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Augue Toxicity Test and Histopathological Study of *Hyoscyamus Muticus* L. Subsp *Falezlez* (Coss) Maire

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ABSTRACT

Hyoscyamus muticus L. subsp *falezlez* (Coss) Maire is a toxic species, highly responded to in the Algerian Sahara, and played an important role in the traditional medicine of the Tuareg. It is one of the Solanaceae richest in tropane alkaloids (especially atropine), which constitute a large group of secondary metabolites.

The objective was to study its toxicity to exploit the data in the valorisation of this endemic species.

The acute toxicity study and the calculation of the LD_{50} were carried out on Wistar mice and using the tinctures of the plant collected from the Adrara Sbaa station (southwest of Algeria). A histopathological examination of the organs after the mice dissection was carried out.

LD_{50} determination from the plant tinctures confirmed its extreme toxicity and was estimated at 0.85± 0.27g/kg dry plant. Functional abnormalities were detected in the heart and liver of mice injected with the lethal dose (6.3g/kg); significant vasodilation was observed.

Content of the results obtained, the toxicological potential of the plant has been highlighted especially in acute. However, it would be interesting to explore chronic toxicity.

1. Introduction

Henbanes are known to Saharan populations for their high toxicity, the most toxic being Desert Henbane (*Hyoscyamus muticus* L. subsp *falezlez* (Coss) Maire)[1]. It was discovered in 1881 by the French army after the poisoning of Colonel Flatters' team by the Tuaregs in the Algerian Sahara. *Hyoscyamus muticus L.* subsp *falezlez* (Coss) Maire is part of traditional Tuareg medicine and is used: in back pain treatment, muscle cramps, lice, and eye inflammation, as well as for spasms, palpitations, and anxiety. Only experienced tradipraticians know how to handle and advise it[2]. Seeds and leaves are used to fatten goats[3].

In Egypt and Morocco, it is employed to relieve painful spasmodic affections and irritable coughs and as an antipyretic, mixed with other plants. The poultice of fresh leaves soothes pain, and the smoke-like leaves in cigarettes are effective against asthma[4, 5].

In pharmacy, the Saharan henbane (*Hyoscyamus muticus* L. subsp *falezlez* (Coss) Maire) interests the pharmaceutical industry thanks to its wealth of tropane alkaloids represented mainly by atropine, an important anticholinergic belonging to the essential drugs on the WHO list [6]. Hysocyamin and scopolamine are the major constituents of this jusquiame extract and are among the most interesting bioactive metabolites of the plant group; they play a role as active ingredients in several medicines despite the rise of synthetic products[6]. The alkaloid content varies depending on the location; the Egyptian henbane exceeds 1%, reaching 5% for cultivated species. However, some jusquiames of Iran do not seem to be very rich (0.027%) or even black jusquiame, which barely reaches 0.05%[1, 6]. For the Algerian Hanebane, it varies from 0.5 to 4%[7-9]. In addition to alkaloids, Saharan Hanebane contains polyphenols, including flavonoids and traces of essential oils[8].

The acute toxicity study establishes the relationship between the administered dose and the intensity of the observed effects and the calculation of the LD_{50} with its confidence limits[10]. It is the first step in toxicological studies to get an idea of the toxicity zone of a given substance through a qualitative and quantitative assessment and their evolution over time following the administration of a single dose[10]. As such, LD_{50} remains an important measure for assessing the plant toxicity[10]. Toxicity studies of tropanic tropane alkaloids have involved certain Solanaceae, mainly Datura and Belladonna ssp[11]. The LD_{50} of Datura was noted at 360 mg/kg[12-14]. *Hyoscyamus albus* LD_{50} was estimated in mice to be 2000mg/kg of Body Weight[15]. For Belladonna, a study using albino Wistar mice of both sexes and from plant tinctures recorded an LD_{50} from 16g/kg tincture and an atropine content of 229mg/kg and 328mg/kg scopolamine[16].

Several studies and treaties have reported the symptoms of Saharan henbane poisoning[2, 12, 17, 18], but no study in Algeria has evoked its acute toxicity. There is a lack of studies evaluating the acute toxicity of this important pharmaceutical plant. It is in this context that our objective is to study the toxicity of the Saharan henbane of Algeria by calculating its LD_{50} and describing the anomalies identified during the test by a histopathological study of the organs.

2. Material and methods

2.1. Material

For the plant material, alcoholic tinctures were prepared, from the powder of the whole plant of Saharan Henbane harvested from the Adrar Sbaa area (South West of Algeria, GPS: 28.205, -0,172) (Figure 1). The plant was identified by Pr KAZI TANI C, a Taxonomist and plant illustrator at Organic Chemistry, Natural Substances and Analysis Laboratory (C.O.S.N.A.), Abou bekr Belkaid University, Tlemcen; which awarded hit the Voucher index (**HSYMU0001)**. After drying and treatment at the Pharmacognosy laboratory of the Faculty of Medicine of Tlemcen, the plant was crushed and kept dry.

