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Comparative Oral, Dermal, and Intraperitoneal Acute Toxicity of *Gaertnera phanerophlebia* (RUBIACEAE) Twig Leaf Extract in Mice

Rakotoarisoa Mbolatiana Abigaila[1]; Nomenjanahary Lalaina[2]; Razafimanantsoa Herimamonjy[3]; Rakotonandrasana Stephan Richard[4]; Jeannoda Victor Louis[5]

[1]Department of Galenic Pharmacy, National Center for Pharmaceutical Research Applications, Antananarivo, Madagascar
[2]Department of Anatomical and Cytological Pathology, CHU-JRA Antananarivo, Madagascar
[3]Central Pharmacy and Laboratory Service, Central Directorate of Military Health Services, Antananarivo, Madagascar
[4]Department of Ethnobotany and Botany, National Center for Pharmaceutical Research Applications, Antananarivo, Madagascar
[5]Laboratory of Applied Biochemistry to Medical Sciences, Fundamental and Applied Biochemistry Department, Faculty of Sciences, University of Antananarivo, Madagascar

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Abstract: The RUBIACEAE family is a promising source in herbal medicine. Many of them are widely used to treat various diseases in folk medicine. This research aims to promote biodiversity in Madagascar and innovate traditional malagasy medicine. The leafy twigs of *Gaertnera phanerophlebia* Baker (RUBIACEAE), a species endemic to the Great Island, have been used to treat pain, fever and wounds. Until now, few studies on its pharmacological and toxicological effects have been published thus so far. So, this study aims to verify the safety of the plant extract and determine the possible side effects by comparing its acute toxicity via three different administration routes in mice. *In vivo* results demonstrated that the leafy twigs crude extract (ST380RF) is non-toxic and non-irritating to the skin according to the Draize index. No mortality or sever effetcs were observed with oral administration up to the maximum dose of 5,000 mg/kg of body weight. Anatomopathological studies of vital organs revealed no adverse effects. However, intraperitoneal injection of the crude extract resulted in the death of some animals, yielding an LD_{50} of 225,007 mg/kg of b.w, which is considered moderatly toxic according to the Hodge et Sterner classification. Additionally, macroscopic and microscopic analysis of vital organs revealed moderate changes in the spleen and kidneys. These results suggested that the oral and dermal administration of the leafy twigs of this plant appeared harmless and safe for medication. Anyway, since no data are available on the effects of repeated exposure, dermal application seems reasonable for drug development based on the crude extract.

Keywords: *Gaertnera Phanerophlebia, Leafy Twigs, Crude Extract, Acute Toxicity, Anatomopathology*.

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I. INTRODUCTION

Acute toxicity is defined as the toxic effect(s) produced by a single exposure to a drug via any route over a short period of time [1]. Acute toxicity studies in animals are commonly used to determine the lethal dose (LD_{50}) of a drug or chemicals [2]. These studies are considered necessary if there is any intention to utilize the plant for pharmaceutical purpose. The main objective is to identify the dose that causes major adverse effects or life-threatening toxicity, which often involves estimating of the minimum lethal dose [3].

Gaertnera phanerophlebia Baker, also known as "Tsitsirontafika", belongs to the RUBIACEAE family, and is endemic to the northeastern part of Madagascar. The decoction of its twigs leaves have been used in traditional medicine to

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treat wounds, pain and fever [4]. Tsitsirontafika is among the endemic medicinal plants of the Malagasy vegetation cover, for which few studies on its pharmacological and toxicological effects have been published thus so far. Despite its traditional use, a critical lack of comprehensive toxicological data limits its integration into modern phytotherapeutic formulations. In his study, the efforts are made to further improve understanding about oral, intraperitoneal and dermal toxicities of the twigs leaves's crude extract. To the best of our knowledge, this is the first investigation on the acute toxicity of the plant extract.

II. MATERIALS AND METHODS

➢ *Plant Material and Identification*

The fresh twigs leave of *Gaertnera phanerophlebia* Baker were collected in December 2019 from the Alaotra Mangoro region of Madagascar in the commune of Ambohibary and the Fokontany of Ampitambe Ambatomainty (Fig 1). Botanists at the National Center for Pharmaceutical Research Applications (CNARP) identified and authenticated the plant. A voucher specimen was archived in the Department of Botany herbarium under the registration number ST380RF.

