AN ECOLOGICAL STUDY OF THE LEBANON MOUNTAIN VIPER *MONTIVIPERA BORNMUELLERI* (WERNER, 1898) WITH A PRELIMINARY BIOCHEMICAL CHARACTERIZATION OF ITS VENOM

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ABSTRACT

This study concentrates on the Lebanon mountain viper Montivipera bornmuelleri endemic to the Lebanese mountains, in terms of ecology, geographic distribution and preliminary biochemical characterization of its venom. Here, one shows that M. bornmuelleri lives in habitats dominated by a variety of mountain plant species at altitudes ranging from 1900 m to 2200 m. This viper exists in association with endemic lacertid lizards in the Oyoun Al Simane area of Kfardebian in the Sannine mountain and with other snakes (such as Platyceps najadum (Müller, 1878) and Hemorrhois ravergieri (Ménétries, 1832) in the Makmel (Bcharre) mountain. Preliminary analysis of this viper's venom was conducted using liquid chromatography coupled to electrospray ionisation mass spectrometry (LC-ESI-MS). The analysis revealed the presence of some bioactive molecules in the crude product. This work constitutes a preliminary study of a research project involving the extraction and characterization of bioactive molecules and potential therapeutic uses of the venom.

Keywords: Lebanon viper, *Montivipera bornmuelleri*, ecology, geographic distribution, venom analysis, liquid chromatography, electrospray ionisation mass spectrometry

INTRODUCTION

In Lebanon, there are three viper species, *Daboia palaestinae* (Werner, 1938), *Macrovipera lebetina* (Linnaeus, 1758) and *M. bornmuelleri* (Figure 1). The former two species are widely distributed in various Lebanese habitats excluding high altitudes (Hraoui-

Bloquet *et al.,* 2002). *M. bornmuelleri* is endemic to the Lebanese mountains (Werner, 1938; McDiarmid *et al.,* 1999; Mallow *et al*., 2003) and found only at altitudes above 1800m (Hraoui-Bloquet *et al.,* 2002).

This endemic species was described for the first time in Lebanon by Werner (1938). It is now listed as endangered (EN) by the IUCN (2006) based on its limited geographic range estimated to be less than 20,000 km². Moreover, its populations are highly fragmented and known to exist at some mountain tops in Lebanon. Several specimens of this snake have been caught and observed by the authors since 1992 mainly in the Oyoun Al Siman region (Mount Lebanon).

Figure 1. A *Montivipera bornmuelleri* specimen near its shelter in Oyoun Al Simane region.

The species has been reported previously from other regions, namely, Bcharré (Werner, 1938) and Mount Hermon (Werner, 1995). Other mountain localities in the Anti-Lebanon have yet to be explored.

The Viperidae venoms are complex mixtures of proteins, nucleotides and inorganic ions (Markland, 1998; Fry, 1999). These combinations confer a formidable array of toxic properties on the venom. These include peptides and polypeptides responsible for a variety of toxic properties including a perturbation of the coagulation cascade, the normal haemostatic system and tissue repair. Consequently, envenomation by these snakes may generally result in

persistent bleeding. These proteins can be grouped into a few major protein families, including enzymes (serine proteinases Zn^{2+}-dependent metalloproteinases, and group II phospholipase A2 isoenzymes) and proteins with no enzymatic activity (C-type lectin-like proteins and disintegrins) (Ménez, 2002; Juárez *et al.,* 2004).

The aim of this work is to make an ecological study of this endemic species *M. bornmuelleri* in Lebanese mountains with a preliminary biochemical characterization of its venom to investigate the presence of main enzyme families involved in the bleeding response and/or some other proteins with distinct biological activities.

STUDY AREAS, MATERIAL AND METHODS

Study areas and material

Investigations of the ecology of this species and its geographic distribution were conducted in late spring of 2009 in Oyoun Al Simane region, and during the spring of 2010 in the mountain area east of Bcharré at higher altitudes (1900m-2200m). The latter area included the region around the cedar forest and Oyoun Orghosh (2100 m) on the eastern slopes of Jabal Al Makmel.