The animal species used in the test is the Mouse Wistar albino variety, weight: 25 +/-gr, male and female. The mice were procured from the Pasteur Institute in Algiers and received at the pet shop of the pharmacology laboratory of the Faculty of Medicine of Oran for the acute toxicity test. They were deprived of power the day before the test, as recom-

mended by protocol[16].

2.2. Methods

The Saharan Henbane toxicity test was conducted between February and March 2022, and only the minimum lethal dose (MDD) and lethal dose 50 (LD50). The protocol followed was that of an optimized method that involved belladonna.

2.2.1. Batch Preparation

Six batches of doses with control were made. Each batch was composed of 8 mice (4 males and females). The total number of mice was 48 (24 males and 24 females)

The dose progression was chosen based on the LD_{50} taken from the belladonna monograph taking into the ratio of the alkaloid content of Saharan henbane to lethal dose 0 (LD_0). The LD_{50} (16g/kg) was divided by a factor (1.5)2 according to the protocol[16]. The rate of dose progression in clinical or toxicological trials is based on multiplication times a 1.5, 2, 3, and 5 rate factors, the lowest factor was chosen. Saharan henbane is eight times more concentrated than Belladonna[19], so we started from the LD_{50} (1.8g/kg). The calculated doses administered to each batch are summarized in the table I.

From the dry drug doses calculated from the table I, tinctures of the plant were prepared for each batch, depending on the weight and volumes to be administered.

The tinctures were obtained from dried and crushed powder of Saharan henbane and those for each batch. 70° ethanol was used following protocol[16]. The extraction residues obtained after filtration and gentle concentration of dyes, were kept in a dry and cool place until the day of the test. Thin layer chromatography was performed for a tincture sample to qualitatively highlight the tropane alkaloids majority (atropine and scopolamine) using controls. The procedure followed is that of the European Pharmacopoeia 10th edition. The mobile phase was composed of a mixture (Acetone/H_2O/NH_4OH), (90:7:3); the mixture was introduced into the chromatography tank (20x15x 10 cm) to saturate it with solvent vapours. Controls: Hyoscyamine sulphate monohydrate (Pharmacopeia reference Standard, Id: 006XO1) and Scopolamine hydrochloride (Sigma–Aldrich, PHR1470) were solubilized in mL of methanol and deposited with the sample on a silica plate (Silica Gel G). The detection was carried out after migration and drying of the plate with Dragendorff reagent (potassium iodobismuthate solution) until the appearance of red-orange spots[20].

2.2.2. Pilot test

The pilot test is carried out on a small animals number (three mice) to determine the DMM (minimum lethal dose), which allows the theoretical rhythmic doses (Table I) to be adjusted to launch the test[10]. The doses involved in the pilot trial were the lowest and highest dose in the table.

2.2.3. Acute toxicity test

The residue extraction (from the six doses) was solubilized in 20° alcohol (1.5ml for each dose). After weighing all the mice and organizing the different batches, the extract of each dose was administered as an intraperitoneal injection, and the volume administered was 0.5 mL[16, 21]. The mice in the batch control were administered by 0.5mL of 20° alcohol.

2.2.4. Exams and Observation

After dose administration, the animals were observed for three hours[10]. The dead underwent a dissection just after for an anatomy-morphological analysis. Those who remained alive were sacrificed after seven days and then dissected to analyse organs and tissues.

An autopsy is mandatory for all animals killed in the trial and some survivors to provide information on vital or target organs[10]. It includes macroscopic and histopathological examinations revealing changes.

Tolerance is judged not only by animal survival or death but also by clinical criteria[16], so the follow-

ing parameters were noted:

-The number of deaths;

-Clinical observations and behaviour (agitation, sedation, abnormal movements, ataxia, light sensitivity, tail straightening);

-Autopsy: Macroscopic examinations of the organs (Heart, liver, and kidneys) of dead and living mice sacrificed after seven days of observation; the sacrificed mice were: dead mice (having been injected by the fatal dose 6.3g/kg), as well as living mice (healthy and low-dose 0.55g/kg mice sacrificed after one week of follow-up). Preparation of histological sections of organs were carried out in the histopathology laboratory BELARBI, Tlemcen, according to a rigorous protocol allowing us the preservation and fixation of histological sections of organs[22].

2.2.5. Calculating the LD50

Two comparative methods were used, numerical and graphical:

-Mathematical method (Karber and Behrens method)[23].

$$LD50 = (LD100 - \sum ab)/n$$

a: Difference between two successive doses
b: Average deaths between two successive doses
n: Average number of animals per lot

-Graphic method of Miller and Trainter[24]

The graphic LD_{50} is obtained after the calculation of the percentages of mortality as a function of the logarithm of the dose from a curve in sigmoid, called the Trevan curve, which can be linearized, by appropriate means (calculation of the problems); the LD_{50} is deduced (expressed in mg per kg of body weight calculated as standard deviation).

To increase the accuracy of the results and make the curve linear, the mortality percentage was represented as a function of the dose log on log-probit paper.

