GSC Biological and Pharmaceutical Sciences

eISSN: 2581-3250 CODEN (USA): GBPSC2

Cross Ref DOI: 10.30574/gscbps

Journal homepage: https://gsconlinepress.com/journals/gscbps/

(RESEARCH ARTICLE)

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Phytochemical and toxicological studies of leaf extracts of *Gambeya boiviniana* Pierre (Sapotaceae) a medicinal plant

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GSC Biological and Pharmaceutical Sciences, 2024, 29(02), 420–430

Publication history: Received on 01 October 2024; revised on 11 November 2024; accepted on 13 November 2024

Article DOI: https://doi.org/10.30574/gscbps.2024.29.2.0419

Abstract

A toxic activity was found in a leaf extract of *Gambeya boiviniana* (*Chrysophyllum bovinianum*), an endemic medicinal Sapotaceae of Madagascar. A purification process including a butanol fractionation and neutral lead acetate precipitation allowed to obtain a partially purified toxic extract (E2) from a clear, brick-red crude hydro-alcoholic extract (CE) with a bitter taste. The toxic principles were bitter, thermostable, soluble in water, in ethanol and in butanol. They were not precipitable by lead neutral acetate. They could be flavonons, unsaturated sterol, triterpenes or phenol compounds. Both CE and E2 were toxic to mice, in which E2 induced symptoms suggestive of central nervous system damage. The 24 h Lethal Dose 50% (LD_{50}) was comprised between 118.15 mg/kg and 122.39 mg/kg of the mouse weight. It caused histological lesions characterized by vascular congestion and hemorrhage in various organs. CE and E2 caused an hemolytic activity on red cells of sheep. E2 was toxic in frog (*Ptychadena mascareniensis*) tadpoles, a dose effect was observed and Lethal Concentration 50% (LC_{50}) was of 41.66 µg/ml. On fish (*Gambusia holbrooki*), at 9.2 mg/ml, the mortality rate was 0% but at 9.34 mg/ml it was 100%. E2 had no effect on mosquito (*Culex quinquefasciatus*) larvae.

Keywords: *Gambeya boiviniana*; Sapotaceae; Toxic; Hemolytic activity; Histological lesions.

1. Introduction

Since immemorial time, medicinal plants have been used as a source of medicines in all cultures. They are widely used in the treatment of acute and chronic diseases [1]. The World Health Organization estimates that up to 80% of the world's developing countries depends on locally available plant resources for their primary healthcare because they are easily accessible and less expensive [2]. It encourages the strengthening of research and evaluation into the safety and efficacy of herbal products. Despite their therapeutic effects, medicinal plants must be used with the utmost caution, as they can be toxic [3]. For example, *Dodonaea madagascariensis* (Sapindaceae) [4] and *Pittosporum ochrosiaefolium* (Pittosporaceae) [5], two Malagasy medicinal plants, have been found to be toxic.

Among the many families dedicated to medicinal purposes, the Sapotaceae family, which comprises several genera, including *Gambeya*, whose synonym is *Chrysophyllum* and which is native to tropical Africa. In addition to their exploitation in food and woodworking techniques, several species of this genus have various uses in traditional medicine. For example, in the Bangui region, a decoction of *Chrysophyllum laurentii* bark serves as a vermifuge against tapeworms [6]. In Cameron, bark and leaves of *Gambeya lacourtiana* are used to treat male sexual impotence and wounds [7]. In Congo, pulp extract of *Gambeya africana* fruits is a good hypoglycemiant and antioxidant agent and could

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be a potential source of compounds for diabetes management [8]. In Madagascar, the healers use the crushed leaves of *Gambeya boiviniana* (*Chrysophyllum bovinianum*) as a plaster against scorpion stings and leaf decoction to cure children suffering from hyperthermic convulsive seizures [9]. This plant is also found in the composition of several herbal recipes used to relieve malarial symptoms, tiredness, muscular aches and pains and poisoning [10]. In the north of Madagascar, decoction of the leaves in steam baths is used to treat fever accompanied by flu-like and neurological symptoms [11].

