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Purification and Toxicity Study of a Saponin from Seeds of *Albizia odorata*, a Fabaceae from Madagascar

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Abstract: In order to continue the research of natural compounds of interest such as pesticides and therapeutic molecules in endemic species of *Albizia* from Madagascar, potentials of *Albizia odorata* seed extract were assessed. A toxic saponin (saponoside), named Albodorine, was isolated by extraction with hot ethanol or distilled water followed by purification procedure comprising n-butanol partition, precipitation by aceton-diethyl ether (50/50), Sephadex LH-20 gel chromatography and silica gel chromatography. All these methods were guided by toxicity tests on mice and homogeneity tests by Thin Layer Chromatography (TLC). Albodorine was thermostable, soluble in water and organic solvents and tasted bitter. Its acidic hydrolysis released glucose, arabinose and rhamnose. Tested on different experimental animal models, it was toxic to warm and cold blooded animals. In mouse, when intraperitoneally administered, it caused acute intoxication mainly presented as hyperpnea, ataxia and terminal seizures before the animal died. Its LD50 was about 9 mg/kg of mouse body weight by intraperitoneal route. In different organs, it caused histopathological lesions characterized by vascular congestions and important hemorrhage in liver, lungs and kidneys. *In vitro*, it reduced the heart rate and force of contraction of isolated rat atria. It had hemolytic activity. Albodorine showed toxicological properties that could be exploited under certain conditions for the control of harmful organisms.

Keywords: *Albizia odorata*, Toxin, Albodorine, Saponin, Hemolysis, Histopathology, Isolated Atria, Inotropic Effect

1. Introduction

The current study is the continuation of the work the authors have undertaken on *Albizia* species endemic of Madagascar [1-6].

Albizia species appear as trees or treelets or shrubs, with 145 species inventoried all over tropical regions [7]. In addition to their use as timbers, a number of important biological activities have been reported on both crude extracts and purified substances from different species of this genus [8]. As examples, *A. lebbeck, A. julibrissin, A. gummifera, A. chinensis, A. adianthifolia* and *A. procera* are important species in traditional medicine. These species are used in folk medicine for the treatment of rheumatism, stomach ache, cough, diarrhea, wounds, anthelmentic and others. Alcoholic and/or hydroalcoholic extracts of *Albizia* species showed anticonvulsant, sedative, anti-inflammatory, antitumor, antifungal, antibacterial and antiparasitic activity [9]. *A. lebbeck* is known to be useful in the treatment of allergic conjunctivitis and atopic allergy [10].

Among the various compounds isolated from *Albizia*, saponosids were the most common and abundant [8]. They were found for example in *A. julibrissin* [11-12], *A. grandibracteata* [13], *A. gummifera* [14] and *A. versicolor* and *A. schimperana* [15]. Saponins are known to have various pharmacological properties [8, 16, 17, 22, 24]. They have membrane-permeabilising, haemolytic, antioxidant, anti-inflammatory, immunostimulant, anticarcinogenic, antidiabetic, anthelmentic, hepato protective activities. They affect feed intake, growth and reproduction in animals, and

they can be used as fungicides, molluscicides and pesticides, as well as against some bacteria and viruses. They have cytoxic activities [18, 19] and they possess clear insecticidal activities: they exert a strong and rapid-working action against a broad range of pest insects [14].

Few studies only were performed on the seeds of *Albizia* species which remained source of valuable compounds [20].

In Madagascar, 30 *Albizia* species are present, 24 of which are endemic, 3 also occur elsewhere and 3 are introduced [7]. In contrast to foreign *Albizia* species, Malagasy *Albizia* species have not been well-known by local populations and scientists. These plants are only known for their use as timbers. Except for works dealing with their botanical aspects, our previous studies are the only ones so far published [1-6]. Up to now, 14 of these plants (*A. androyensis, A. arenicola, A. aurisparsa, A. bernieri, A. boinensis, A. boivini, A. divaricata, A. greveana, A. mahalao, A. masikorurum, A. polyphylla, A. sp., A. tulearensis* and *A. viridis*) were already subjected to chemical and toxicological investigations.

A. odorata is a tree up to 30 m tall with a trunk up to 1.50 m in [7, 21]. It is a rare tree growing in north-west and central-west regions of Madagascar in forests on limestone. It flowers in January-February and its fruiting period is July-October. Its wood is used for furniture-making and no other uses of this plant have been known.

