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EVALUATION OF THE LETHAL POTENCY (LD_{50}) OF MOROCCAN SCORPION VENOMS OF THE GENUS *BUTHUS*

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ABSTRACT
Scorpion stings are major health hazards that lead to suffering of victims and high mortality. In Morocco it is the first cause of poisoning it represents 30% of all cases recorded by the Poison Control Center of Morocco, with an average incidence of 1.06% and a general case fatality rate of 0.19%. Determination of scorpion venoms (LD_{50}) is an important step to evaluate and compare venom toxicity. Thus, through this study we will determine by probit method the LD_{50} in male mice Swiss by subcutaneously root values of five scorpion species of *Buthus* genus considered as dangerous in Morocco. Our result shows that the median lethal doses SC (Subcutaneous) of venoms studied *Buthus lienhardi*(0.27mg/kg), *Buthus atlantis* (0.40mg/kg), *Buthus paris* (0.45mg/kg), *Buthus occitanus* (0.52mg/kg), *Buthus mardochei* (1.00mg/kg), the values obtained indicate that *B. lienhardi* possess the most toxic venom followed by *B. atlantis* venom whereas, the latter is more toxic than *B. paris*, which is more toxic than *B. occitanus*. It is interesting to note this venoms mentioned before are much more toxic than *B. mardochei* venom. In addition, our data showed that *B. lienhardi* venom is about three times more toxic than *B. mardochei* venom. Conclusively, determination of the LD_{50} of scorpions venom studied allowed us to deduce the degree of their toxicity, which is important for neutralization capacity of antivenom treatments.

KEYWORDS: Scorpion; Venoms; *Buthus*; LD_{50}; Toxicity; Morocco.

INTRODUCTION
Around the world and especially in summer, the envenomation of scorpions is a real public health problem[1], because scorpions are a group of nearly 2584 species whose dangerous species belong essentially to a single family Buthidae, which includes a thirty species really dangerous for man.[2]

Morocco possesses the richest scorpion fauna and varied dangerousness species in the Western Mediterranean and it characterized by high species endemism[3], 56 different scorpion species identified in Morocco.[4] Scorpion species belonging to *Buthus* genus are considered as the most dangerous species in Morocco and are generally suspected as the major causes of several human fatalities.[5]

According to the poison control center of Morocco, scorpion envenomation remains among the main causes of intoxication.[6] In fact, 29,816 scorpion stings are recorded each year including about 81 deaths.[7] In Morocco, its gravity is due to the diversity of genera of this family of Buthidae whose venom is potentially fatal. The area's most affected by this problem are the center and the south of Morocco. The risk of mortality by scorpion envenomation is much higher in young patients less than 15 years old.[8]

Hence, fight against envenoming is a priority-public health issue in several countries.[9] Appropriate assessment of scorpion venom 50% lethal doses (LD_{50}) is an important step for an accurate evaluation of the toxic activity of venoms according to the World Health Organization; venom lethality is expressed as median lethal dose (LD_{50}) defined as the amount of a substance (venom) causing death of 50% injected mice.[10;11] Venom median lethal dose assessment is useful for the determination of the scorpion venom toxicity which is important for neutralization capacity of anti venom treatments and for scientifically improving the traditional treatment.[12,13]

Five distinct routes can be used for venom injection, intracerebro-ventricular (ICV), intramuscular (IM), intravenous (IV), intraperitoneal (IP), and subcutaneous (SC). The lowest LD_{50} value[14], in this work we studied the relative toxicities of scorpion *B.lienhardi*, *B.occitanus*, *B.paris*, *B.atlantis*, *B.mardochei* species in mice, based on the determination of their LD_{50} values in subcutaneous route of venom injection.

Abbreviations

B.atlantis= Buthus atlantis
B.paris=Buthus paris
B.lienhardi=Buthus lienhardi
B.mardochei=Buthus mardochei
B. occitanus=Buthus occitanus
BSA= Protein Bovine Serum Albumin
ICV= Intracerebro-ventricular
IV= Intravenous
IP= Intraperitoneal
IM= Intramuscular
LD_{50}= Median Lethal Doses 50
SC= Subcutaneous

MATERIAL AND METHODS

Animals

Mice male Swiss Albino

Mice (25±2g) were used for determining the LD_{50} by subcutaneous injection. They were housed in macrolon cages on 12h light/12h dark cycle. The temperature is maintained at 25°C. Water and food were offered ad *libitum*.