For this study, eight specimens were captured during May and June in the Oyoun Al Simane region of Sannine Mountain (spring 2009) and four specimens from the Bcharré Cedars region of Jabal Al Makmel (spring 2010). All the snakes were captured before 12 noon. The captured snakes were handled with tongs and were immediately transferred into large plastic jars and then were transported to the laboratory.

The snakes were kept in special glass terraria where they were housed individually. A small rock was provided for shelter. Heating lamps were installed whereby the snakes were able to freely thermoregulate. They were provided with drinking water and were fed mice every two weeks.

Snake venom collection and biochemical studies

The venom milking was made on alternate weeks, *i.e*., one week for feeding followed by one week for venom collection. The vial used for venom collection was sterilized under ultra violet for at least 6 hours. The venom quantity given by each snake was very small and so, venom was pooled from several snakes each time. After venom collection, the sample was placed in a freeze drier and the resulting sample was kept at -20ºC until further analysis.

For the biochemical characterization of the venom collected, LC-ESI-MS was used to analyze a small amount of the venom sampled, with the specific aim of examining some bioactive molecules. The LC-ESI-MS used is a string of Agilent 1260 type equipped with an automated sampler of Agilent G 1329A type and a pump of Agilent G 1311A type interfered with detector UV / VIS of G Agilent 1314D-VWD type. The analytical column from Restek Ultra II is C18 and is 15 cm long with an inner diameter of 3 microns and a diameter of grafted silica particles of 3.5 micrometers. The chromatographic gradient used was a binary gradient consisting of solvent A (water + 0.1% formic acid) and solvent B: Acetonitril (ACN). LC is directly connected to mass spectrometry for online analysis. As a measure of their elution, the different molecules reach the ESI source where they are ionized and analyzed.

This coupling has the advantage of automating the analysis process. The ESI-MS analysis of the venom is done with an extended scan mode between 200-6000 m/z. The software used were: 44.0 Hystar Version 3.2, Hystar Post Processing, Esquire Control Version 1.3 and Data analysis compass. For LC elution, ACN, water and 0.01% formic acid were used in different ratios as the mobile phase.

1 mg of crude venom was dissolved in 1 ml of water (0.05% formic acid and 5% CAN). The insoluble material was removed by centrifugation using an Eppendorf centrifuge at 1300 g for 10 min at room temperature. The supernatant was recovered and the pellet containing the insoluble matter was removed. Samples were injected automatically into the LC-ESI-MS system and analyzed in positive mode. A 90 min linear gradient from 0 to 100% of buffer B [0.1% (v/v) formic acid/ACN] in buffer A [0.1% (v/v) formic acid/H2O], at a flow rate of 1ml/min (λ = 215 nm) was applied to the column after injection of the sample. After separation by LC the supernatant was characterized by ESI-MS.

RESULTS

Biogeography and ecology

In the Oyoun Al Simane region, the snake's habitat is mainly rocky slopes and plateaux with a variety of mountain plant species especially the *Berberis libanotica* Ehrenberg small shrubs (Figure 2). Here, the snake is associated with sheep and goat herds and their shepherds. The grazing activity leads to a substantial effect on the vegetation in the area reducing the density of soft herbaceous plants and enhancing the dominance of thorny mountain vegetation and cushion plants. The habitats, especially the hilltops, become quite dry after the snowmelt leaving some moisture in the depressions, in and around sinkholes, become markedly dry.

Figure 2. A landscape view of Oyoun Al Simane showing the hilly habitats of *Montivipera bornmuelleri.*

In the Cedar region, the physical structure of the snake's habitat resembles that of Oyoun Al Simane. The structure of the vegetation, however, is different due probably to the lack of grazing activity that dominates Oyoun Al Simane. In the cedar region, grazing is limited and restricted to a few sites only. It is markedly more moist and with more herbaceous cover (Figure 3).

Figure 3. The Cedars region in North Lebanon, the hills around the cedar forest. The foreground shows the habitats where *Montivipera bornmuelleri* specimens were captured.