Fig 1 Twigs leaves of *Gaertnera phanerophlebia* (RUBIACEAE)

- Photo by Rakotoarisoa Mbolatiana Abigaila, Andasibe, 2019;
- Richardson Razakamalala, MBG-Madagascar)

➢ *Animals*

Healthy male and female mice (*Mus musculus*) weighing between 20 and 35 g, were obtained from the animal house of the Department of IMVAVET (Malgache Institute of Veterinary Vaccines) and used as the animal model. They were allowed to acclimate for seven days prior to the start of the study. The animals were fed with standard livestock pellets and had unrestricted access to clean drinking water. Toxicological study was conducted in alignment with the OECD guidelines and the 3Rs principle. The experimental protocols were approved by the Pasteur Institute of Madagascar (IPM) Ethics Committee and aligned with the established standards [5,6].

➢ *Preparation of Crude Extract*

Dried twings leaves of *G. phanerophlebia* were reduced to a fine powder using a mechanical grinder. The powdered plant material (350 g) was extracted by maceration with methanol (1,800 ml) at room temperature for 24 x 3 hours. After cooling, the mixture was filtered and evaporated to dryness [7]. The resulting crude extract (ST380RF) was labeled and stored in a refrigerator (4° C) for further use.

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Fig 2 Preparation of the Crude Extract (ST380RF) from *Gaertnera phanerophlebia* Leafy Twigs: Extraction (1) and Evaporation (2)

➢ *Acute Toxicological Studies*

An acute toxicological study is a qualitative and quantitative analysis of toxic phenomena that may occur following a single administration of the substances being teste. Then, the acute toxicity study of *G. phanerophlebia* twigs leaves (ST380RF) was carried out according to the Organization for Economic Co-operation and Development (OCDE) instructions (423, 425) [8,9,10].

- *Preparation of Extract:*

The substance to be administered was prepared by diluting the crude extract (ST380RF) in 5 % DMSO. The dose was calculated based on each mouse's weight and expressed in milligrams of active ingredient per kilogram of body weight (mg/kg bw). The volume to be administered to each animal was 1 ml per 100 g of body weight. Three routes of administration were used to compare the acute toxicity of *G. phanerophlebia* leafy twigs extract in mice: oral, intraperitoneal, and dermal.

- *Preparation of Animals:*

Before substance administration, the animals were deprived of food for 18 hours, weighed, divided into groups of three per sex (n = 3/group/sex). The groups included : a control group which received distilled water, and treated groups, which were administered with prepared solution of ST380RF. Doses ranging from 10 to 5, 000 mg/kg of b. w of the crude extract were used in animals [11].

- *Animal Observation:*

Within the first 30 minutes, during the sixth hour, and then every one hour for 24 hours and finally daily for three days, the animals of both sexes were observed for changes in behavior, signs of toxicity, and mortality. Surviving animals were determined for behavioral activities, including variations in skin, membrane, and pupils colors, changes in body posture ; changes in body weight ; mouvements ; rearing ; tremors ; and histological tissue damage [12,13,14,15,16]. The median lethal dose (LD_{50}) for the oral and intrapreritoneal routes was calculated using the following formula :

$$\textit{Oral Median Lethal Dose } (DL_{50}) = [M0+M1]\ /2$$

- M0 : the highest dose of the test substance that produces no mortality
- M1 : the lowest dose that produces mortality

The obtained LD_{50} values were interpreted according to the classification rate, as shows in table 1. Lower numbers indicate higher toxicity (below 5 mg/kg), while higher numbers indicate lower toxicity (above 15,000 mg/kg) [17,18].

- *Oral Acute Toxicity (p.o):*

The objective of this experiment was to calculate the oral median lethal dose (LD_{50}p.o) of *G. phanerophlebia* twigs leaves (ST380RF). The study was conducted using the method described by Enegide *et al*. (2013) with some modifications [19]. Animals received the ST380RF extract via gavage.