To use the percentages 0 and 100% whose probits tend towards infinity, they were replaced by corrected values. Since the products of the 0% and 100% mortality statistically tend towards infinity, these are replaced by corrections:

0% correction: Y0= 50/n

correction of 100%: Y100= (100n-50)/n is the number of animals used in each of these batches.

n: the number of animals per batch

-Calculation of standard deviation S

It improves the meaning of LD50 (confidence limits)[15]. Standard deviation is given by the following formula:

$$S = (LD84\% - LD16\%)/2$$

-Calculation of deviation from average δ

For greater accuracy, the deviation from the mean is calculated using the formula below:

$$\delta = 2S/\sqrt{2n'}$$

n' is the total number of animals in the batches with mortality percentages between 7% and 93%.

7% is the No Observed Adverse Effect Level (NOAEL)

93% is the percentage corresponding to the last dose giving stable effects.

So the graphic LD_{50} is calculated as follows:

LD_{50}= LD_{50} graphique±

3. Results

3.1. Alkaloids identification

Thin Layer chromatography of the dye has highlighted, after detection by Dragendorff reagent, a large spot corresponding to hyoscyamine, accompanied by scopolamine and other unidentified compound probably corresponding to polyphenols, since the developer used, reacts with polyphenols (Figure 2).

3.2. Pilot Test Result

After the pilot test, a dilution of ½ was required at the theoretical initial doses. At the end of the pilot test, the DMM was deduced and was equal to 0.266g/Kg[10]**.** From this dose it was possible to choose a low-

Figure 1. *Hyoscyamus muticus L. Subsp falezlez (Coss.) Maire, Adrar Sbaa harvesting station*

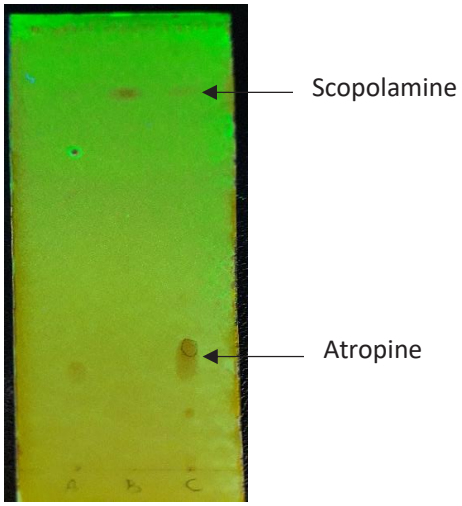


Figure 2. *Chromatography of Hysocyamus muticus L. subsp falezlez (Coss.)Maire, observed at 365 nm A: Atropine, B: Scopolamine, C: Plant achoholic extract*

Figure 3. *Mouse treated with 0.55g/kg IV dose of plant extract*

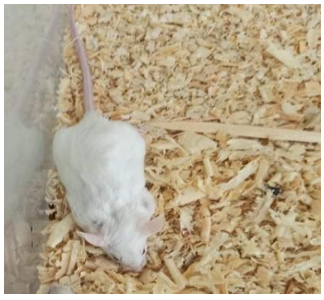


Figure 4. *Mouse treated with 6.3g/kg IV dose of plant extract*

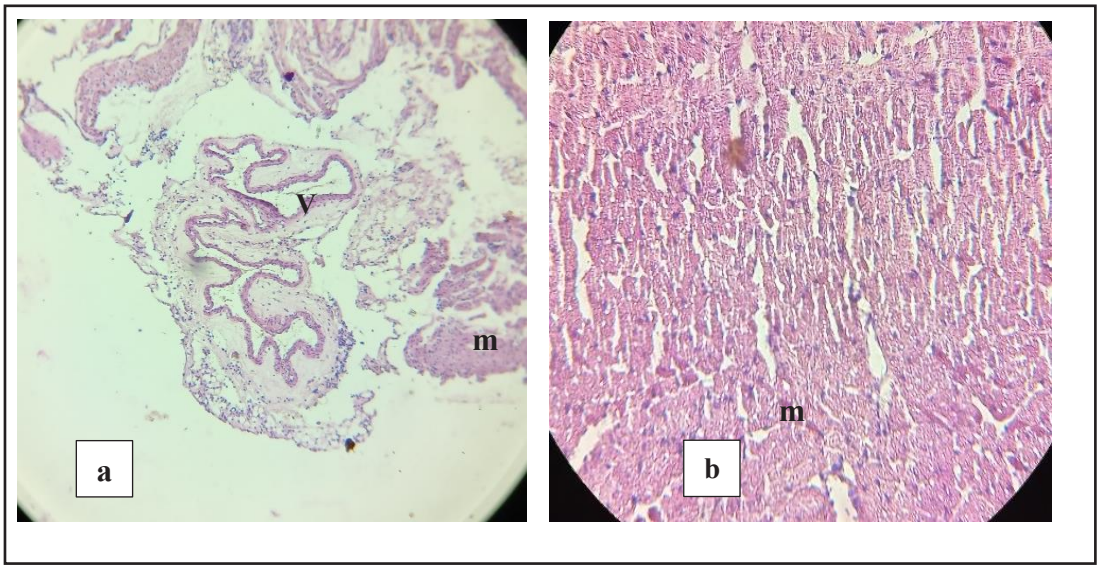


Figure 5. *Microscopic observation of a slice of the heart of the control mouse. a: 10x10, b: 10x40, V: vessel, m: heart muscle*