Gambeya boiviniana was chosen as study material. In addition to its various uses in traditional medicine, preliminary tests on mice demonstrated the toxicity of leaf extracts on mice. Moreover, this plant is endemic to Madagascar and grows in several regions, making it easy to find and to harvest.

This work is part of our laboratory program to assess the potential toxicity of traditional Malagasy medicinal plants. The aim was to determine the phytochemical composition of *Gambeya boiviniana* leaf extracts and to assess their toxicity on different animals.

2. Materials and methods

2.1. Materials

2.1.1. Plant material [12, 13]

Gambeya boiviniana (Figure 1) is known in Madagascar by several vernacular names including Rehiaka, Reheky, Famelo, Famelomana, Fakamboly, Famelomana fotsy, Hazomahogo, Hazomiteraka, Voantsikidy, Voampamelona and Voandrahiaka. This medium-altitude tree is the only endemic species of the *Gambeya* genus in Madagascar.

Figure 1 *Gambeya boiviniana*

Leaves were harvested in the Manombo natural reserve (south of Farafangana) in January. Voucher specimens of *Gambeya boiviniana* were deposited in the herbarium of Plant Biology and Ecology Department of the Faculty of Sciences of the University of Antananarivo.

Fresh leaves were air-dried for a week. They were then ground with a mortar. The fine powder obtained by sieving the crushed material was the starting material. It was stored at room temperature in a well-sealed container.

2.1.2. Mice

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

2.1.3. Frog tadpoles

The legless frog tadpoles (*Ptychadena mascareniensis*) were captured in rice fields on the campus of the University of Antananarivo. They were adapted in an aquarium containing rainwater a few days before the tests.

2.1.4. Fishes

Fishes (*Gambusia holbrooki*) came from the rice fields near the campus of the University of Antananarivo. The tests were carried out after the fish have adapted in an aquarium for a few days.

2.1.5. Mosquito larvae

Mosquito larvae (*Culex quinquefasciatus*) were caught in rice fields near the campus of the University of Antananarivo. The larvae were captured only on the day of the test.

2.2. Methods

2.2.1. Hydro-alcoholic extraction

The leaf powder was suspended in hydro-alcoholic solution (75%) in a 1:10 (w/v) ratio. The mixture was heated under reflux with magnetic stirring for 3 h at 70°C, then left to macerate overnight at +4°C. The macerate was filtered through 4 layers of gauze. The filtrate obtained was centrifuged at 16.000 rpm for 15 min at +5°C. The supernatant was concentrated by evaporation and the pellet discarded.

2.2.2. Fractionation with n-butanol

The extract to be treated was mixed volume by volume with n-butanol in a separating funnel. The mixture was stirred vigorously, then left to settle completely, resulting in the formation of two distinct phases: an upper butanol or organic phase, and a lower aqueous phase. Both phases were recovered separately. The aqueous phase was subjected to three further successive fractionations. The four butanol phases thus obtained were combined and filtered on filter paper to remove saturation water droplets. A large volume of distilled water was added to the filtrate, then the butanol was evaporated. Traces of butanol were removed from the aqueous phase by evaporation after the addition of distilled water.

2.2.3. Precipitation with neutral lead acetate (NLA)

NLA, like most heavy metal salts, enabled the defecation of biological extracts by precipitating large molecules such as proteins, nucleic acids, polysaccharides and other substances such as organic acids.

A 20% (w/v) aqueous solution of NLA was added dropwise to the magnetically stirred extract to be purified. The precipitate formed was removed by centrifugation at 3000 rpm for 10 min. The addition of a specific volume of a 10% (w/v) aqueous disodium phosphate solution removed excess lead from the supernatant. Another centrifugation at 3000 rpm for 10 min was performed to remove the precipitate formed. The supernatant was recovered.

2.2.4. Phytochemical screening

The reactions of chemical group detection were those developed by Fong *et al.* [14] and Marini-Bettolo *et al*. [15].