Montivipera bornmuelleri in the Oyoun Al Simane region is associated with the lizard species *Phoenicolacerta kulzeri* (Müller & Wettstein, 1932), *Parvilacerta fraasi* (Lehers, 1910), *Trachylepis vittata* (Olivier, 1804) and *Laudakia stellio* (Linnaeus, 1758). No other snake species were observed yet in this region since 1992. In the Cedars region, the snake is associated with the skink *T. vittata* and at least two other snake species, *P. najadum* and *H. ravergieri* both of which are observed in the surveyed area. Its association with *Ph. kulzeri* is not as extensive as in Oyoun Al Simane region while its association with *P. fraasii* is restricted to Oyoun Orghosh but not in the Cedars region. Within the same vicinity of the study area in the Cedars region, other lizard species were observed, namely, *Lacerta media* (Mertens, 1922), *Ophisops elegans* (Ménétries, 1832), *L. stellio* and even *Testudo graeca* (Forsskal, 1775).

The snakes caught range in size (snout-vent length) from 20 cm to 55 cm. It is much smaller than the other two vipers, *D. palaestinae and M. lebetina* which are found in Lebanon at lower altitudes. The specimens of *M. bornmuelleri* caught show a range of color patterns illustrating some polymorphism in the populations. Observations show that these snakes become active in early May but their activity is restricted to emergence to basking spots very

close to their burrows, among rocks and vegetation which provide shelter from cold winds and where the ambient temperatures could be around 14-18ºC during the day.

The snake's diet comprises various small animals including small mammals and lizards. The snake is associated with a few lizard species and one small mammal species, the snow vole, *Microtus nivalis* (Martins, 1842) which shows many burrow entrances in the snake's habitat.

Mating most probably occurs in May-June during which courtship behavior has been observed. This species is viviparous and live births occur between mid-August and early September. One of the snakes captured in early June from Oyoun Al Simane gave birth to 5 snakes in mid-August. Another female from the Cedars region gave birth to one in early September. Thus, the gestation period is about 2-2.5 months.

Venom study

High-performance liquid chromatography (HPLC) analysis of the crude venom of *M. bornmuelleri*: Figure 4 shows the crude venom profile of *M. bornmuelleri* obtained by a HPLC/C18 column analysis with UV detection at λ = 215 nm. The X-axis in the figure represents the retention time of the venom fractions and the Y-axis is the relative intensity of each peak. This shows 9 major compounds eluted with different retention times, according to the hydrophobicity of the venom's components.

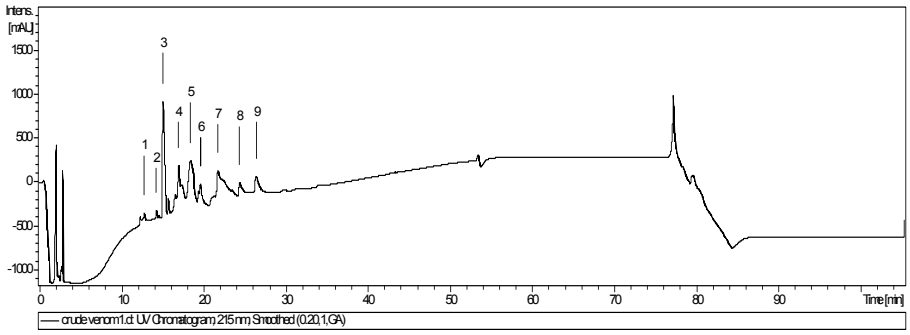

Figure 4. Analytical C18 reversed-phase profile of the crude venom showing 9 major compounds eluted at different times of retention. Chromatographic conditions were: 90 min linear gradient from 0 to 100% of buffer B [0.1% (v/v) formic acid/ACN] in buffer A [0.1% (v/v) formic acid/H2O], at a flow rate of 1ml/min (□ = 215 nm). Fractions were automatically characterized by ESI-MS.