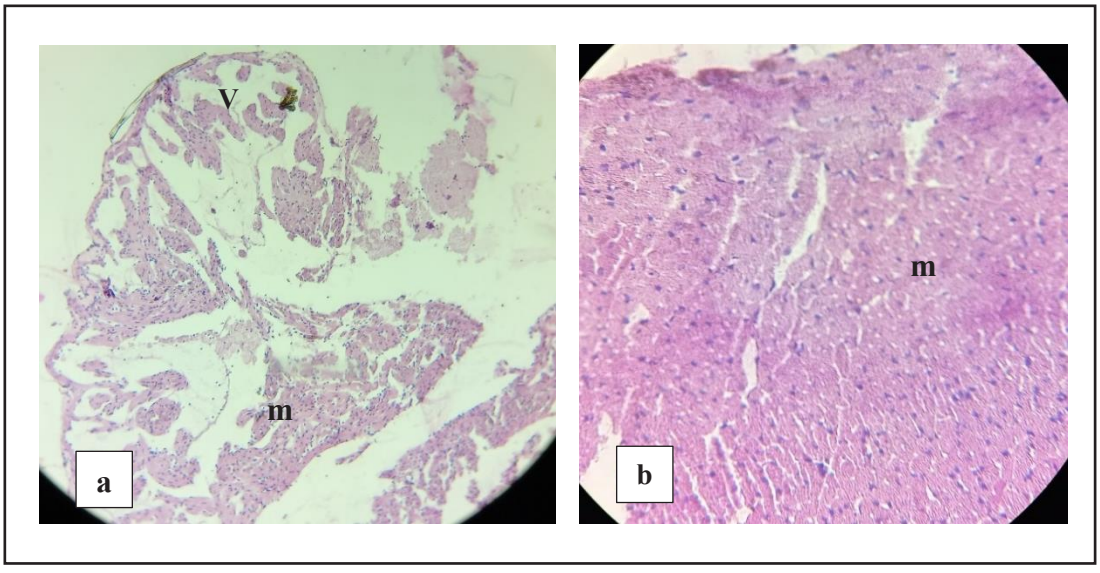


Figure 6. *Microscopic observation of a slice of the heart of a mouse injected by the dose 0.55g/kg. of plant extract*

a: 10x10, b: 10x40, V: vessel, m: heart muscle

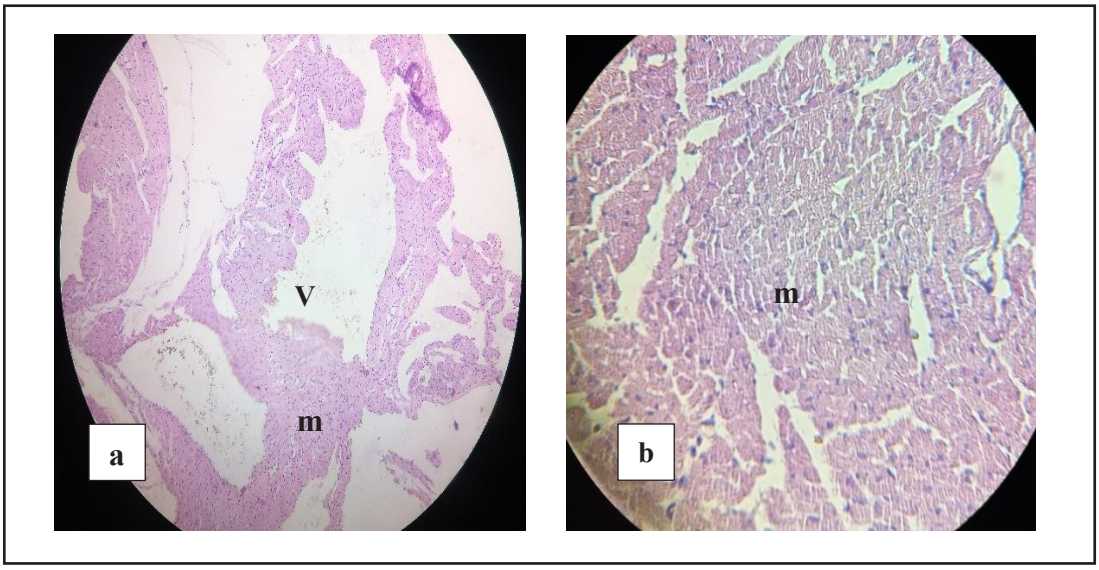


Figure 7. *Microscopic observation of a slice of the heart of a mouse injected by the fatal dose 6,3g/kg of plant extract*