- *Intraperitoneal (i.p) Acute Toxicity:*

The intraperitoneal acute toxicity experiment referred to the adverse effects or lethality of the substance after a single, short-term exposure (24 h). The substance was injected directly into the peritoneal cavity (the abdomen), to rapidly assess the lethal dose for 50 % of subjects, and to identify immediate systemic reactions that are not seen with oral route [20,21].

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Table 1 Classification of LD_{50}

LD_{50} value	Classification
<5 mg/kg	Extremely toxic
5-50 mg/kg	Highly toxic
50-500 mg/kg	Moderately toxic
500-5,000 mg/kg	Slightly toxic
5, 000- 15, 000 mg/kg	Practically non-toxic
>15, 000 mg/kg	Relatively harmless

LD_{50} : Lethal dose

- *Histopathological Studies of Organs :*

A Histopathological study is a standard preclinical research method used to understand the effects of a new drug or substance on the histological, damage, and toxicity of organs [22]. This procedure helps to establish safe dosage ranges and identify potential toxicities to target organs, such as the liver, kidneys, heart, and spleen, early in development. Animals that survived oral and i.p administration from the control and treated group were killed after 24 hours. The macroscopic and microscopic features of the vital organs (liver, kidneys, spleen and heart) of male and female mice were compared in the control and the treated groups.

- *Macroscopique Feature :*

After euthanizing the animals with an injection of Nembutal® (0.140 g/kg, i.p.), a necropsy was performed to analyze the external macroscopic features of the heart, liver, spleen, lungs, kidney, adrenal glands, esophagus, stomach, small intestine, hypophysis, hypothalamus, brain and reproductive organs (uterus and ovaries or testicle, prostate, epididymis, seminal vesicles, and vas deferens) [23]. These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (P_r) (g and g/100 g of body weight, respectively), calculated using the following formula :

$$Pr(g)=Po/Pa \times 100$$

Where, P_o : the weight of the organ ; P_a : the animal's body weight, both in grams

- *Microscopic Feature:*

A histological examination was performed using the method described by Hould (1984) [24]. The animals were anesthetized with Nembutal®, perfused with a saline solution to removal the blood. Then, a buffered formalin solution (10 %) was used for 10 minutes. The same organs were removed and fixed in a Bouin solution for 48 hours at room temperature [25]. The fixed tissue was sectioned into five micrometers with a rotary microtome. Then, it was stained with hematoxylin and eosin before examined with a light microscope [26]. Anatomopathological studies are carried out at the Anatomical Pathology Laboratory of the Joseph Ravoahangy Andrianavalona University Hospital Center (CHU- J.R.A).

- *Dermal Acute Toxicity:*

The dermal acute toxicity test evaluates and categorizes the effects of substances that come into contact with the skin [27]. Experimentation was carried out according to the classic Draize test, following OECD guidelines 402 and 404, with some modifications [28,29]. The Draize acute primary irritation procedure was carried out on male mice weighing between 20 and 30 grams [30,31]. The total study duration was 72 hours from the day of application [32].

For the Draize test, the application site (approximately 10 % of the total area in the mid-dorsal region) was shaved and cleaned with distilled water. Then, 150 mg/kg of the crude extract (ST380RF) was evenly applied to the site and covered with sterile gauze and adhesive tape to maintain continuous contact with the skin. The procedure was performed on both intact and abraded skin, without causing bleeding or scarring of the stratum corneum [33] (Fig. 2). The animals were then placed in individual cages to prevent ingestion of the product by other animals.