2.2.5. Tests on warm-blooded animals

Acute toxicity tests on mice

Toxicity was assessed by injecting 0.3 ml per mouse of extract intraperitoneally (i.p.). 3 male mice weighing 25 ± 2 g were used for each test. Another batch of 3 male mice, receiving 0.3 ml saline, served as a control.

Results were interpreted using the Hodge and Sterner scale [16].

The dose that kills 50% of mice in 24 h or LD_{50} was determined by calculation and graphical methods (Reed and Muench, 1938) [17]. Seven doses of the extract, in geometric progression of reason r =1.09, ranging from 84.69 mg/kg (0% mortality) to 159.36 mg/kg (100% mortality), were injected intraperitoneally into seven batches of 5 mice. A batch of 5 mice injected with 0.3 ml of saline solution served as a control.

Histopathological examination

Mice that have developed symptoms of intoxication were sacrificed. Hearts, livers, kidneys, brains, lungs and stomachs were rapidly removed and immersed in fixative or BOUIN fluid (picric acid 5 ml, formol 40% 20 ml, glacial acetic acid 5 ml). After 48 h, each organ was fragmented into thin 5 mm thick sections using a scalpel. More details of the method used are given in our previous article [18].

Tests on sheep red blood cells

In the presence of a hemolytic substance, red blood cells are lysed, releasing hemoglobin and turning the supernatant red. Intact red blood cells sediment.

A suspension of fresh sheep blood was used. The suspension was washed three times in succession. After each wash, the suspension was mixed with saline and centrifuged at 3000 rpm for 5 min. The pellet was recovered, while the supernatant was removed by aspiration. The pellet obtained from the third wash constituted 100% red blood cells. A 50% red cell suspension was obtained by mixing the previous pellet and saline solution volume by volume. A 2% suspension was prepared by diluting the 50% suspension with Phosphate Buffered Saline (PBS) buffer of the following composition: sodium chloride 7.65 g; disodium phosphate 0.724 g; monopotassium phosphate 0.210 g; distilled water 1000 ml. The test extract was diluted in cascade with PBS (geometric progression of reason 0.5). 50 µl of 2% red cell suspension was poured into the wells of a V-bottom microplate, each containing 50 µl of test extract in decreasing concentrations. Two controls were made: a positive control (C+) containing 50 µl of distilled water mixed with 50 µl of 2% red cell suspension (hemolysis appeared) and a negative control (C-) containing 50 µl of PBS mixed with 50 µl of 2% red cell suspension (no hemolysis appears). The microplate was incubated at 37°C for 3 h, then at 4°C overnight. Results could be read with the naked eye. The reading criteria were: no hemolysis (negative test) if the solution became colorless with a red cell pellet at the bottom of the well; total hemolysis (positive test) if the solution remained stained red; partial hemolysis if the solution was stained red and there was a red cell pellet at the bottom of the well.

2.2.6. Tests on cold-blooded animals

Experiments on cold-blooded aquatic animals involved testing their resistance to environmental intoxication. The animals were placed in batches of 7 in crystallizers containing rainwater. The extract was added in such a way as to have final concentrations following a geometric progression of determined reason, from LC_0 (maximum concentration at which all animals survive) to LC_{100} (minimum concentration killing 100% of animals). An untreated batch served as a control. The experiment lasted 24 h. Determination of the 24 h LC_{50}, or concentration that killed 50% of the animals tested in 24 h, was carried out by graphical method [19].

Tests on legless frog tadpoles

Six batches of seven legless tadpoles were tested with six different concentrations of test extract, in geometric progression. These concentrations were determined on the basis of preliminary test results.

Tests on fish *Gambusia holbrooki*

Seven different concentrations of the extract in geometric progression were tested on seven batches of seven *Gambusia holbrooki*.

Tests on mosquito larvae [20].