a: 10x10, b: 10x40, V: vessel, m: heart muscle

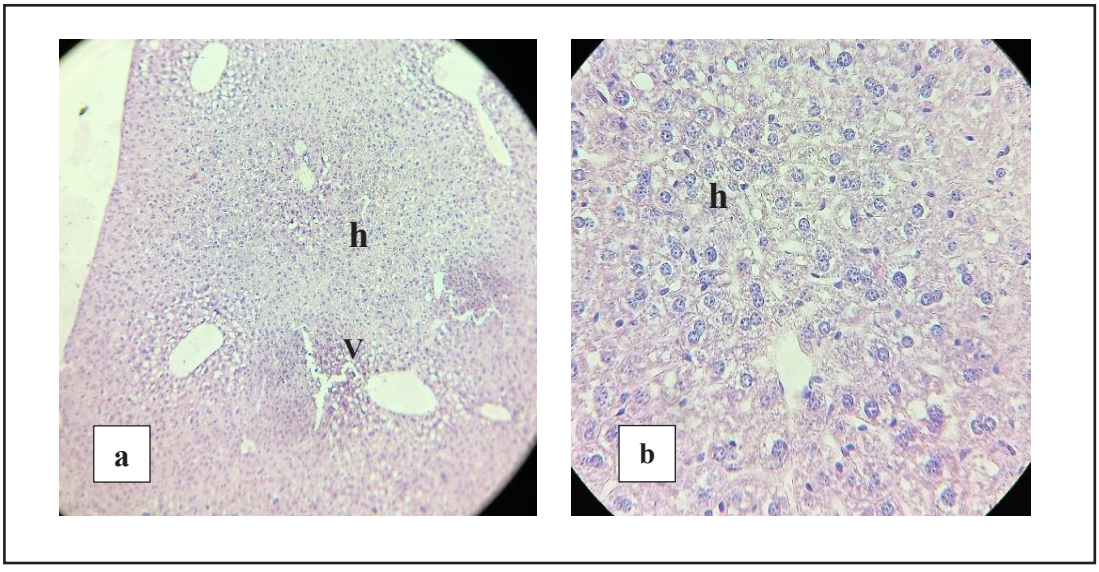


Figure 8. *Microscopic observation of a slice of the liver of a control mouse a: 10x10, b: 10x40, h: hepatocytes, V: vessel*

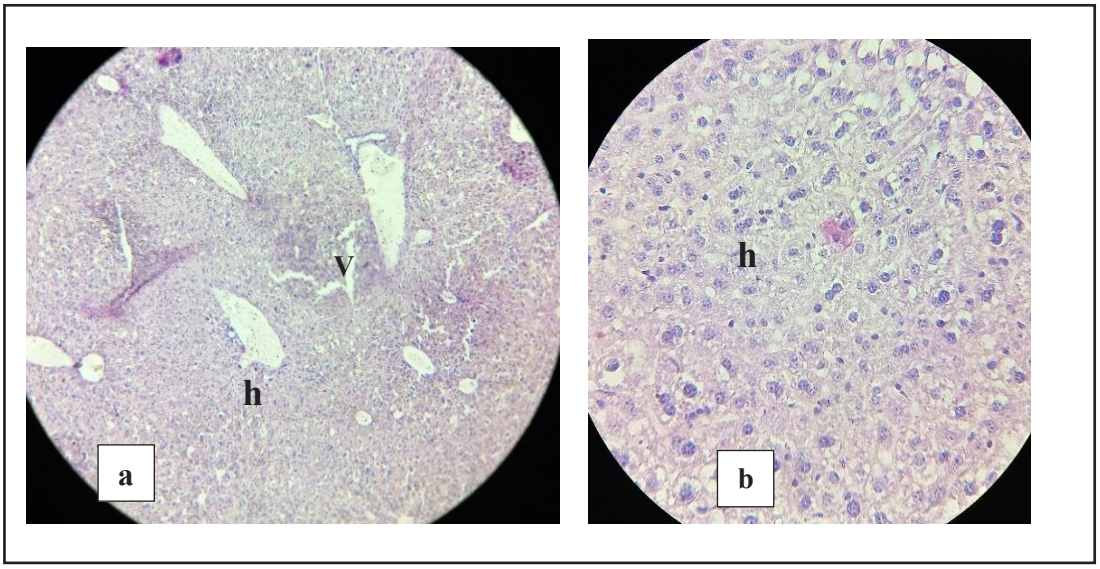


Figure 9. *Microscopic observation of a slice of the liver of a mouse injected by the dose 0.55g/kg. of plant extract a: 10x10, b: 10x40, h: hepatocytes, V: vessel*

