Toxicity of hypaphorine from *Astragalus lusitanicus*.

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Abstract:
While looking for the toxic principle of *Astragalus lusitanicus* Lam., an herb highly toxic in livestock especially in small ruminants, hypaphorine, the N,N,N-trimethyl tryptophan betaine was isolated. This alkaloid was identified, for the first time in *Astragalus lusitanicus*, using NMR and MS methods. It was synthesized in sufficient amount and tested for potential toxicity in mice and lambs. In this study it is show, with good evidence, that hypaphorine could not be hold responsible for *Astragalus lusitanicus* poisonings in small ruminants.

Keywords: *Hypaphorine, Astragalus lusitanicus, toxicity, small ruminants.*

Astragalus lusitanicus (fabaceae) is found in some Mediterranean countries, where it was reported to be toxic for livestock, especially in Spain, Portugal and North Africa [1, 2, 3, 4]. In Morocco, this plant is frequently responsible of intoxication in sheep and goats, especially in young animals [1, 5, 6].

The poisonous species of *Astragalus* may be classified, on the basis of their toxic principles, into three main groups: (a) The group of the species termed locoweeds which is known to contain the alkaloid swainsonine [7, 8]. (b) The group of those containing the nitro compound: miserotoxin [9, 10, 11, 12]. (c) The group of species that are selenium accumulators [13].

The toxic *A. lusitanicus* in Morocco does not contain neither swainsonine nor miserotoxin and contains only very low concentrations of selenium [14]. However, the clinical signs and lesions produced in intoxicated animals are dominated by epileptic crisis and cytoplasmic vacuolization in brain and other tissues [5, 6, 15], and are similar to those caused by locoweeds [16].

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Knowing that swainsonine is highly water soluble, it was found in the aqueous extract of the plant a compound, which gives with Erlhich reagent a purple color on TLC like swainsonine. This compound was isolated and identified as hypaphorine. In the literature, this alkaloid was the subject of conflicting reports on its toxicity. On one hand it is considered as neurotoxic agent [17] and on the other hand it was reported to have only little pharmacological activity [18].

In order to verify if hypaphorine plays a role in the toxicity of *Astragalus lusitanicus*, it was synthesized in sufficient amount and administered at relatively high doses to mice and lambs. Herein are reported the results of this study.

Materials and methods

Plant materials

Astragalus lusitanicus was collected in December 2001 in the region of Sidi Bettach, about 30 km south of Rabat, in Morocco. In this area, frequent animal poisoning, by this plant species, are recorded each year in sheep herds.

Isolation and purification of hypaphorine.

One kg of fresh plant material was macerated in 2 liters of water, under continuous shaking, during 24 h. The filtrate was evaporated under reduced pressure to yield 30 g of a dry extract.

6 g of the extract were dissolved in a small volume of water and chromatrogaphed on reversed phase Silica gel RP2 (0.063-0.2 mm Merck), eluted with water and water methanol mixture. Thirty fractions were obtained. They were controlled using TLC under UV light and after spraying with the Erlhich reagent. The fraction number 14 contains a major compound **X**, which shows a purple color with Erlhich reagent. It was purified by preparative TLC using: Chloroform: methanol: ammonia (7/2.6/0.4) as a solvent system for migration. This compound, identified to hypaphorine (21 mg) is colorless, mp 254-255 °C.

Synthesis of hypaphorine

Hypaphorine was synthesized according to the method of Van Romburgh and Barger [19]. The L(-)-S-tryptophane 2.04 g (1 mmol) was dissolved in 25 mL of methanol and added with a solution of 0.4 g of sodium hydroxide in 2 mL of water. Then 8.52 g of methyl iodide (6 mmol) were added to the mixture under magnetic stirring at 20-25 °C. The solution was maintained alkaline, by addition of small portion sodium hydroxide (0.85 g) in 2 mL of water, during 24 h. After evaporation of methyl iodide (bp. 42.5°C), the mixture was heated at 65°C during 1h to hydrolyze the methyl ester to an acid. The solution was then acidified by addition of HCl (10%). The hypaphorine chloride precipitate was washed with methanol, dissolved in methanol-ammonia and then chromatographed on Sephadex LH20 (30 g), eluted with methanol. The crystals formed in the first fractions are pure hypaphorine (1.8g): mp 252-255°C, $[\alpha]_D$ +140°C (C 1, H_2O), MS (Electro-spray): m/z 247 $[MH]^+$, 1H and ^{13}C NMR (table 1). These data agreed with those in the literature for this compound [20].

Animals

Three local breed lambs were used. They were purchased from the animal market of Sale and housed in a sheep box in the barn of the Institute of Agriculture and Veterinary Medicine, Rabat. They were fed good quality forage and water *ad libitum*. After one week of acclimatizing, they received one administration of hypaphorine solution in water (100 mg/mL) by oral route.