Larvae were collected using a sieve and distributed in batches of 10 in small crystallizers containing spring water. In beakers, different volumes of extract were added so as to have concentrations in geometric progression of reason r, ranging from LC_0 to LC_{100}. Mosquito larvae were then introduced into the medium by pouring the contents of a crystallizer into the beaker containing the extract. Dead larvae were counted after 24 h. Larvae are considered dead when they are touched by a needle in the cervical region and no longer moved, and moribund when they were unable to dive or rise to the surface when the water was agitated.

3. Results

Diagram summarizing the extraction and the various purification stages is presented in Figure 2. The crude extract, butanol-free organic phase and the clear yellow supernatant obtained after precipitation with neutral lead were called CE, E1 and E2 respectively.

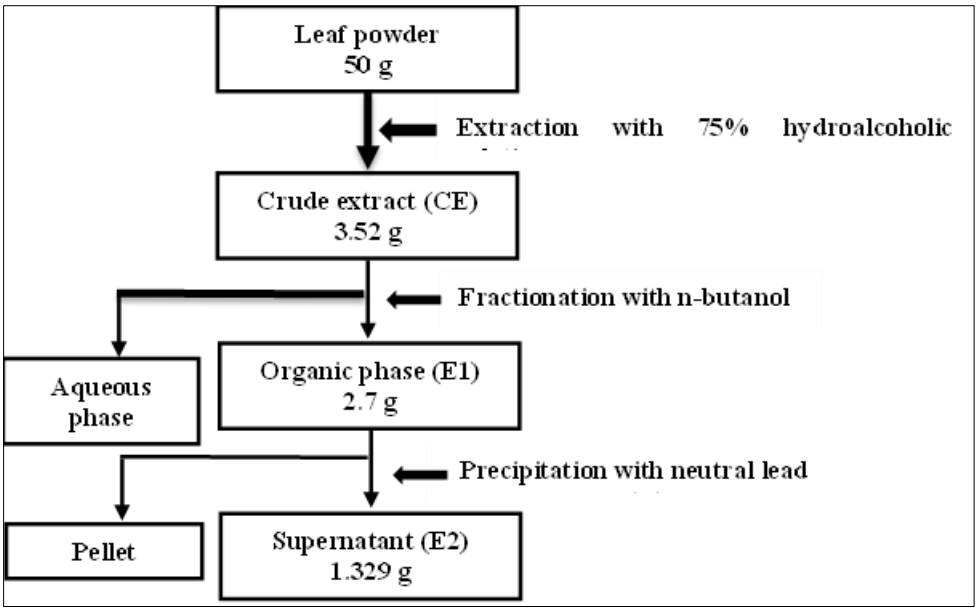

Figure 2 Diagram summarizing the extraction and the various purification stages

3.1. Degree of product homogeneity

The evolution of extract homogeneity during the different purification steps was monitored by thin layer chromatography, using the solvent system: butanol/acetic acid/water B/A/E (60/20/20; w/w).

Revelation with vanillin sulfate reagent (Figure 3) showed that E2 has fewer bands than the crude extract.

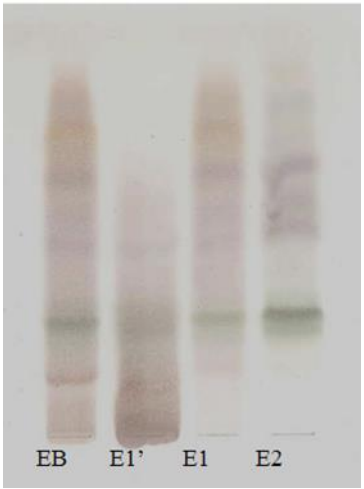

Figure 3 TLC in the B/A/E system (60/20/20; w/w) of extracts obtained during the various purification steps

CE: crude extract; E1: organic phase; E1': aqueous phase; E2: semi-purified extract

3.2. Chemical nature of E2 semi-purified extract components

Phytochemical screening results of semi-purified extract E2 are presented in Table 1.