The application site was evaluated for cutaneous reaction scoring, such as erythema (redness), and edema (swelling), as presented in table 2 within 24-72 hours [34,35]. The skin reaction was assessed according to the Draize scale based on the Primary Irritability Index (PII) value by the following formula, and the result was interpreted using the IIP value showed in table 3.

$$PII = average\ value\ (Edema + Erythema)\ /24$$

Table 2 Method of Scoring the Erythema and Oedema Reaction in Acute Dermal Toxicity

Types of injury	Degree of injury/irritation	Score
Erythema and pressure sore formation	No erythema	0
	Very light (barely perceptible)	1
	Well defined	2
	Moderate to severe	3
	Severe pressure ulcer formation	4
Edema formation	No edema	0
	Very light (barely perceptible)	1
	Lightweight (well-defined edges)	2
	Moderate (height approximately1 mm)	3
	Severe (more than 1 mm in height and extending beyond the exposure area)	4

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Table 3 Interpretation of the Primary Irritability Index (PII) value according to Draize scale

PII value	Categories	PII value	Categories
PII < 0.5	Non-irritating	2.1 < PII < 5	Irritant (Moderate Reaction)
0.5 < PII < 2	Slightly irritating	5.1 < PII < 8	Very irritating (Severe reaction)

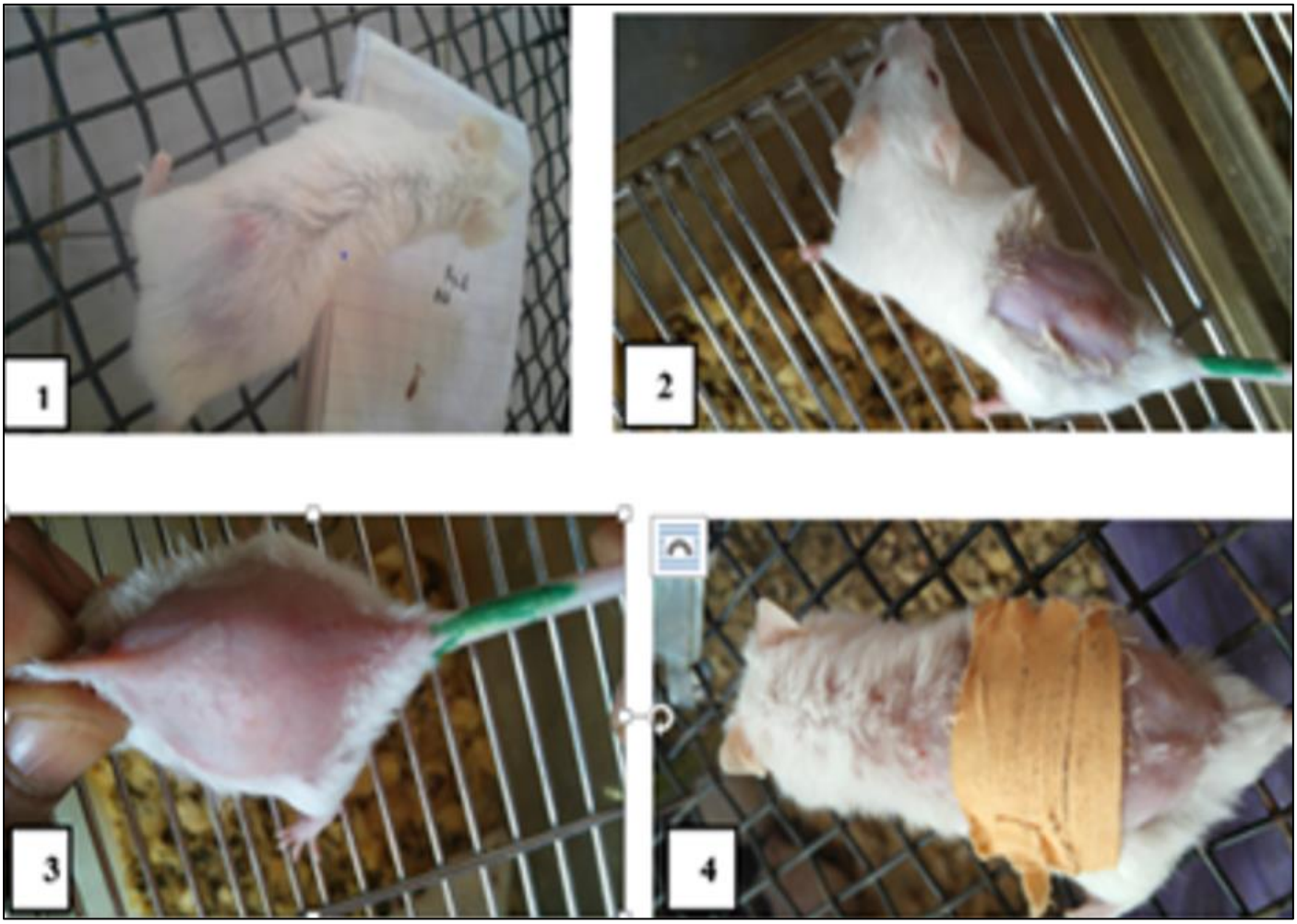

Fig 3 Method of Acute Dermal Toxicity Test on Mice

(1: male mice; 2: mid-dorsal region shaved, intact skin; 3: shaved and abraded skin; 4: region covered with a sterile gauze and adhesive)

- *Statistical Analysis*

All experimental measurements were carried out in triplicates and all measured variables were expressed as the mean ± standard error of the mean (SEM). The statistical significance of each test was estimated by Student's t test. Significance of the differences was established at the 5 % level ($p<0.05$).

III. RESULTS

➢ *Extraction Result*

The yield and characteristics of the crude extract are given in table 4.

Table 4 Crude extraction yield from leafy twigs

Plant Material	Crude Extract	Mass (g)	Yield (%)	Appearance
*Gaertnera phanerophlebia*350 g (leafy twigs)	ST380RF	27.78	7.36	Green paste

A yield of 7.36 % of crude extract in methanol was obtained from leafy twigs. This result indicates the successful extraction of polar compounds, which is consistent with methanol's high polarity. Methanol efficiently dissolves polar substances, such as phenolic compounds. This explains its higher yield compared to less polar solvents for this type of extract. These results suggests that the raw material is rich in polar phytochemicals and that methanol's strong affinity for these molecules allows for their efficient separation. Therefore, methanol is a good choice for isolating these specific bioactive compounds [36].