N°	^{13}C δ (ppm)	^{1}H δ (ppm)	
1	-	NH	10.17 s
2	125.11	CH	7.11 s
3	108.96	C	-
4	128.27	C	-
5	119.05	CH	7.50 d
6	120.04	CH	7.02 t
7	122.60	CH	7.05 t
8	112.45	CH	7.35 d
9	138.04	C	-
10	24.65	CH_2	3.20 d
11	80.56	CH	3.72 t
12	171.62	COO-	-
13	52.66	$N(CH_3)_3$	3.07 s

Table I : ^{1}H and ^{13}C NMR data of compound x (CD_3OD).

Six ten weeks old balb-C mice were kindly received from Pharmacology Laboratory (Faculte de Medecine of Rabat). They were housed 2 per cage in polyethylene cages and fed pellet food (Cicalim n°47) and water ad libitum. After 1 week of acclimatation they were given hypaphorine solution.

The solution of hypaphorine to be administered to mice by ip injection was prepared at concentration of 25 mg/mL in PBS solutions and sterilized by filtration through a membrane (porosity 0.22 µm) and that to be administered by oral route was at concentration of 100 mg/mL in tap water.

Results

Identification of the compound

The ESI-TOFMS spectrum of the compound **X** showed the protonated molecular ion [MH]+ at m/z 247 suggesting formula $C_{14}H_{18}N_20_2$ (M=246) in agreement with ^{14}C NMR data (table1). The J-modulated ^{13}C spectrum

presented 9 CH or CH3 carbons, one CH2 and 4 quaternary carbons. Bidimensional NMR spectra ^{1}H-^{13}C HSQC and ^{1}H-^{1}H COSY, assigned the 4 aromatic vicinal CH at δ 7.50, 7.02, 7.05, and 7.35 of benzene ring, 3H in -CH2-CH- system at δ 3.20 and 3.72 and 3 CH3 in singulet at δ 3.07. The HMBC spectrum showed long-range correlations of 3 quaternary sp^2 carbons at δ 108.96 with H-11; 128.27 H-10 and H-8 and 138.04 with H-2, H-5 and H-7, indicating the presence of the indolic skeleton and one remaining quaternary carbon at δ 171.62 assigned to carboxylic group.

The structure of compound **X** was thus identified to that of hypaphorine, N,N,N-trimethyl tryptophanium betaine (figure 1).

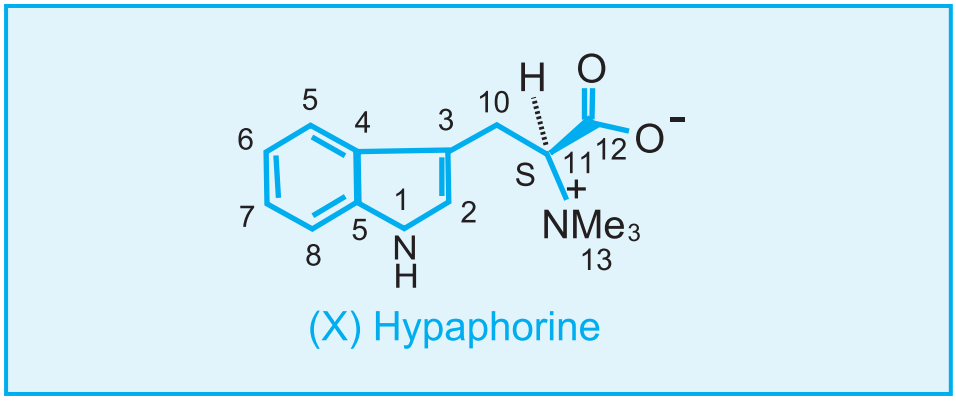
In vivo toxicity assay

The ip administration of hypaphorine to mice at doses of 0.5 and 1 g /kg body wt (table 2), did not produce any sign of toxicity in animals. They tolerated even high doses of 2 g/kg by oral route without any apparent toxic effect and survived until they were given an euthanasia solution 3 months later.

The oral administration of hypaphorine to young lambs at doses of 0.5, 1, and 1.5 g/kg body wt (table 3) did not produce any abnormal sign in these animals.

Discussion

Greshoff first discovered Hypaphorine in the seeds of *Erythrina hypaphorus* Boel. (E. *subunbrans* (Hassk.) Merr.) [19]. Its chemical structure was described by Van Romburgh [19] as an α-N,N,N-trimethyl-β-tryptophanium betaine ($C_{14}H_{18}N_2O_2$). Hypaphorine was later found in other plant species of the genus Erythrina [18, 21, 22, 23] and Abrus [18, 23].
The literature on physiological activity of hypaphorine was carefully examined and found that even though the compound was reported to be a convulsive poison in the Merck Index [17] and other websites, there was no strong evidence to support this affirmation. In fact, hypaphorine, the betaine of tryptophan was first described to have no pronounced (but little) pharmacological activity [19, 22].



(X) Hypaphorine

Figure 1 : Structure of Hypaphorine

Mouse ID#	Body weight (g)	Dose (g/kg)	Total amount administered (mg)
1	25	0.5[a]	12.5
2	22.7	0.5[a]	11.4
3	25.4	1[a]	25.4
4	24.7	1[a]	25
5	23.8	2[b]	47.6
6	18.8	2[b]	40

[a]: ip administration [b]: po administration

Table II : Toxicity assay in mice of hypaphorine solution by ip and oral routes.