Table 1 Phytochemical screening results for extract E2

Chemical groups	Tests	Results
Alkaloids	Mayer	-
	Wagner	-
	Dragendorff	-
Steroids and triterpenes	Libermann-Burchard	+
	Salkowski	+
Desoxyoses	Keller-Kiliani	+
Flavonoides and leucoanthocyanins	Wilstater	+
	Bate-Smith	+
Anthraquinones	Bornsträger	-
Tannins and polyphenols	Gelatin test	-
	Salted gelatin test	-
	Ferric chloride test	+
Saponins	Foam test	-
Iridoids	Hot HCL	-

E2 contained steroids, triterpenes, flavonoids, leucoanthocyanins, tannins and polyphenols, but no alkaloids, anthraquinones, saponins or iridoids.

3.3. Effects on animals

3.3.1. Effects on mice

Symptoms of intoxication

At the lethal dose of 159.36 mg/kg of E2 extract, immediately after injection, mice run in circles in their cages. After 5 min, they were breathing deeply, with the respiratory rate rising from 90 to 150 per min. The earlobes were turned backwards and hyperemia was observed. After 20 min, the mice remained motionless and showed piloerection. After 2 h 30 min, a progressive decrease in respiratory rate and enophthalmos were observed. Ataxia set in. After 8 h, the mice showed spaced clonic convulsions which became increasingly strong, leading to death after 9 h.

At the sub-lethal dose of 84.69 mg/kg, an increase in respiratory rate was also observed after injection. Their ears were pulled back. After 30 min, the mice rarely moved and showed piloerection and enophthalmos. The mice remained motionless for several hours. Eight hours after the injection, a progressive remission set in and the mice returned to a normal state.

Histopathology

Table 2 Main histological lesions induced by E2 at 159.36 mg/kg weight on various mouse organs

Organs	Observed lesions
Heart	Dilated capillaries, hemorrhagic areas
Stomach	Dilated capillaries in muscularis and serosa
Kidneys	Hemorrhagic areas
Liver	Dilated sinusoidal capillaries, hemorrhagic areas
Brain	Hemorrhagic areas in the brain parenchyma
Lungs	Hemorrhagic areas

The effects of the E2 extract at the tissue level were studied at a lethal dose of 159.36 mg/kg of mice, i.p. The main lesions caused by E2 on each organ are summarized in the Table 2 and shown in the Figures 3.

