareniensis* tadpoles and *Cyprinus carpio* alvins with LC_{50} values of 52.59 µg/mL and 16.96 µg/mL, respectively.

3.4. Toxic Effects of TAE

The effects of TAE on mice were evaluated for 24 h at the doses corresponding to LD_0 (28.56 mg/kg), LD_{50} (43.57 mg/kg) and LD_{100} (81 mg/kg) by i.p. route, and its activities were compared with those of LME. The experimental results are shown in Table 5. At a dose of 28.56 mg/kg, all mice injected with TAE died. Therefore, TAE was more toxic than LME, as LME did not cause any mortality at this dose.

Table 5 Comparison of the effects of TAE and LME on mice via the i.p. route

DOSE (mg/kg)	Mortality rate (%)	
	LME	TAE
28.56	0	100
43.57	50	100
81	100	100

4. Discussion

Phytochemical screening showed the presence of various secondary metabolites, but alkaloids and flavonoids were the most important compounds. These compounds are relatively abundant in the genus *Crotalaria*, especially *C. emarginella* [33], *C. juncea* [34], *C. pallida* [11], *C. grahamiana* [35] and *C. lachnophora* [36]. Saponins, leucoanthocyanins, quinones, iridoids and triterpenes were absent.

The effects of LME on mice showed that toxicity depended on the route of administration. Injection by the i.p. route was much more effective than the oral and subcutaneous routes. For example, to obtain the same effect by the i.p. route, a dose 70 times higher (6000 mg/kg) was required by the oral route. This could be explained by the fact that the oral route requires the compounds to pass through the digestive tract before being absorbed and reaching the target organs or tissues. During this passage, the toxic principles of LME would probably be degraded by digestive enzymes or bacterial flora, thus reducing the number of molecules absorbed from the gastrointestinal tract before reaching the various target organs. This low oral toxicity of *C. bernieri* justified, at least in part, its use as food for zebus, which was often mentioned in ethnobotanical surveys. For this reason, no cases of poisoning have yet been reported, as the plant must be ingested in very large quantities to be toxic. However, any chronic toxicity should be investigated. Similarly, LME administered subcutaneously caused symptoms only at doses that were lethal by the i.p. route. This observation may be explained by the hypothesis that absorption is difficult or absent.

The symptoms developed by LME on mice were dominated by disorders of the nervous, respiratory and cardiovascular systems, such as clonic convulsions and increased respiratory rate. These symptoms were similar to those observed

with extracts from the organs of other *Crotalaria* species: *C. spectabilis* [37], *C. pallida* [38] or *C. juncea* [39]. This diversity of symptoms could be due to the involvement of more than one toxic principle, which is normal for a crude extract such as LME.

According to Hodge and Sterner scale [28] (1 mg/kg ≤ DL50 ≤ 50 mg/kg), LME can be classified as "highly toxic". It is difficult to compare the toxicity of *C. bernieri* with that of its foreign congeners because the conditions for assessing this effect are not the same: the animals, organ extracts and routes of administration used are different. To illustrate, the hydroethanol extract of *C. madurensis* had an LD_{50} = 4000 mg/kg orally in mice [40]. In contrast, the LD_{50} for *C. lachnosema* LME is 1300 mg/kg [41] and 154 mg/kg for *C. assamica* LME [42]. In comparison to the toxicity of other malagasy plants we studied, LME was less toxic than the aqueous extract of *Albizia greveana* seeds (LD_{50} = 1.3 - 2.30) [31] and more toxic than *Rhodocodon madagascariensis* bulbs (LD_{50} = 170 mg/kg) [43]. However, the toxicity of LME was comparable to that of *Pittosporum ochrosiaefolium* (LD_{50} between 46.24 and 47.15 mg/kg) [44].

Histopathological studies of the organs treated with different doses of LME revealed many lesions that varied depending on the organ and route of administration. These were thought to be due to the activity of toxic molecules, particularly the alkaloids, which were present in significant quantities in the extract. The presence of neutrophils, especially in lungs and liver, indicated an immune defense mechanism of the tissues against chemical aggression. This aggression could be caused mainly by acidic or basic compounds such as alkaloids [45].

The high toxicity of TAE compared to LME suggested that alkaloids were the main cause of LME toxicity.

The effects of LME on renal and hepatic function at a dose of 28.56 mg/kg/day for 30 days did not induce significant changes in plasma levels of ALAT, ASAT, creatinine and urea in treated mice compared to controls. This result may indicate that no functional or structural changes were observed in the kidneys or liver. It may be that at the dose used, the damage was not sufficient to cause a functional disturbance in these organs.

In cold-blooded animals, LME was toxic to *Ptychadena mascareniensis* tadpoles (LC_{50} = 52.59 µg/mL) and *Cyprinus carpio* alvins (LC_{50} = 16.96 µg/mL). Compared to methanolic extracts of *Albizia* (Fabaceae) seeds from Madagascar, whose toxicity was assessed under the same conditions [31], LME was less toxic on *Cyprinus carpio* alvins than seed methanolic extract (SME) from *Albizia androyensis* (LC_{50} = 3.86 µg/mL) and *Albizia masikororum* (LC_{50} = 4.15 µg/mL). It was also less toxic to *Ptychadena mascareniensis* tadpoles than LME from *Albizia tulearensis* (LC_{50} = 15.04 µg/mL) and *Albizia mahalao* (LC_{50} = 21.87 µg/mL), but more toxic than LME from *Albizia aurisparsa* (LC_{50} = 60 µg/mL). The high toxic effects of LME to cold-blooded animals were probably due to saponins. The toxicity of these compounds to cold-blooded animals is well-known [46, 47]. However, phytochemical screening of the extracts (leaf powder and LME) did not reveal the presence of these molecules. This suggested that the toxic activity on cold-blooded animals was related to molecules other than saponins like polyphenols. It should be noted that several of *Crotalaria* species of Madagascar (*C. craspedocarpa*, *C. coursii*, *C. cytisoides*, *C. incana*, *C. mahafalensis*, *C. xanthoclada*) are known to be toxic to cold-blooded animals, which explains their widespread use as fish poison [48].

5. Conclusion

The results obtained in this study provided the first scientific data on the toxicity of *C. bernieri* to various organisms and showed its possible use in the control of harmful organisms. However, given its high toxicity to fish and tadpoles, and possibly other cold-blooded animals, its use in aquatic environments is not recommended. This data should also draw attention to the precautions to be taken in medicinal uses of this plant.

These new data have contributed to increasing knowledge of the Madagascar plant biodiversity, especially poisonous plants and their biological valuation on the other hand.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interests.

Statement of ethical approval

All the tests on animals were approved and in line with the standard established by Ethics Committee of the IPM.

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