➢ *Acute Toxicity Results*

The result of acute toxicity experiments via oral and itraperitoneal routes are given in table 5.

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Table 5 Toxicity in of the Crude Extract of *G. phanerophlebia* Leafy Twigs Following Oral and Intraperitoneal Administration

Administration	**Oral (p.o)**	**Intraperitoneal (i.p)**
Parameters	LD_{50} >5.000 ± 00.4	LD_{50} = 225.007 ± 0.33
Extract classification	Practically non-toxic	Moderately toxic
Toxicity class	5	3

- *In Oral Administration:*

The acute toxicity studies were performed on male and female Swiss mice. The doses ranged from 10 to 5, 000 mg/kg of b. w via the oral route. No animales died within three days of observation during the experiment. Clinical observations began immediately after the administration of the ST380RF extract. Monitoring the treated animals compared to the control group allowed the collection of symptoms of intoxication that occurred after ingestion of the extract at different doses. All animals exhibited symptoms such as tremors, piloerection, abdominal contortion, and venous dilation when administered orally. These symptoms resolved within four hours, and the mice returned to normal after six hours. These effects are considered signs of mild, transient toxicity. The oral LD_{50} value of ST380RF could not be determined. Therefore, the LD_{50} p.o is then greater than 5,000 mg/kg of b.w. According to the Hodge et Sterner classification (1943), the crude extract of *G. phanerophlebia* leafy twigs is pratically non-toxic (class 5) [37]. These results showed that the oral administration of the leafy twigs of *G. phanerophlebia* is not toxic to mice, suggesting a safety use by humans.

- *In Intraperitoneal Administration:*

Intraperitoneal injection of the crude extract ST380RF caused decreased mobility (nervous disorder), increased respiratory rate (respiratory disorder), loss of reflexes (motor disorder), abdominal contortions, fatigue, anorexia (digestive disorder), and enophthalmos in animals of both sexes. These symptoms were more severe and resulted in the death of some mice. Deaths occurred between 5 and 24 hours after treatment. The calculated LD_{50} i.p was 225.007 mg/kg of b.w in mice.