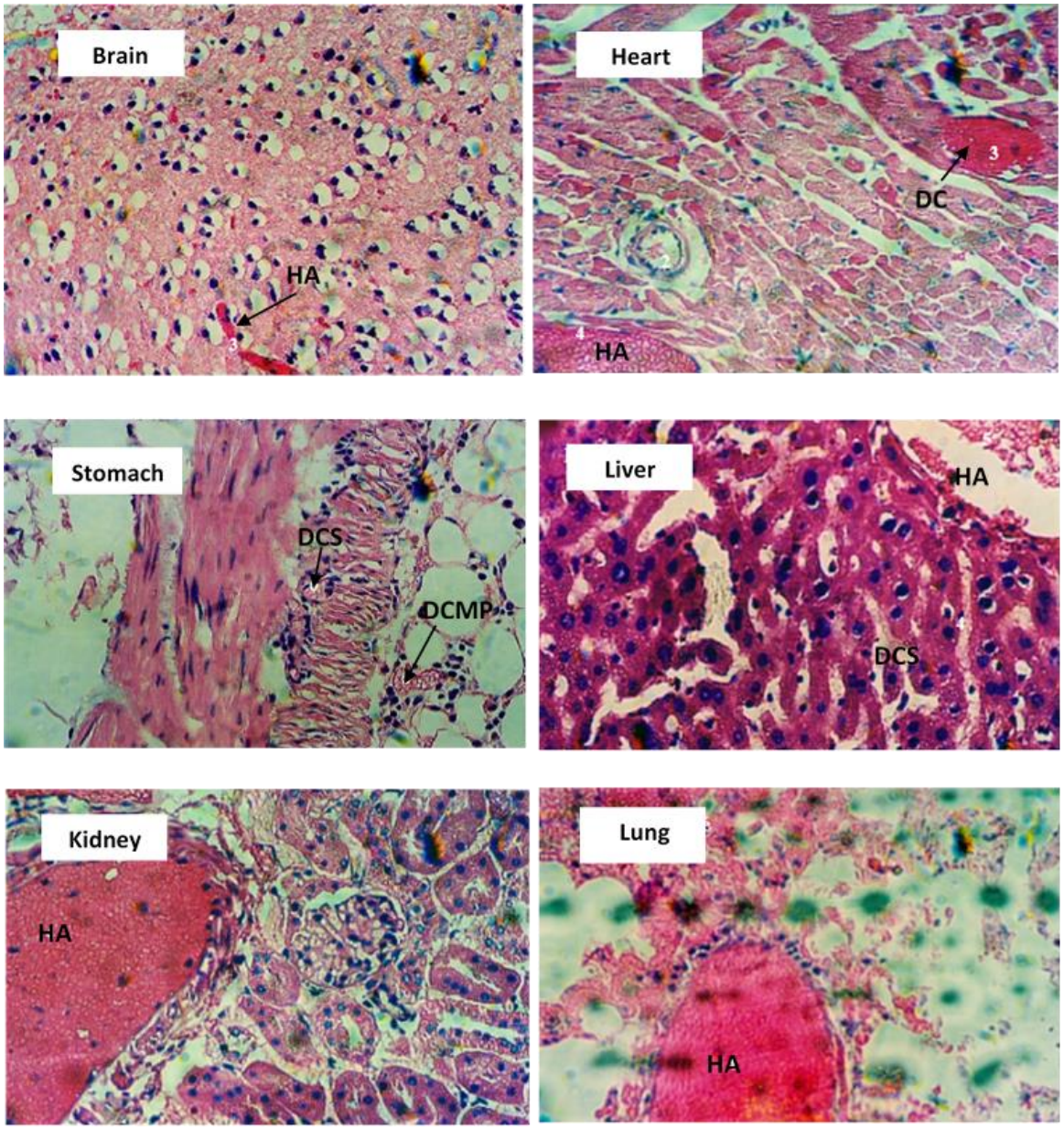

Figure 3 Main histological lesions in brain, heart, stomach, liver, lungs and kidneys caused by i.p. administration of E2 extract at a dose of 159.36 mg/kg body weight (magnification X 400).

DC: dilated capillary; HA: hemolytic area; DCS: dilated capillary in serosa; DCMP: dilated capillary in the muscularis propria.

Effects of CE and E2 on sheep red blood cells

The hemolytic power of the extracts was tested on a 2% sheep red blood cell suspension. The effects of CE were compared with those of E2. The test was carried out in the presence of two controls: negative control (PBS) and positive control (distilled water). The results are shown in Table 3 and Figure 4.

Table 3 Effects of different concentrations of CE and E2 on sheep red blood cells

Concentration (mg/ml)	2	1	0.5	0.256	0.125	0.062	0.031	0.015	0.008	0.004
CE	+++	+++	+++	+++	+	+	+	+	+	-
E2	+++	+++	+++	+	+	+	-	-	-	-