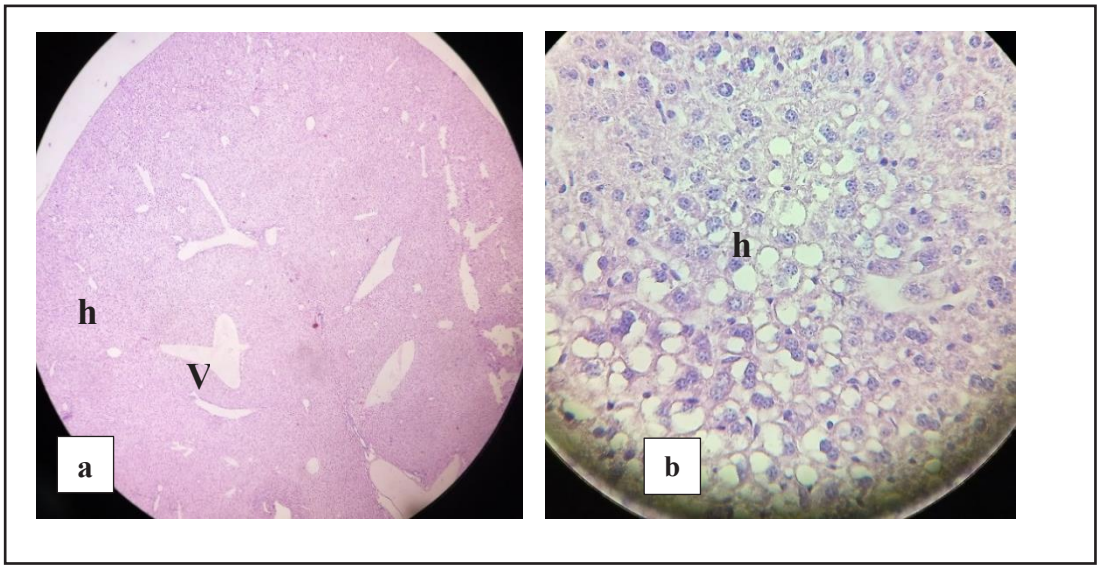


Figure 10. *Microscopic observation of a slice of the liver of a mouse injected by the lethal dose 6.3g/kg of plant extract*

a: 10x10, b: 10x40, h: hepatocytes, V: vessel

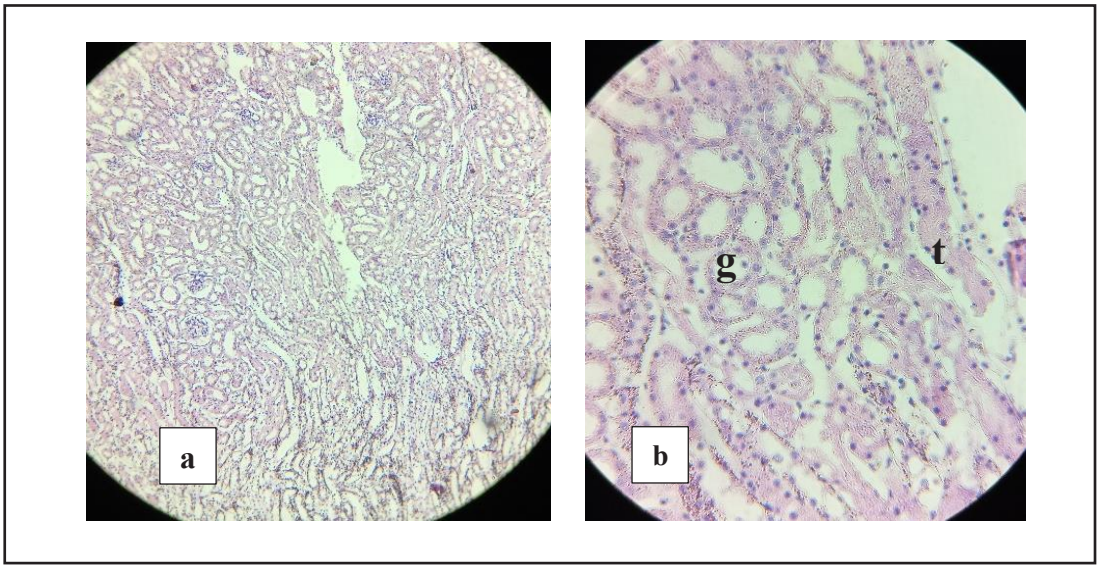


Figure 11. *Microscopic observation of a kidney section of a control mouse a: 10x10, b: 10x40, t:bypassed tube, g: glomerulus*

er dose and 4 higher doses, they are represented in Table II.

3.3. Acute Toxicity Test Results

Mortality was assessed after 1 hour of dye administration[10].The mortality rate is summarized in (Table III). The mice in the batch control stayed alive.

3.2.1. Result of the mathematical method (Karber and Behrens method)[22]

The mathematical LD_{50} was estimated to be 0.833g/Kg, and the difference between two successive doses (a), the mean death between two successive doses (b), and the mean number of animals per batch (n), for calculation are summarized in Table IV.

3.2.2. Result of Miller and Trainter's Graphical Method[24]

The graph LD_{50} was obtained from the Trevan curve (see Appendix 1) after the calculation of the correction of percentages of 0 and 100% mortality, standard deviation (S), and deviation from the mean and are respectively: 6.25, 93.75, 0.55, 0.27. The graphic LD_{50} is estimated at 0.85± 0.27g/kg. The number of deaths and the percentage of mortality are summarized in Table V.