ESI-MS analysis of the crude venom of *M. bornmuelleri*: the preliminary ESI-MS analysis of the total crude venom eluted by LC shows a variety of different molecular mass classes in the crude venom. The mass spectrum analysis of peak 5 (Figure 4) shows that the most abundant ions have molecular masses of approximately 13.700 Daltons ($1953.8 \times 7H^+$ or $2278.7 \times 6H^+$). This mass corresponds to the range of mass reported for the vast majority of snake venom phospholipase A2 (PLA2). For the *M. bornmuelleri* venom, this mass is shown in Figure 5*a*. The mass spectrum of compounds eluted at a retention time range between 19 min and 24 min which includes peaks 6 and 7 of HPLC chromatogram shows a mass of 25.091 Daltons ($2282.0 \times 11H^+$) which corresponds to serine protease represented by

Figure 5b. While mass spectrum of the compounds eluted at a retention time range between 25 min and 27.5 min including peaks 8 and 9 of LC chromatogram shows molecular masses of 57.500 Daltons (2213.0 × $26H^+$) and 57.700 Daltons (2748.9 × $21H^+$) which corresponds to metalloproteinase III represented by Figure 5*c*.

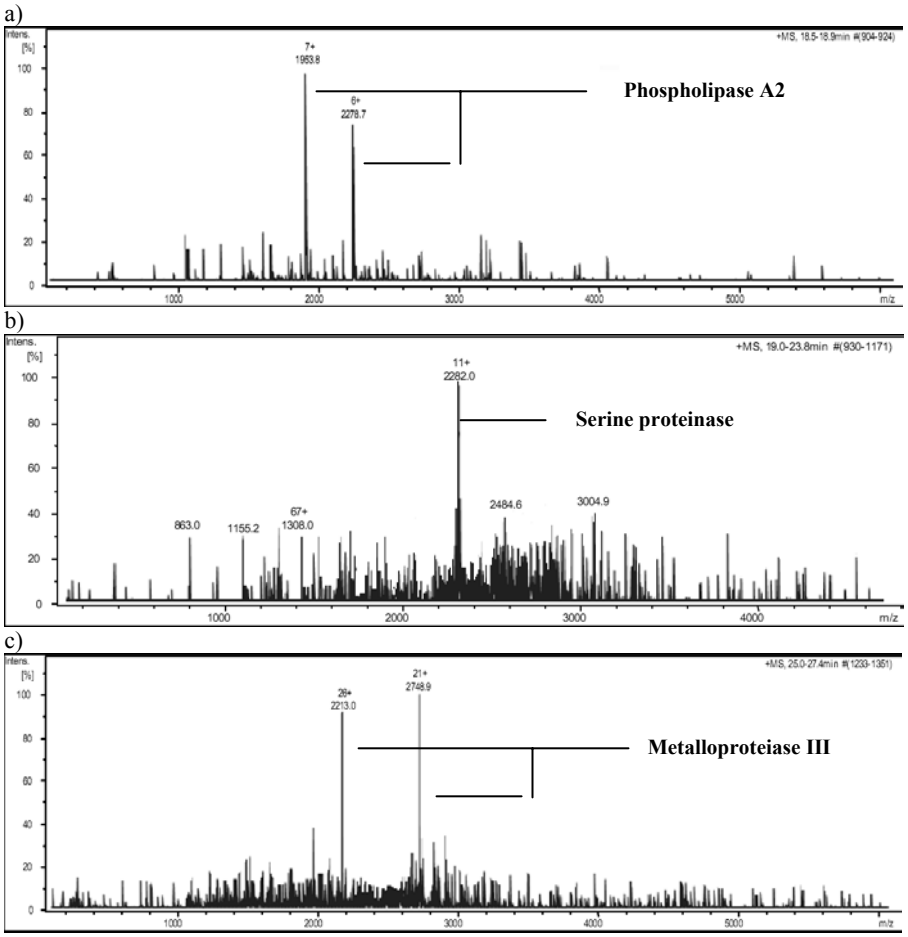

Figure 5. Mass spectrum (MS) profiles of the three families of proteins identified by ESI-MS. a) MS profile corresponding to PLA2 characterized; b) MS profile corresponding to serine proteases characterized; c) MS profile corresponding to metalloproteinase III characterized.