The present work focused on the purification of the toxic substances found in crude extract of *Albizia odorata* seeds and the study of their chemical nature, toxicological effects on various experimental animal models of cold and warm blooded animals and pharmacological effects on isolated rat atria.

2. Experimental

The methods used but not described in this work were detailed in our previous paper [1-6].

2.1. Plant Materials

Fruits were collected in western dry forest of Ampijoroa. Voucher specimen of the plant was deposited in the herbarium of Plant Biology and Ecology Department of the Faculty of Sciences of the University of Antananarivo.

The seeds from dried fruits of *A. odorata* were ground into fine powder. Seed coats were separated from almond powder by sieving.

2.2. Chemicals

All the chemical products used in this work (solvents and others) were essentially from MERCK, PROLABO, RIEDEL de HAEN and high quality (pure or analytical grade).

The chromatography supports used were Sephadex LH20 gel (PHARMACIA), silica gel 60 (70-230 mesh) (MACHEREY-NAGEL), silica gel 60F254 plates (MERCK), ion-exchange resins DOWEX 50W X 8 and DOWEX 1 X 8 (BDH) and WHATMANN n°3 paper.

2.3. Animals

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

Wistar strain white rats and guinea pigs were provided respectively by National Centre of Pharmaceutical Research Application (CNARP) and Madagascar Applied Research Institute (IMRA).

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

Apode and two-legged 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.

Mosquito larvae, *Culex quinquefasciatus*, bovine filariasis vector, were furnished by Entomology department of Pasteur Institute of Madagascar (IPM).

Shellfish, *Biomphalaria pfeifferi*, a human intestinal bilharziasis vector, was also provided by IPM.

2.4. Extraction and Purification Methods

Chemical and biochemical methods based on physicochemical difference properties were used in order to establish extraction and purification of the toxic compounds.

Procedure was guided by toxicity on mice and by homogeneity tests on thin layer chromatography (TLC).

2.5. Phytochemical Screening

All reactions of chemical group detection are those developed by Fong *et al.* [22] and Marini-Bettolo *et al.* [23].

2.6. Acid Hydrolysis

The method used was that of [24]. Dry material (250 mg) was dissolved in 25 mL of H2SO4 (4N). The solution was heated 4 hours at 110°C under reflux. After cooling at room temperature, the addition of distilled water (25 mL) resulted in the formation of an abundant precipitate. After centrifugation, the pellet containing the aglycone was separate from the supernatant containing the sugars. The supernatant was first neutralized and deionized by ion exchange chromatography.

2.7. Sugar Identification

Sugars were identified by characteristic reactions and TLC.

2.7.1. Sugar Identification Tests
Classic methods for sugar analysis as described in [25] were used i.e. chemical tests for the presence of carbohydrates (Molisch test), pentoses (Bial test) and ketose sugars (Seliwanoff test).

2.7.2. Identification of Sugars Via Paper Chromatography
Acid hydrolysate of the purified extract was first neutralized and deionized by ion exchange chromatography. After those treatments, it was then submitted to a chromatography on

Whatman paper n°3 using glucose ribose, arabinose, xylose, fructose, rhamnose, mannose, galactose as references and mixture of n-butanol, acetic acid and water (4/1/1, v/v/v) as developing solvent. Spots were revealed by silver nitrate followed by heating at 100°C for 1 hour.

2.8. Haemolytic Test

A suspension (50 µL) of sheep red blood cells (SRBC) in Phosphate Buffered Saline pH=7.2 (PBS) was distributed in 24 wells of microtiter plate. The first well received 100 µL of albodorine solution (1 mg/mL). Successive dilutions in geometric progression of ratio 0.5 were performed from the second well. A positive (SRBC 2 % in distilled water) and a negative (SRBC 2 % in PBS) controls were used. After a gentle stirring, plate was incubated in an oven at first at 37°C for 3 hours and then at 4°C for 24 hours.