Scorpions

All scorpions belong to the family Buthidae, which has surprising abilities to resist environmental stressors[15], resistant to dehydration, microbial infections and ionizing radiation (gamma rays).[16] The Buthidae family remains the largest in number of species and the best known species from a medical point of view.[5;17] The toxicity of the Buthidea venoms is due to toxins acting essentially on ionic channels, in particular on nerves for long toxins and on many tissues for short toxins.[18] Studied species of scorpion was collected from different regions of Morocco. So *Buthus occitanus*, *Buthus paris*, *Buthus atlantis*, *Buthus lienhardi* and *Buthus mardochei* were collected respectively in Ben El Abbadia forest, Igunane, Ain Kaicher, Ghazoua and Adrar (Fig. 1). They were housed in well ventilated wooden cages with free access to food and water. The species was determined according to an appropriate identification key.[19]

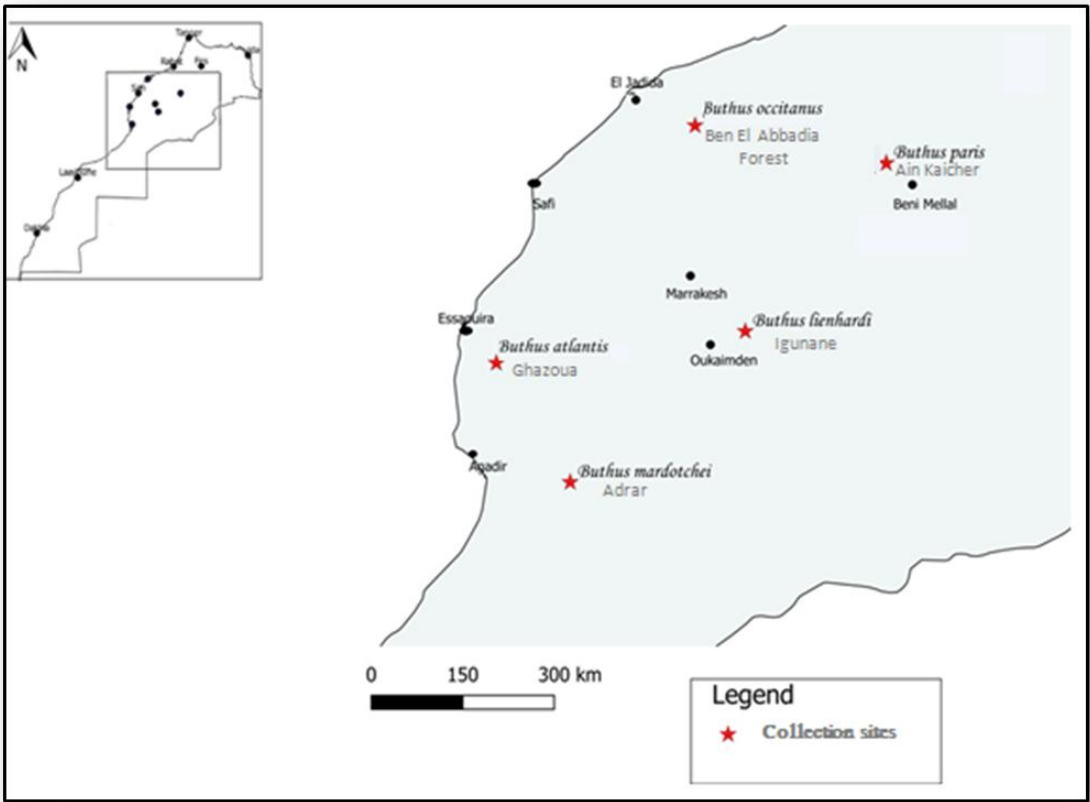

Fig.1: Distribution of collection sites of studied scorpions in Morocco.