DISCUSSION

This study represents the first attempt to provide detailed information about the viper *M. bornmuellerri*, from the Lebanese mountains. Several differences can be observed between *M. bornmuelleri* and the two other vipers species recorded in Lebanon (*M. lebetina* and *D. palaestinae*) (Hraoui-Bloquet *et al.* 2002). The size of this viper barely exceeds 60 cm,

while those of *M. lebetina* and *D. palaestinae* can reach 1.50 m. The size differences reflect differences in the geographic distribution of these three species. *M. bornmuelleri* is found only at very high altitudes from 1900 m and up. The others two viper species of Lebanon live in forests and rocky areas with an altitude range extending from the coast up to 1600 m. The small size is one of the adaptations of *M. bornmuelleri* to a colder mountain habitat.

Being endemic to the mountains of Lebanon, *M. bornmuelleri* has been placed on red list of species as "endangered" (IUCN Red List 2006) due to its limited and fragmented geographic distribution. The other two vipers are more abundant and have been recorded in Lebanon and other regions in the Middle East, namely, Turkey (Joger, 1984), Jordan (Amr *et al*., 1994; Disi *et al*., 1988), Palestine (Werner, 1938) and Syria (Joger, 1984). They could be found close to human habitation in villages and towns.

Given its existence in alpine climatic conditions, *M. bornmuelleri* remains in hibernation for 7 to 8 months and has a limited period of activity (4 to 5 months). It is viviparous giving live births of a maximum observed number of 5 individuals. Among the lizards that are sympatric with this snake species, only the skink *T. vittata* is also viviparous. The other sympatric lizard species lay eggs and can reproduce two to three times per season while *M. bornmuelleri* breeds only once a year. Studies of the reproductive cycles of the lizard species living in association with *M. bornmuelleri* are in progress.

M. bornmuelleri's habitat is rocky with sparse vegetation that is limited to thorny cushion plants and grasses. Moreover, there are habitat differences between the two localities studied in this work: Oyoun Al Simane is in the central part of Mount Lebanon while the Cedars region is located in the northern part of Mount Lebanon. The differences in the habitats between the two localities might be due either to topography, climate or impacts of grazing. Temperatures are lower in the northern Mount Lebanon than in its central part during the same periods of the year. In Jabal Al Makmel there is a tendency for cloud accumulation at the noon period after which the weather becomes foggy and the temperature drops. There is intensive grazing in Oyoun Al Simane but not in the Bcharré area.

Many more individuals were collected and observed, for the same effort, in the region of Oyoun Al Simane than Jabal Al Makmel which might reflect differences in the snake density. This could be due to one or more of the following reasons. The first one could be related to the climatic differences between the two sites. The second could be the coexistence with other snake species in the Jabal Al Makmel region with the consequent competitive pressure on *M. bornmuelleri.* This is not the case in Oyoun Al Simane where there is no other snake species. Further ecological studies will be needed to resolve this question.

In terms of venom characterization, this work demonstrates the usefulness of the LC-ESI-MS for the preliminary proteomics decryption of *M. bornmuelleri* venom. The passage of the crude venom through an HPLC/C-18 column allows separating components of different times of retention. The ESI-MS analysis of the LC eluted compounds shows a variety of different molecular masses. Comparing a part of results obtained with previous studies concerning the characterization of viper venoms (Fry *et al.,* 2003; Tsai *et al*., 2010), the identities of three families of proteins (phospholipase A2, serine proteinase and metalloproteinase III) were proposed, which have masses matching with the theoretical masses found in the bibliography (Table 1) (Fry *et al*., 2003). However, definitive assignment

of a protein family detected in *M. bornmuelleri* venom can only be done through demonstrated structural studies such as N-terminal sequencing.