3. Results

3.1. Chemical Study

3.1.1. Developed Extraction and Purification Protocol
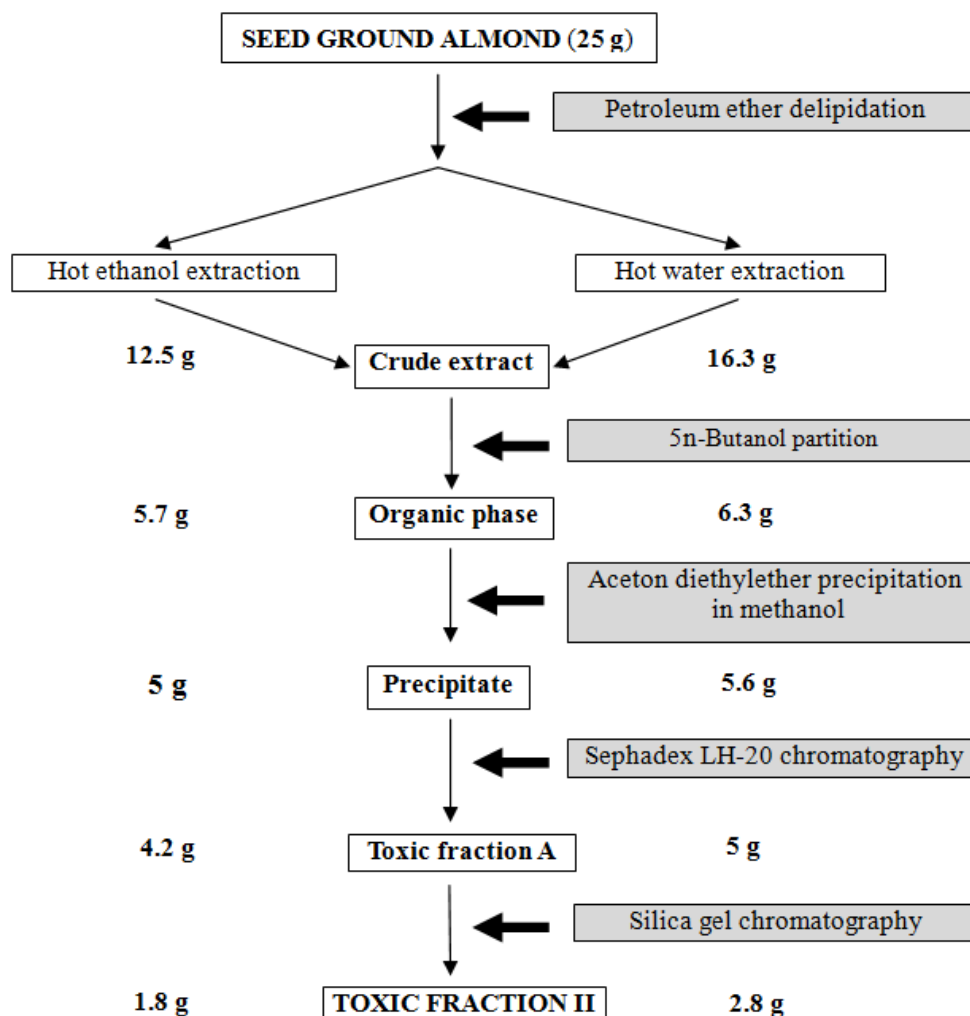

Figure 1. *Summary diagram of the different extraction and purification steps of toxic principle from ground almond: numbers represent the residue weights obtained at each step.*

Seed ground almond (25 g) was defatted by treatment with petroleum ether at 60 – 80°C in a Soxhlet extractor. The solvent free powder was extracted again in a Soxhlet extractor at boiling temperature during 24 hours in either ethanol or water. The extract was filtered and evaporated under reduced pressure to yield dry residue. The latter, redissolved in water (50 mL), was partitioned with n-BuOH (3 x 50 mL). The organic phase was evaporated to dryness and precipitated with aceton/diethylether in methanolic solution, giving limpid brown solution which was evaporated

to dryness under reduced pressure. The resulting residue was chromatographed on Sephadex LH-20 column (1.5 x 80 cm) with n-butanol/acetic acid/distilled water (60/20/20, w/w/w) as eluting solvent with a flow rate of 3 mL/h/cm^2. Two fractions (A and B) were identified according to the TLC behaviour. The toxic fraction A was chromatographed on silica gel column (2.5 x 120 cm) and eluted with n-butanol/acetic acid/distillated water (75/20/05, w/w/w) with a flow rate of 6 mL/h/cm^2. Toxic fractions (I, II and III) were identified. Only the fraction II, displaying a single spot in

TLC, was toxic.

The extraction and purification protocol developed to obtain purified extract is summarized in Fig. 1.

Purified extract was obtained with yields of 16.6 % and 14.4 % when extraction was done respectively with hot water or hot ethanol.