Always in the ancient works, Folkers & Unna [22] reported the toxic curare-like action of the alkaloids in the seeds of Erythrina species, without mentioning precisely hypaphorine: one of the major alkaloid constituents of the seeds of these plants (2-3% depending on the species).

Some studies have shown the curare-like paralysis in frogs of natural alkaloids from Erythrina, such as erythramine [18] and others like erythraline, β-erythroidine (LD_{50} 24 mg/kg, ip, in mice) [23] But also some alkaloids isolated after hydrolysis of an aqueous extract known to have curare-like action in frogs, such as erysodine, erysopine, erysicine and erisivine. This fact suggested the presence of glycosides of alkaloids, which are very water soluble [23]. Later, it was isolated, on one hand the glycosides: 15-O−β-D-glucoerysovine [24], and 16-O−β−D-glucoerysovine [25], and, on the other hand the esters of hypaphorine: erysophorine (=15 hypaphorinoxyerysovine) [26] and erysodinophorine (=16-hypaphorinoxyerysovine) [27] (figure 2).

It should be noted that a derivative of hypaphorine, the methyl α-dimethylamino-β-(3-indole)-propionate methiodide has an LD50 of 450 mg/kg in mice [18, 22].

On the basis of this historical data, it was decided to test hypaphorine in mice by injection of high doses 500 mg/kg up to 2000 mg/kg. No toxic effects were observed.

Been aware of any species response variation, hypaphorine was also tested in lambs. Despite the oral administration of relatively high doses of pure alkaloid (0.5; 1 and 1.5 g/kg) compared to lethal dose of the whole plant (5 g/kg dry weight) [6], these doses didn't produce any toxic effect. Thus, by testing hypaphorine in lambs,

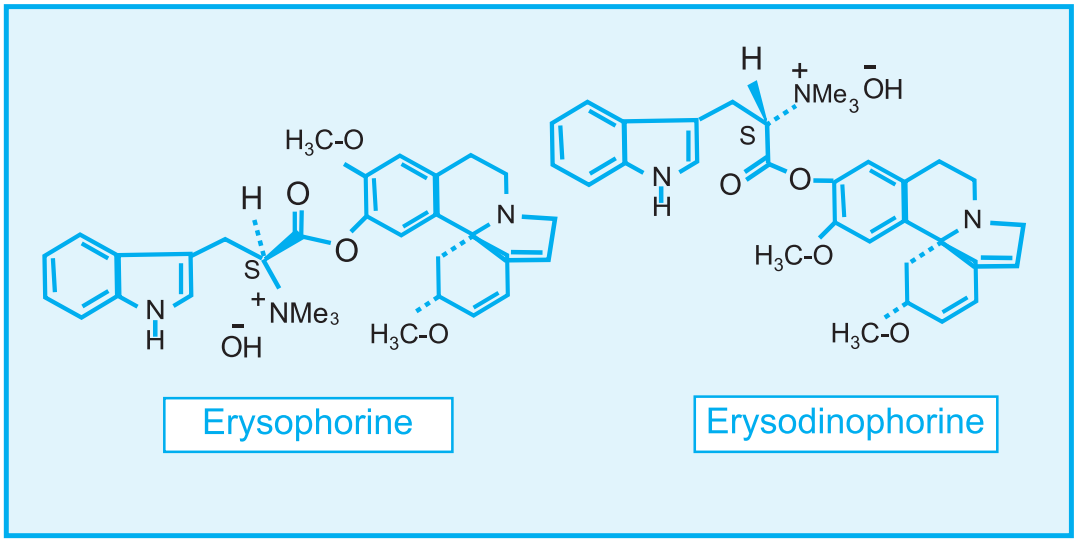


Figure 2 : Some toxic alkaloids of Erythrina

the most sensitive animals to *A. lusitanicus* poisoning, in the field and experimental conditions, this study bring here good evidence that hypaphorine could not be responsible for the toxicity of this plant species. Moreover, it can be suggested that the affirmation saying that hypaphorine is a convulsive agent, as it is stated in the Merck Index [17], should be reconsidered. It may also be concluded that the confusion about the toxicity of hypaphorine came from the fact that hypaphorine part in erysophorine and erysodinophorine molecules (figure 2), makes these alkaloids more water soluble and thus enhances their curare-like activity in the frogs.

Lamb ID#	Body weight (kg)	Hypaphorine	
		Dose (g/kg)	Total amount administered (g)
1	7	0.5	3.5
2	7	1	7
3	6	1.5	9

Table III : Toxicity assay in lambs of hypaphorine solution by oral route.

But free hypaphorine produced no toxicity, whereas the other parts of erysophorine and erysodinophorine were toxic. Thus, the results showing that hypaphorine has no toxic effect in mice and lambs are in agreement with the literature data.

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Summary

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