TABLE 1

Protein Families Previously Characterized in Snake Venoms (Fry *et al*., 2003)

Family	MW (KDa)
Bradykinin-potentiating peptide	1
Waglerin	2.5
Sarafotoxin	2-3.5
Natriuretic peptide	3-4
Myotoxic peptide	4-5
Disintegrin	4-8
BPTI/Kunitz-type protease inhibitor	6-7
Three-finger toxin	6-9
Prokinecitin	8-9
Thaicobrin/Ohanin	11-13
Cystatin	12-15
Pancreatic-type PLA2	13-14
Synovial-type PLA2	13-14
C-type lectin	14-19
CRISP	23-26
Peptidase family M12B	20-25
Peptidase family S1	25-28
Prothrombin activator	48-50
L-amino oxidase	55-59
Acetylcholinesterase	55-59
P-III Metalloprotease	40-70

Concerning the phospholipase A2 (PLA2), in similarity with other species of *viperidae* snakes (Serrano *et al*., 2005; Guércio *et al*., 2006; Calvete *et al.,* 2007; Sanz *et al*., 2008; Angulo *et al*., 2008; Lomonte *et al*., 2008; Gutiérrez *et al*., 2008), the venom of *M. bornmuelleri* contains a proportion of PLA2s enzymes with approximate molecular masses of 13.700 Daltons. Previously, several isoforms of PLA2 have been isolated in the snake venom (Alape-Girón *et al*., 2008). All of them have been shown to damage skeletal muscle tissue within minutes of their intramuscular injection in mice, reproducing the drastic myonecrotic picture induced by the whole venom (Gutiérrez *et al*., 1989; Gutiérrez & Lomonte, 1995). Thereby, the possible effect(s) of the PLA2 isozymes isolated from *M. bornmuelleri* venom will be evaluated in future studies.

As for the metalloproteinases, the Zn^{2+}-dependent metalloproteases are abundantly found in snake venoms, and classified into four structural groups, from P-I to P-IV, on the basis of their domain composition (Bjarnason & Fox, 1994; Fox & Serrano, 2005; 2008; Ramos & Selistre-de-Araujo, 2006). In *M. bornmuellerai* venom, the presence of metalloproteinases of the 3rd groups has been demonstrated by LC-ESI-MS. Similarly, this protein family was isolated from *Bothrops asper* (Linnaeus, 1758), venom (Aragon-Ortiz & Gubensek, 1987). This enzyme hydrolyzes a number of protein substrates *in vitro* such as casein, hemoglobin, gelatin and fibrinogen (particularly its alpha-chain). Other activities displayed by this molecules include dermonecrosis and blistering (Rucavado *et al*., 1998; Jiménez *et al*., 2008), complement activation (Farsky *et al*., 2000), and induction of an inflammatory response including leukocyte recruitment, hypernociception, and synthesis of matrix metalloproteinases and cytokines (Rucavado *et al*., 2002; Fernandes *et al*., 2006; 2007). In future studies, the possible activities of metalloproteinases III found in the *M. bornmuelleri* venom will be evaluated.

Finally, the serine proteinases are abundant constituents in snake venom. They account for 5–18% of the proteins, depending on age and geographic region variations (Alape-Girón *et al*., 2008). Of these enzymes, those with thrombin like clotting activity have been isolated and studied. Asperase, the first serine proteinase with thrombin-like activity isolated from *Bothrops asper* venom, was described by Aragon-Ortiz and Gubensek (1976, 1978). This enzyme is glycosylated, with an estimated molecular mass of 26 k Daltons. In similarity, this family of protein is presented in *M. bornmuelleri* venom and characterized by LC-ESI-MS. Future studies of the serine proteinases ability to induce coagulopathy will be investigated. This toxic enzyme also could cause behavioral alterations such as loss of the righting reflex, opisthotonus, and intermittent rotations over the long axis of the body (Pérez *et al*., 2008).

CONCLUSION

In future research will focus on the structural identification (sequencing of the N-terminal sequence) and biological characterization of bioactive proteins derived from the *M. bornmuelleri* venom. Thereby, one will evaluate the possible effect(s) of the PLA2 isozymes and other enzymes isolated from the *M. bornmuelleri* venom in the pathophysiology of envenoming. However, histological studies of the effect of the crude venom on mouse tissues are in progress.

Finally, a solid initial platform was provided in Lebanese University upon which to build further research on venomous animals and potential clinical effects of their liquid

secretions. This study will be the starting point for subsequent launch of a research program to study the potential benefit of natural sources existing in Lebanon. However, one is aware of the considerable number of medicinal plants in Lebanon but one has no idea what are the therapeutic molecules involved!

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