- *Histopathological and Organ Weights Effects*:

The effects of ST380RF on vital organs weight, and histopathological aspects were studied in both sexes. The table 5 shows the average weights of the livers, spleens, and kidneys of animals treated with the crude extract (ST380RF).

Table 6 Average Weight of Vital Organs in Mice Treated with ST380RF

Parameters	**Average Weight of Vital Organs (g)**		
Treated group			
Vital organs	Liver	Spleen	Kidneys
Male	1.75 ± 0.5	*0.32 ±0.04	*0.31 ±0.01
Female	1.74 ±0.67	*0.39 ±0.6	*0.28 ±0.08
Control group			
Male	1.74 ± 0.77	0.11 ±0.2	0.21 ±0.03
Female	1.74 ±0.54	0.16 ±0.05	0.19 ±0.12

Values are expressed as mean ± SD; *significatify different from the control at $p<0.05$

When compared to the control group, oral and intraperitoneal administration of the plant extract (ST380RF) had no significant effect on liver weight in mice of both sexes (male: $p = 0.724$; female: $p = 0.0983$). The livers appeared normal. However, treatment with *G. phanerophlebia* extract after i.p injection caused an increase in organ weight in the animals, at the level of the spleen and kidneys, with a significant difference (Fig. 4). The obtained p-values were very low (spleen: $p = 0.000$; kidneys: $p = 0.0316$). These results suggest potential toxicity or adaptive changes after i.p administration of the plant extract in animals. Increased weight might indicate inflammation or fat accumulation (steatosis). Additionally, macroscopic and microscopic observation of vital organs in decreased mice reveals a few small intracellular vacuoles.

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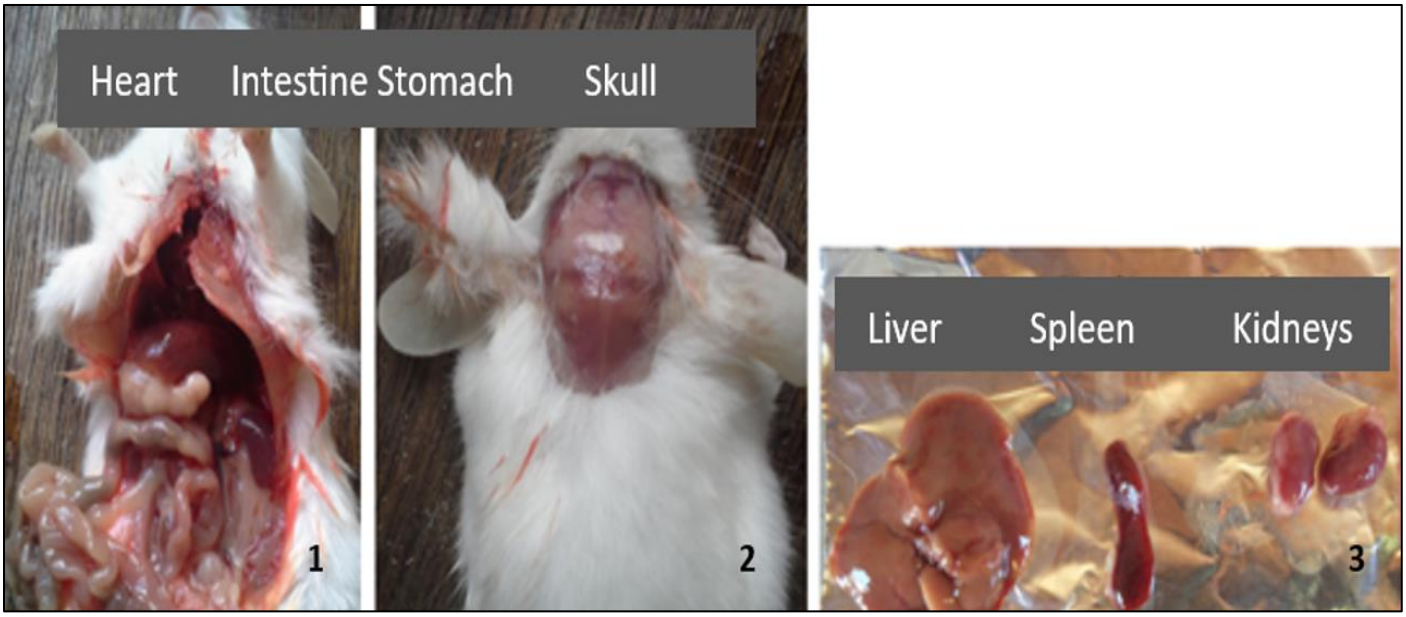