(GPS Coordinates: Ain Kaicher: (32.731734,-6.584153); Igunane: (31.236062,-7.807972); Ghazoua: (31.444830, -9.740321); Ben El Abbadia forest: (32.968663,-8.485489); Adrar: (30.426312,-9.562464)).

Venoms extraction

Venoms of studied species were obtained from mature scorpions of each species by electrical stimulation of the telson as described by.[20] The venoms were diluted with sterile bidistilled water and centrifuged at 15.000g for 15min until use the diluted venom was stored at -20°C.

Measurement of protein concentration

Protein concentrations were accurately determined according to the method of[21], Using as reagent Coomassie blue and as reference protein bovine serum albumin (BSA). The optical density of the proteins contained in the venom is then measured at a wavelength of 595nm to determine the protein content of the scorpion venoms supernatant using the standard calibration curve. The solution for each venom was prepared at a final protein concentration of 5 mg/ml.

Determination of the Median Lethal (LD_{50}) Dose

A preliminary test is carried out to determine the response range of envenomed mice between 0% and 100% mortality. Then, a definitive test of the lethal potency (LD_{50}) of venom in micrograms of dry weight per mouse was determined according to World Health Organization recommendations.[22; 23] Lots of eight mice were used each group received a subcutaneous injection of increasing doses of venom diluted in 200 ml of saline physiological solution. An equivalent volume of saline solution was injected into the mice in the negative control group. After the envenomation, the animals were observed for 24 hours and the number of dead animals was recorded at the end of the experiment. The dose that killed 50% of the animals (LD_{50}) was calculated by probit analysis.[24; 25]

RESULTS AND DISCUSSION

The toxicity evaluation of scorpion venom is a critical step an efficient determination of the venom activity. The LD_{50} of venom is the prime parameter to determine the toxicity and lethality of venom extracted from the five poisonous scorpions present in the Morocco. Several animal models have been used but the most common model for venom toxicity analyses in the LD_{50} value determination in mice.[26;27;28 29]

The LD_{50} is frequently used to express the acute toxicity threshold and to classify and compare toxic substances. It is however, of very limited value because it concerns only mortality and gives no information on the mechanisms involved and the nature of the lesions. This is a rough and preliminary assessment, which can be influenced by several factors such as scorpion species, sex, age, individual, seasonal and geographical variability of venom, the body weight, and the route of venom injection.

In this study, the LD_{50} mean values of venoms were assessed by subcutaneous route using 25±2g *Swiss* mice.

Determination of the protein content of scorpion venoms

The absorbance of all tubes is then measured. The values obtained from the tubes of the standard range make it possible to draw a standard straight line: absorbance = f (quantity). This proportionality makes it possible to determine the amount of protein contained in each venom of the species to be studied.

From the graphic presentation below, it can be see that the protein concentration contained in each venom of different species of scorpion. Venom of *B.lienhardi* with a protein content value of 7.85µg/µl, followed by the *B.atlantis* with a value of 7.7µg/µl, then by *B.paris*, *B.occitanus* and *B.mardochei* with an respectively values of 7.62µg/µl, 6.66µg/µl and 6.4µg/µl. (Fig.2).