The homogeneity of fraction II was checked by TLC using different solvent systems: ethyl acetate/ethanol/distilled water (60/30/10, v/v/v); n-butanol/acetic acid/distilled water (60/20/20 and 75/20/05, w/w/w); chloroform: methanol: distilled water lower phase (65/25/10 and 75/20/05, v/v/v). Each chromatogram showed a single spot, meaning that fraction II contained only one component we named albodorine (Fig. 2).
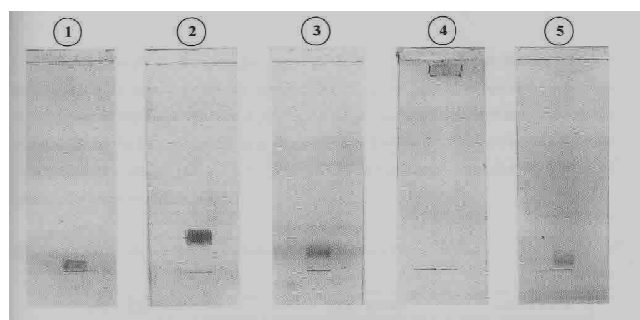

Figure 2. *Thin layer chromatography of fraction.*

II in different solvent systems:
1: ethyl acetate/ethanol/ water (60/30/10, v/v/v)
2: n-butanol/acetic acid/water (60/20/20 w/w/w)
3: n-butanol/acetic acid/water (75/20/0.5, w/w/w)
4: chloroform/methanol/water (65/25/10, v/v/v)
5: chloroform/methanol/water (75/20/05, v/v/v).

3.1.2. Chemical Nature of Albodrine

Table 1. *Results of chemical nature determination of albodorine.*

CHEMICAL GROUPS	TESTS	RESULTS
	Mayer	-
Alkaloids	Wagner	-
	Dragendorf	-
Flavonoids, leucoanthocyanins	Willstätter	-
	Bate-Smith	-
Insatured lactones	Kedde	-
Deoxyoses	Keller-Kiliani	+
	Gelatin 1%	-
Tannins and polyphenols	Salted gelatin 10%	-
	FeCl$_3$	-
Quinones	Borntrager	-
Steroids	Liebermann-Burchard	+
Triterpenes		-
Cyanogenic glycoside	Grignard	-
Coumarin		-
Iridoïds	Hot HCl	-
Saponins	Foam test	+

+: positive test; - : negative test

Albodorine appeared as an amorphous powder when dried. It was thermostable, soluble in water and organic solvents. Its bitter taste was another argument for its saponosidic nature.

Albodorine reacted positively to the tests of saponin, steroids and desoxyose (Table 1). These results suggested that albodorine was a saponin with a steroidic aglycone. Acidic hydrolysis liberated three sugars identified as glucose (hexose), arabinose (aldopentose) and rhamnose (deoxy-hexose) (Table 2 and Fig. 3).