+ + +: Total hemolysis; +: Partial hemolysis; -: No hemolysis

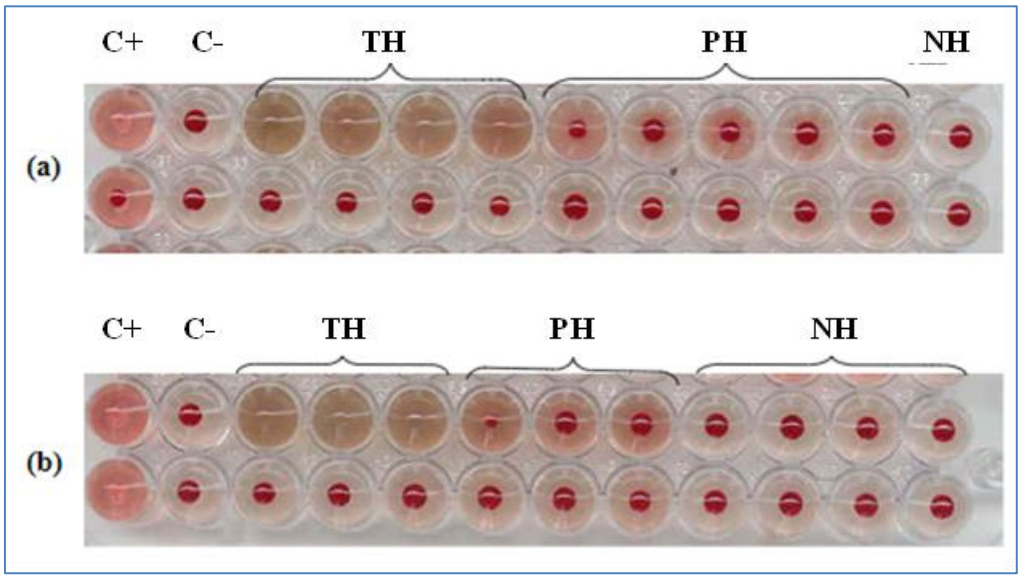

Figure 4 Effects of different concentrations of CE (a) and E2 (b) on sheep red blood cells.

C+: Positive control; C-: Negative control; TH: Total hemolysis; PH: Partial hemolysis; NH: No hemolysis.

Effects varied according to extract concentration. The result was: total hemolysis at concentrations of CE ≥ 0.25 mg/ml and E2 ≥ 0.5 mg/ml; partial hemolysis at concentrations between 0.125 mg/ml and 0.008 mg/ml for CE and between 0.25 mg/ml and 0.062 mg/ml for E2; no hemolysis at EC and E2 concentrations ≤ 0.004 mg/ml and 0.031 mg/ml respectively.

3.3.2. Effects of E2 on frog tadpoles

Six batches of 7 frog tadpoles were tested with six concentrations of E2, in geometric progression of reason r = 1.05, the first term of which was 48.62 µg/ml. The results are summarized in Table 4.

Table 4 Effects of E2 at different concentrations on frog tadpoles

Concentrations (µg/ml)	logC	Number of dead	Number of survivors	% of death
48.62	1.686	7	0	100
46.30	1.665	6	1	85.71
44.1	1.644	5	2	71.42
42	1.623	5	2	71.42
40	1.602	3	4	42.85
38.09	1.589	0	7	0

According to the results, the extract had a toxic activity on tadpoles. A dose-effect was observed. Based on these results, the LC_{50} (24 h) was estimated at 41.66 µg/ml.

3.3.3. Effects of E2 on fish Gambusia holbrooki

Seven concentrations of the extract E2 ranging from 8.93 µg/ml to 9.77 µg/ml, with a geometric reason of 1.015, were each tested on a batch of 7 *Gambusia holbrooki*. The results obtained are shown in Table 5.

Table 5 Effects of E2 on *Gambusia holbrooki*

Concentrations (µg/ml)	logC	Number of dead	Number of survivors	% of death
9.77	0.989	7	0	100
9.62	0.983	7	0	100
9.48	0.976	7	0	100
9.34	0.970	7	0	100
9.20	0.963	0	70	0
9.06	0.957	0	7	0
8.93	0.950	0	7	0

Due to the very narrow range between the concentration causing 100% mortality (9.34 µg/ml) that causing 0% mortality (9.20 µg/ml), it was not possible to calculate the LC_{50} (24 h) because the mortality rate was 0% at 9.2 mg/ml but at 9.34 mg/ml it was 100%: the effect of the extract obeyed the "all-or-nothing rule".

Effects of crude extract on mosquito larvae

The 2 mg/ml crude extract was tested on a batch of ten mosquito larvae. After 24 h, the larvae were still alive. The crude extract therefore had no effect.