3.2.3. Symptoms Observed in the Toxicity Test

The injection of the lowest dose (0.55g/kg) during the pilot test, in IV (to assess the effect quickly) caused from the first minute a partial paralysis and a reduction in activity as well as tachycardia and a cyanotic tense tail. Mydriasis occurred after 10 minutes and caused the mouse to close its eyes and curl up. Paralysis lasted 10 minutes and then activity was slowly resumed (Figure 3).

Injection of the lethal dose of 6.3g/Kg in IP resulted in complete paralysis within 30 seconds of administration, tachycardia and then swelling of the body, death occurred after 10 min, and the animal became

Table I: Theoretical Doses To Be Administered To Each Lot Of The Acute Toxicity Test

Batch n°	Rhythmic doses	Dry drug doses (g/kg)
1	$LD_{50}/ (1.5)^3$	0.55
2	$LD_{50}/(1.5)^2$	0.8
3	LD_{50}	1.8
4	$LD_{50} x (1.5)^2$	4.2
5	$LD_{50} x (1.5)^3$	6.3
6	$LD_{50} x (1.5)^4$	9.5

Table II: Doses Administered To Each Lot Of Acute Toxicity Test After Batch Test

Batch n°	Doses	Dry drug dose (g/kg)
1	$LD_{50}/ (1.5)^3$	0.183
2	$LD_{50}/(1.5)^2$	0.266
3	LD_{50}	0.6
4	$LD_{50} x (1.5)^2$	1.4
5	$LD_{50} x (1.5)^3$	2.1
6	$LD_{50} x (1.5)^4$	3.16

bluish. IV injection of the same dose resulted in death within seconds of administration (Figure 4).

3.2.4. Results of organ dissection and histopathological study

-Macroscopic review

After dissection of dead mice (injected with lethal dose 6.3g/kg), as well as live mice (healthy and low dose 0.55g/kg mice sacrificed after one week of follow-up), macroscopic organ analysis showed no difference, so no significant difference was observed about organ weight or morphological abnormality. The organs were smooth, non-fibrous, and shiny, retaining their colour for the sacrificed mouse holes.

-Microscopic examination

After staining the organs of the sacrificed mice (Control, live injected at low dose, dead at high dose), pathological anatomy analysis showed the following results:

-For the heart: The cardiac muscle is normal without necrosis for the three mice (Figures 5-7), concerning the vascular tissue, the vessels of the mice (control and low dose) are comparable, however, significant dilation was noted for the lethal dose in mice (Figure 7).

-For the liver: Preservation of hepatocyte nuclei, chromatin is not dense and tissue is well preserved, no dilatation of vessels or infiltration of polynuclear was observed, and no evidence of nucleus hypertro-

Table III: Mortality Rate After One Hour Administration

Batch n°	Dose	Dry drug dose (g/kg)	Number of animals	Number of dead
1	$LD_{50}/ (1.5)^3$	0.183	8	0
2	$LD_{50}/(1.5)^2$	0.266	8	0
3	LD_{50}	0.6	8	5
4	$LD_{50}x (1.5)^2$	1.4	8	6
5	$LD_{50}x (1.5)^3$	2.1	8	8
6	$LD_{50}x (1.5)^4$	3.16	8	8

phy or chromatin condensation was observed for all three mice (Figures 8-10), however vessel dilation in the low dose and lethal dose and which is greater in the lethal dose.

A discrete steatosis was observed for the 3 cases without tissue lesions or necrosis, due to the diet of mice.

-For the kidney: No abnormalities in the nuclei of the cells of the bypassed tubes or glomeruli were observed (Figures 11-13).

4. Discussion

LD_{50} is an index that is frequently used to express acute toxicity as well as to classify toxic substances however, it gives little information on the mechanisms involved and the nature of the lesions generated during the test since it concerns only mortality. Only functional abnormalities such as vasodilation were revealed during the microscopic examination of organs without signs of necrosis however, it allows a preliminary assessment of the toxicity of the tested substance.

In our acute toxicity test, the LD_{50} was estimated to be 0.85± 0.27g/kg of dry drug *Hyoscyamus muticus* L. subs *falezlez* (Coss.) Maire, which is significantly higher than belladonna[16], which makes sense given the richness of Saharan henbane compared to belladonna. This is consistent with the levels obtained in the belladonna study[16] and those reported in other studies[25, 26]. The results confirmed its extreme toxicity.

Symptoms of poisoning observed in Wistar mice after administration of toxic doses were marked by paralysis and reduced activity as well as tachycardia and mydriasis, the animal curled up and died bloated and bluish. This description was reported in the notes on Saharan henbane[3,27].