Table 2. *Identification of sugars in albodorine acid hydolysate.*

SUGARS	REACTION	RESULTS
Oses	Molisch	+
Aldopentoses	Bial	+
Ketose	Seliwanoff	-

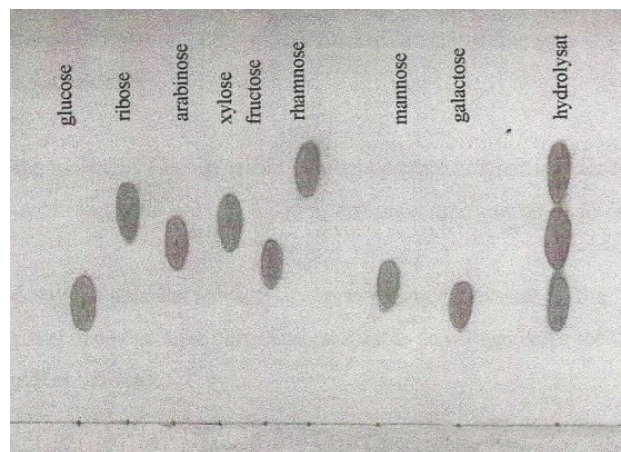+: positive test; - : negative test

Figure 3. *Paper chromatography of albodrine acid hydrolysate.*

3.2. Toxicological Study

3.2.1. Acute Toxicity of Albodorine on Mice

(i). Symptoms and LD50

Albodorine was toxic on mice by both intraperitoneal and oral routes. The symptoms developed by intoxicated animals were mainly nervous system disorders (hypoactivity, ataxia, paralysis of the hind legs, piloerection, clonic convulsions), respiratory disorders (hyperpnea, cyanotic extremities), cardiovascular disorders (vasodilatation, ear hyperemia) and other disorders (abdominal contortion, exophthalmos).
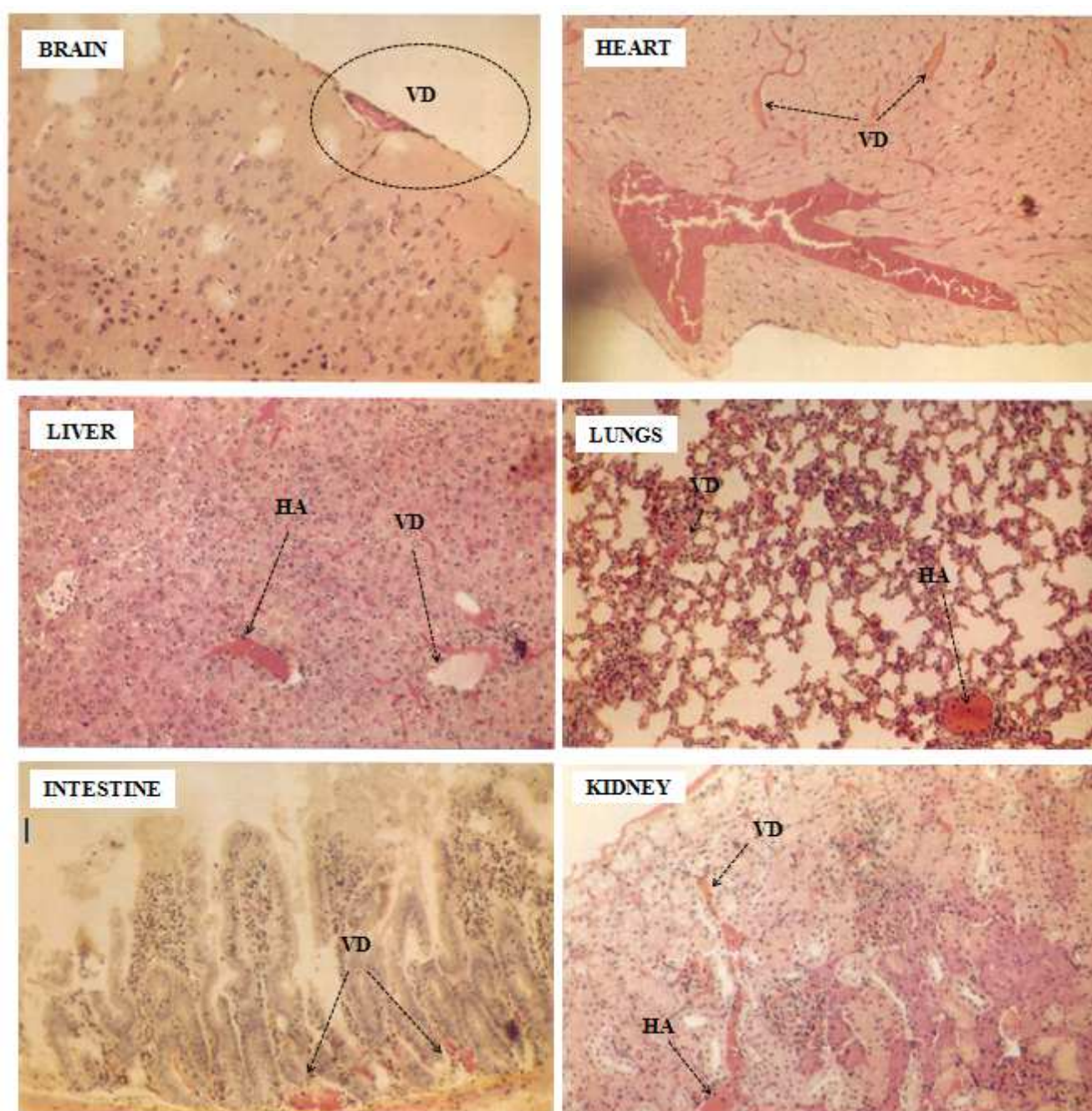
The signs of the nervous system disorders were the earliest and most often observed.

The developed symptoms by both intraperitoneal and oral routes were generally similar but doses used were higher for the latter.

The LD$_{50}$ (24h) of albodorine was assessed at 9 mg/ kg body weight by intraperitoneal route.