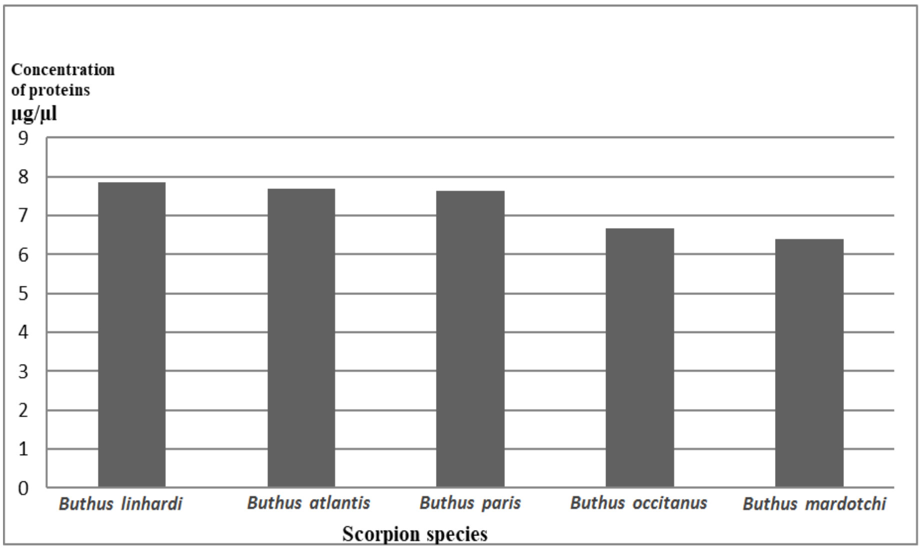

Fig.2: The protein content of the studied scorpion venoms.

Lethal potency of scorpion venoms

The LD_{50} values of collected scorpion venoms were assessed by SC route in 25±2g Swiss mice and summarized in Table 1.

Experimental data indicate that the most toxic is the venom of *B.lienhardi*, while the last toxic is the venom of *B.mardochei*, *B.lienhardi* venom is more toxic than that of *B.mardochei*'s venom.

Ethics Committee Approval

Acute toxicity study of scorpion venoms on animal model (Male Swiss mice) strictly followed the ethical principles in animal research adopted by the World Health Organization for the use of experimental animals. The procedure was conducted (8-17h) in accordance with approved institutional protocols and provisions for care of animals and for the use prescribed in scientific life procedures. Animals ACT 1986 (European Council Directive: 86/609 EEC). All efforts have been made to minimize the suffering of animals, and the number of animals does not exceed the statistical requirements of the experiment. They were approved by a local ethics committee of Research Laboratories of the Faculty of Sciences, Cadi Ayyad University, Marrakech.

Table1. LD_{50} values of studied species venom through subcutaneous mode of injection calculated by probit method.

Venom of the scorpions	Family	Dose in (µg/kg)					Weight of Mice (g)	NO. of survival after 24Hrs					Probit					DL_{50} mg/kg
Buthus lienhardi	Buthidae	160	200	250	270	320	25-27	0	3	4	5	8	3.25	4.67	5	5.31	6.75	0.27
Buthusatlantis	Buthidae	400	500	600	650	700	25-27	0	2	5	6	8	3,35	4,33	5,31	5.67	6.75	0.40
Buthus paris	Buthidae	450	500	550	600	650	25-27	0	2	4	7	8	3,35	4.33	5	6.13	6.75	0.45
Buthusoccitanus	Buthidae	300	350	400	500	600	25-27	0	4	5	6	8	3,25	5	5.31	5,67	6,65	0.52
Buthusmardochei	Buthidae	900	1000	1200	1300	1500	25-27	0	3	4	6	8	3,35	3,25	5	5,67	6,65	1.00

According to the results of the table above we note the median lethal doses SC of venoms studied *B.lienhardi* (0.27mg/kg), *B.atlantis* (0.40mg/kg), *B.paris* (0.45mg/kg), *B.occitanus* (0.52mg/kg), *B.mardochei* (1.00mg/kg). Our results are different with those found by other authors as follows *B.occitanus* (0.9mg/kg)[30; 31], *B.paris* (4.15mg/kg)[32], *B.mardochei* (1.50mg/kg)[33], this supports the idea that venom toxicity varies according to several factors such as genus, species, age, physiology, feeding state and region of the scorpion.

The result show that most of the venom obtained from species of same genus with geographical barrier has different LD_{50} values depicting the effect of geographical regions, and several other factors so it is the need of time for in-depth research in this area, to develop antivenom serum for specific species based upon their exact lethality. In addition, the LD_{50} value of these assays must be noticed with caution since these are obtained using a mouse model. *B. lienhardi* venom proved more toxic than the venoms *B.atlantis*, *B.paris*, *B.occitanus*, *B.mardochei* this toxicity is attributed to *B.lienhardi* richness in low molecular weight proteins compared to others venoms.

This is in agreement with the results obtained in our study showing that the values of the protein concentrations contained in the species of scorpions studied are proportional to their LD_{50}.