Fig 4 Histopathological Features of Vital Organs After ST380RF Administration

- *Dermal Acute Toxicity Results:*

The results of the acute skin toxicity test of the *G. phanerophlebia* crude extract of on scarified and unscarified skin are summarized in the table 6.

Table 7 Skin Toxicity Rating in Mice Treated with ST380RF from 24 h to 72 Hours

Skin Observation	**Scarified**				**Unscarified**			
Parameters	Er	Ed	Score	PII	Er	Ed	Score	PII
ST 380RF	0	0	0	0	1	0	1	0.5
Control group	0	0	0	0	1	2	3	1.5
Classification	Non- irritating							

Er: Erythema; Ed: Edema; PII: Primary Irritation Index

The findings indicate that applying ST380RF topically to mouse skin for 24-72 hours caused no irritation. The treated skin appeared smooth and hydrated, similar to the control group. According to Draize scores, the product is non-irritating (PII of 0 for unscarred, 0-0.5 for scarred skin), meaning it is safe for short-term dermal use without causing redness (erythema) or swelling (edema).

IV. DISCUSSION

While the medicinal properties of *G. phanerophlebia* described in the Malagasy pharmacopoeia are interesting, they are not sufficient to validate its use in human therapy. Investigating potential toxicity thresholds and related adverse effects is essential. To this end, acute toxicity tests were performed on mice via three routes of administration (oral, intraperitoneal, and cutaneous) to define the safety profile of the compound based on specific routes of administration [3,38].

Typically, an acute toxicity study is conducted on laboratory animals using sufficient doses of substance in question to produce death or morbidity. [39].

Determining the lethal dose helps to establish the tolerance limits for using plant extract as a natural remedies. Based on toxicity experiments, the lethal dose (LD_{50}) and other essential parameters such as histopathological changes, time of death, and the number of deaths within 24-72 hours, were determined according to the routes of administration of the *G. phanerophlebia* twigs leaf extract.

As the results above show, the lethality parameters LD_{50} value of the crude extract of *G. phanerophlebia* varies depending on the route of administration.

The plant extract is non-toxic and non-irritating in oral and dermal route, respectively. The acute effects were much less severe with oral exposure. However, after intraperitoneal injection, the toxicity effects were the most pronounced and fatal ; the crude extract (ST380RF) was considered as moderately toxic. Consequently, the results of the histopathological studies after i.p route indicate alterations in the weight of the spleen and kidneys. Theses results may reflect immune responses or changes in blood cell production [40] or they may indicates issues such as congestion or tubular damage [41]. McMahon *et al.* (1991) and Gallez *et al.* (1999) have previously mentioned this difference in toxicity depending on the route of administration with capsaicin and manganese, respectively [42]. However, comparing the acute toxicity of *G. phanerophlebia* with that of its foreign congeners is difficult because few toxicological studies have been carried out on other species. Thus, the oral administration of the crude extract (ST380RF) of the leafy twigs appeared to be harmless, safe for medication but since no data are available on his effects of repeated exposure, dermal application seems reasonable for drug developpement based on this plant. Study

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on oral and intraperitoneal repeated exposure will be undertaken to elucidate its mechanism of action. Acute toxicity studies in animals are considered necessary if there is any intention to utilize the plant pharmaceutically.

V. CONCLUSION

The acute toxicity study conducted in this research work has provided more information and details on the pharmacological use of G. phanerophlebia. However, despite the absence of toxicity in oral and dermal routes, spleen and kidney damage has been observed with intraperitoneal administration. This necessitates further toxicity studies before considering the use of this plant in medicine. These results constitute the first toxicological studies on the plant.

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