(ii). Histopathological Lesions

From a macroscopic point of view, in mice, a larger liver size with blackish hemorrhagic staining was observed, but no visible alteration of brain, heart, lungs, kidneys, liver and intestines was detected. At the tested dose (12 mg/kg), all the examined viscera were affected by albodorine but, purifier organs (lungs, liver and kydney) were the most concerned. Histologic damages resulted in vasodilatation with sometimes hemorrhagic areas (Fig. 4).

HA: Hemorrhagic area; VD: Vasodilatation

Figure 4. Main lesions induced by albodorine (12 mg/kg) on brain, heart, liver, lungs, intestine and kidney.

3.2.2. Effects of Albodorine on Isolated rat Atria

Six concentrations of albodorine in geometric progression of ratio 2 (8 to 256 µg/mL) were tested. At concentrations lower than 64 µg/mL, albodorine had no or non-significant effect. At higher concentrations, albodorine reduced the contractive force of isolated rat atria up to 100 %, demonstrating negative inotropic effect (Table 3). This effect was both concentration and contact time dependent.
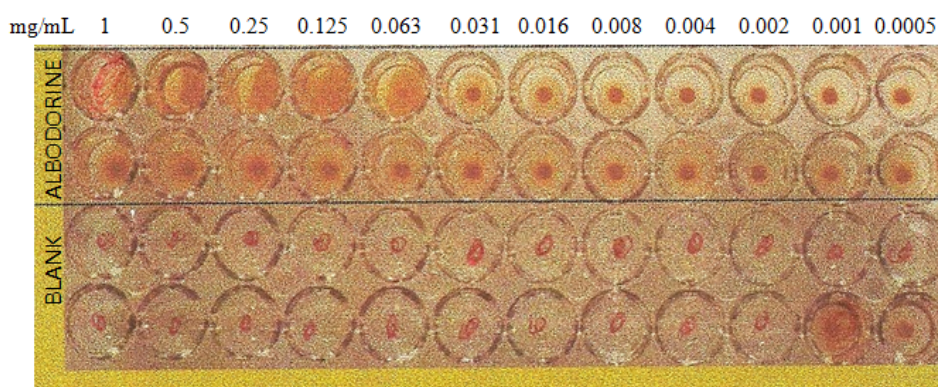
3.2.3. Effects of Albodorine on Sheep Red Blood Cells

Albodorine exerted a lytic activity on sheep red blood cells. The effect varied according to concentration: i) at a concentration lower than 0.016 mg/mL, the toxin had no effect; ii) at a concentration greater than or equal to 0.125 mg/mL, sheep red blood cells were totally altered; iii) at intermediary concentrations, a partial lysis with a sedimentation of sheep red blood cells was observed (Fig. 5).

Table 3. Effect of different concentrations of albodorine on the contractive force of isolated rat atria.

Time in seconds	10	20	30	40	50	60	120	180	300	600	900	1200	1500	1800
Albodorine (µg/mL)														
Blank	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	-2.5	-2.5	-2.5	-2.5	-2.5
16	0	0	0	0	0	0	0	0	0	-2.5	-2.5	-2.5	-2.5	-2.5
32	0	0	0	0	0	0	0	0	0	-2.5	-2.5	-2.5	0	0
64	-16.7	-16.7	-16.7	-16.7	-16.7	-33.3	-33.3	-37.5	-41.7	-58.3	-62.5	-66.7	-75	-83.3
128	-4.16	-4.16	-4.16	-4.16	-4.16	-33.3	-41.7	-66.7	-66.7	-70.8	-75	-100	-100	-100
256	12.5	12.5	8.33	-25	-29.2	-29.2	-54.2	-70.8	-100	-100	-100	-100	-100	-100

Numbers represent inhibition percentage of the contractive force

Figure 5. Haemolytic activity of albodorine on sheep red blood cells.

3.2.4. Effects of Albodorine on Other Warm Blooded Animals and Cold Blooded Ones

Albodorine was lethal to rat and guinea-pig at mice LD100 dose (16.6 mg/kg). It was also toxic to the frog *Ptychadena mascareniensis* tadpoles, the fish *Cyprinus carpio* alvins and the shellfish *Biomphalaria pfeïfferi*. On the contrary, it had no effect on the insect *Culex quinquefasciatus* larvae (Table 4).

Table 4. Toxicity of albodorine on different animal species.