Comparison of Lethal Dose (LD_{50}) of venom

All species of scorpions are toxic. However, only a very small number of the 1050 known species are dangerous to humans. We can note that *Leiurus quinquestriatus*, also known as the stalking scorpion, is one of the most dangerous scorpion species.[5] And most of these species belong to genera Buthus, Parabuthus, Mesobuthus, Tityus, Leiurus, Androctonus, Centruroides family of Buthidae.[34; 35] Among these species, *Tityus serrulatus*, *T. bahiensis* are common and lethal scorpion species in South America, especially in Brazil; *Centruroidessuffusus*, *C. limpidus*, *C. sculpturatus* in Mexico; *Leiurusquinquestriatus*, *Androctonuscrassicauda*, *A. mauretanicus*, *A. australis*, *A. amoreuxi*, *Buthusoccitanus* in the Middle East and North African countries; *Parabuthusgranulatus*, *P. transvaalicus* in South African countries; *Mesobuthustamulus* and *Palamneusswammerdami* in India.[36; 37]

In Table 2 below we have tried to compare the toxicity between the venom of the studied scorpions and other venoms of the scorpions with the reference we pointed that shows an all species of scorpions belong to the same family of Buthidea.

Until now, investigations on the degree of scorpions have certainly been given priority to the twenty or so species identified as deadly for humans. Almost all of these lethal species belong to the family Buthidae where *B.lienhardi* is specifically classified among the most toxic scorpions with a LD_{50} of 0.27mg/kg, followed by the scorpion *B.atlantis* which has a LD_{50} (0.40mg/kg) less than Brazilian scorpion *Tityus serrulatus* (0.43mg/kg). Whereas *B.paris* (0.45mg/kg) reveals toxicity more than *Androctonus crassicaulla* (0.5mg/kg), *B.occitanus* (0.52mg/kg)and the toxicity of *B.mardochei* (1.00mg/kg). All the studied scorpions they exhibit a weaker toxicity than the one described for the venom of the black scorpion *Androctonus mauritanicus* (LD_{50} ranging from 0.050e0.2mg/kg), which could be considered the most toxic Moroccan scorpion venom.[38] (Table 2).

Table 2: Comparaison between the venom of scorpions in our study and the venom of other known scorpions by their venom toxicity.

Scorpions of our study	DL_{50} mg/kg mice	Locality	Mode of injection	Reference
B.lienhardi	**0.27**	**Igunane(Morocco)**	Subcutaneous	**The present work**
Androctonus australis	**0.3**	Brazil	Subcutaneous	[39]
Centruroides santa maria	**0.39**	Central America	Subcutaneous	[32]
B. atlantis	**0.40**	**Ben El Abbadia Forest (Morocco)**	Subcutaneous	**The present work**
Tityus serrulatus	**0.43**	Brazil	Subcutaneous	[39]
B.paris	**0.45**	**Ain Kaicher (Morocco)**	Subcutaneous	**The present work**
Androctonuscrassicaulla	**0.5**	Middle Ariary	Subcutaneous	[40; 31]
B.occitanus	**0.52**	**Ghazoua(Morocco)**	Subcutaneous	**The present work**
Buthacusarenicola	**0.99**	North Africa	Subcutaneous	[32]
B.mardochei	**1.00**	**Adrar (Morocco)**	Subcutaneous	**The present work**
Hottentotta saulcyi	**1.01**	Iran	Subcutaneous	[30]
Buthusoccitanusmardochei	**1.5**	Guelmin(Morocco)	Subcutaneous	[38]

CONCLUSION
In the present work, we determined experimentally the LD_{50} values of reference scorpions venoms in mice, and evaluated the venom potency with relevant to previous literature. This study aims at evaluating the lethal potency of a series of scorpion venoms injected in mice by the subcutaneous route. The results show that the lethality of scorpion venom in mammals differs with the age, species and different environmental factors. The determination of LD_{50} of each venom is important not only to produce potent antivenom serum but also for determination of neutralization capacity of antivenom treatments. Our work should be extended to a greater number of scorpion venoms for a more complete analysis. Also, other representative routes of venom injection (i.e., intracerebro-ventricular, intramuscular, intraperitoneal and intravenous) might be examined *in vivo* to assess the actual toxic potential of various venoms.

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