Concerning the pathological examination of the organs of the sacrificed mice, the tissues of the organs of the three mice (control, died by the high dose, and those sacrificed), were preserved without structural abnormality or signs of necrosis. This is due to immediate death since the trial only involved the assessment of acute toxicity, but functional abnormalities were detected in the heart and liver of the mouse injected with the lethal dose; significant vasodilation was observed. Theoretically, hyoscyamine and especially atropine is a parasympatholytic at the vascular level, there is no para-sympathetic tone but its action blocks that of acetylcholine, so in low or therapeutic doses, it leads to vasoconstriction[10]. At high doses, the noted vasodilation can be explained by a pharmacodynamic mechanism by indirect strengthening synergy and by the antagonistic function of acetylcholine suppression. This experiment has been demonstrated only on dogs by studying the hypertensive action of adrenaline after injection of atropine at high doses

Table IV: Calculation Of LD50 By Mathematical Method

Doses of dry drug administered (g/kg)	Number of dead	a	b	ab
0.183	0	0.083	0	0
0.266	0	0.334	2.5	0.835
0.6	5	0.8	5.5	4.4
1.4	6	0.7	7	4.9
2.1	8	1.06	8	8.48
3.16	8	-	-	-

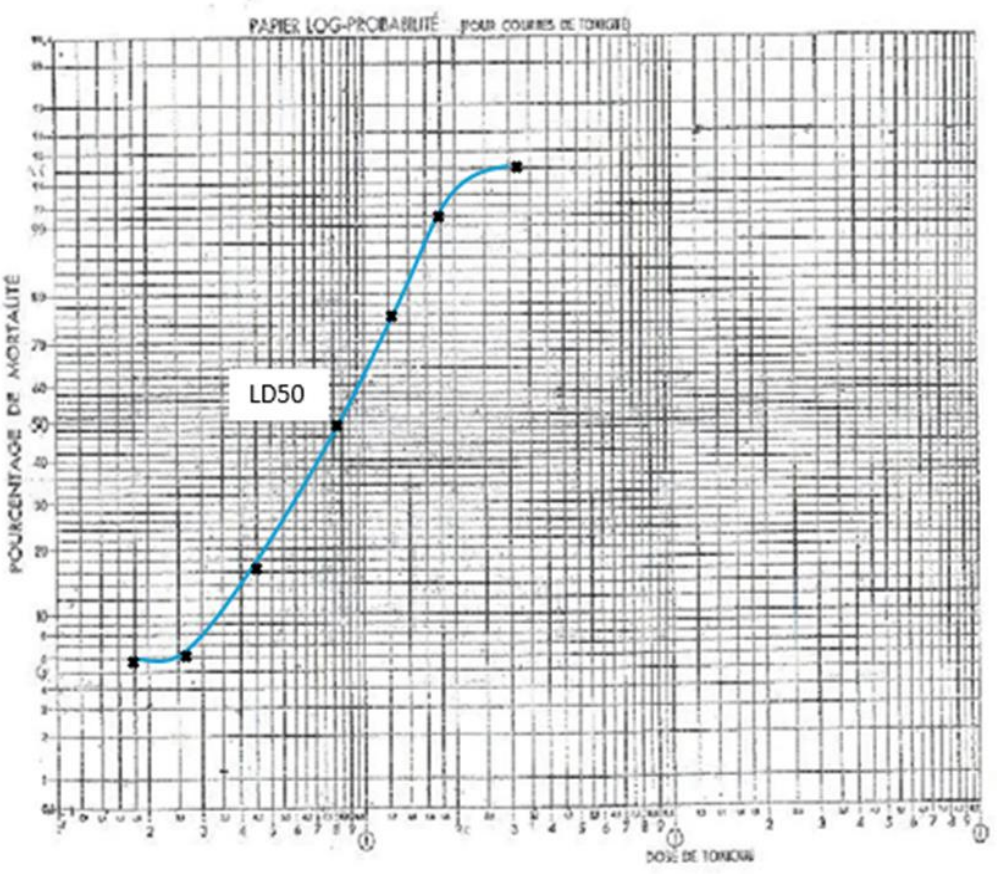
Table V: Percentage Mortality

Doses of dry drug administered (g/kg)	Number of dead	% Mortality
0.183	0	0
0.266	0	0
0.6	5	62%
1.4	6	75%
2.1	8	100%
3.16	8	100%

and the appearance of effects by bradycardia vagal reflex abolition[22]. No other tests were performed on other mammals. The observation may be an addition to the plant pharmacotoxicology.

4. Conclusion

The LD50 index is used to express the acute toxicity threshold and to classify and compare toxics. However, it has limited value since it concerns only mortality and gives no information on the reaction mechanisms involved and the nature of the lesions caused. It is therefore, a preliminary assessment however the test carried out on the Saharan Henbane of Algeria could confirm once again its high toxicity in acute. It would be interesting to explore chronic toxicity. The LD50 was estimated to be 0.85± 0.27 g/kg dry plant. Histopathological examination revealed significant heart and liver vasodilation at the injected dose of 6.3 g/kg dry plant. □



Appendix 1. *LD_{50} graph on the Trevan curve*

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