Animal class	Species	LD50 or LC50	Observations
Mammals	Mouse	9 mg/kg	
	Rat	nd	Animals died at mice LD100 dose (16.6 mg/kg)
	Guinea-pig	nd	Animals died at mice LD100 dose (16.6 mg/kg)
Amphibians	*Ptychadena mascareniensis* (apode frog tadpoles)	16.71 µg/mL	-
	Ptychadena mascareniensis (two-legged frog tadpoles)	34.19 µg/mL	-
Fishes	*Cyprins carpio* (alvins)	5.62 µg/mL	-
Shellfishes	*Biomphalaria pfeïfferi*	75.01 µg/mL	LC95 à 245.45 µg/mL
Insects	*Culex quinquefasciatus* (larvae)		No observed effect at concentrations as high as 500 µg/mL

nd: not determined

4. Discussion

4.1. Chemistry

The results obtained concerning the physicochemical and biological properties of albodorine were among saponosides characteristics [26]. The molecule comprised sugars commonly encountered in saponins. The genine (aglycone) behaved like steroid. Those data suggested that albodorine was a steroidal saponin. Most of the seed extracts from *Albizia* Madagascar we have studied so far reacted positively to steroid test but not to triterpene one [4]. This was somewhat surprising because according to literature, the main saponins isolated from *Albizia* genus are triterpenoidal saponins and there are no reports on the literature about the isolation of steroidal saponins from species of this genus [8]. In addition, triterpenoid saponins are mostly found in dicotyledonous species, while many of the major steroidal saponins are synthesized by monocots, such as members of the Liliaceae, Dioscoreaceae and Agavaceae families [27, 28]. However, steroidal saponins were isolated from some dicotyledonous Angiosperms as *Tribulus pentandrus (Zygophyllaceae)* [29], *Lysimachia paridiformis* (Primulaceae) [30], *Solanum chrysotrichum* [31] *Solanum melongena, Solanum lycopersicum* [32] *Capsicum frutescens* [33] (Solanaceae) and *Paullinia pinata* (Sapindaceae) [34]. There is no clear relationship between the plant origin and the type of saponin, nor is there evidence that specific saponins are associated with particular parts of plants [35].

According to Deore *et al.* [36], the structural complexity of saponins results in a number of physical, chemical, and biological properties only a few of which are common to all members. So, it is permitted, given the endemicity of *Albizia odorata* to expect that albodorine is a new saponin.

Albodorine was isolated from seed of *A. odorata*. Further investigations will inform whether it is specific of seed or also found in other parts of the plant.

4.2. Toxicological Properties

From a toxicological point of view, compared to *Albizia* species from Madagascar so far studied, *A. odorata* is one of the most toxic on mice. Its toxicity was near that of seed methanolic extract from *A. viridis* (LD50 of 6.72-8.04 mg/kg) and *A. divaricata* (LD50 of 5.33-7.39 mg/kg) [1-6]. In comparison to mouse intraperitoneal LD_{50} of saponins from foreign *Albizia* species, albodorine was more toxic than proceranin A from *Albizia chinensis* seeds (LD50 of 15 mg/kg) but less toxic than albitocin from *A. adianthifolia* root bark (LD_{50} of 6 mg/kg) [37]. Finally, compared to well-known non saponosidic toxins [37], albodorine was as toxic as nicotine (LD50 of 10 mg/kg) but much less toxic as strychnine (LD50 of 0.98 mg/kg) and rotenone (LD50 of 2 mg/kg).

The main lesions found in all organs and the high toxicity of albodorine to cold blooded animals confirmed the saponosidic nature of albodorine.

5. Conclusion

In the light of the chemical and pharmacological data obtained, it can be concluded that the toxic principle of *A. odorata* seeds could be a saponin with a steroidal aglycone group and sugar moiety comprising glucose, arabinose and rahmnose. Comprehensive chemical studies, which are in progress, will precise the exact albodorine structure and provide answers to some questions raised above.

Albodorine was the first saponin isolated from Malagasy *Albizia*. A rational use of albodorine in pest control must take account of its high toxicity and its broad enough activity spectrum.

Keeping in mind the various pharmacological properties concerning the saponosides isolated from foreign *Albizia* and saponosides in general, investigations are ongoing for exploring other properties of albodorine.

Our results contributed to a better knowledge of poisonous plants from Madagascar, especially the endemic *Albizia* species.

Acknowledgment

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