4. Discussions

Several extraction and purification techniques, guided by toxicity tests on mice and thin layer chromatography were tested. Those that were not selected, such as cold and aqueous extractions, gel filtration and dialysis, nevertheless provided some informations on the physicochemical properties of extract components. The hydro-alcoholic extraction method was chosen to extract the toxic principles, as the crude extract (CE) obtained was sterile and free of hydrolytic enzymes, and the extraction yield was high (37.75%). Two purification techniques were selected: fractionation with n-butanol and precipitation with neutral lead acetate. These methods, based essentially on the difference in solubility, enabled us to obtain a partially purified toxic extract (E2). According to available informations, the active ingredients in the extracts were thermostable, soluble in water, ethanol and butanol, and were not precipitable by neutral lead acetate. The results of phytochemical screening showed that E2 contained flavonones, triterpenes, unsaturated sterols and phenolic compounds but neither saponosides nor alkaloids.

E2 has been shown to have toxic activity on various animal organisms. In mice, i.p. administration of E2 induced intoxication symptoms characterized mainly by piloerection, ataxia and clonic convulsions. All the reactions developed by the mice suggested that the toxin(s) attacked the central nervous system. The LD_{50} value for E2 was estimated to be between 118.15 mg/kg and 122.39 mg/kg. It was within the range of moderately toxic (50 mg/kg ≤ LD_{50} ≤ 500 mg/kg) according to Hodge and Sterner scale [16].

Compared with the few data available in the literature on the toxicity of plants in the Sapotaceae family, E2 is less toxic than *Mimusops commersonii* seed extract. (LD_{50} = 44.5 mg/ml) [20] but much more toxic than the hydroethanolic extract of *Gambeya africana* fruit. (LD50 >2000 mg/ml [8].

Compared with the toxicity of Malagasy medicinal plants such as *Dodonaea madagascariensis*, a Sapindaceae, (LD_{50} = 36.12 mg/ml) [4] and *Pittosporum ochrosiaefolium*, a Pittosporaceae, (LD_{50} = 46.69 mg/ml) [5], the toxicity of *Gambeya boiviniana* leaves was much lower.

Histopathological examination revealed that E2 caused lesions characterized in particular by hemorrhagic areas and dilated capillaries in all the organs of treated mice. These results were probably due to the vasoactive properties of

certain active principles. Both CE and E2 extracts lysed sheep red blood cells, but E2 was less active than CE. It should be noted that saponosides, well known for their hemolytic properties, were not detected in the extracts tested.

However, no allusions to any form of intoxication or adverse effects in the medicinal uses of *Gambeya boiviniana* have been reported by the local populations surveyed or in the literature. Toxic principles may be produced in small quantities or not at all during the harvesting season. Other possible explanations were that the active compounds were not toxic when taken orally, or that negative interactions between existing secondary metabolites prevented or reduced the effects.

In cold-blooded animals, a dose-effect was observed in frog tadpoles and the LC_{50} value was around 41.66 µg/ml. In fish, no dose-effect was observed, but at a concentration as low as 9.34 µg/ml, E2 caused 100% mortality. In contrast, CE, at a concentration as high as 2 mg/ml, had no effect on mosquito larvae.

In future work, it will be necessary to work on material harvested in all seasons, in order to check for variations in secondary metabolites composition and toxicity. The active ingredients will be isolated and tested separately. As far as toxicity is concerned, we need to assess oral toxicity and study prolonged exposure. Further investigation of possible side-effects among users of the plant extract will be carried out.

5. Conclusion

The results obtained in the present work, although preliminary, have made it possible to determine the nature and physico-chemical properties of the chemical groups present in *Gambeya boiviniana* leaves. They highlighted the toxicity of leaf extracts on different animal organisms. These data should draw attention to the precautions to be taken in medicinal uses of this plant.

Compliance with ethical standards

Acknowledgments

The authors are grateful to the Pasteur Institute of Madagascar (IPM) for providing animals for toxicity assessment and to the Laboratoire Central d'Anatomie Pathologique du Centre Hospitalier Universitaire (CHU) Joseph Ravoahangy-Andrianavalona for work on histopathological studies.

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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