PRELIMINARY STUDY OF *VIPERA URSINII MACROPS* VENOM COMPOSITION AND BIOLOGICAL ACTIVITY

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INTRODUCTION

Meadow vipers (*Vipera ursinii*) form a group of five subspecies, but their distribution area is highly fragmented and covers Europe, Western and Central Asia. Karst viper, *Vipera ursinii macrops* (*Vum*) inhabits high mountain grasslands in Croatia, Bosnia and Herzegovina, Serbia, Macedonia, Montenegro and northern Albania. In Croatia it is a highly threatened viper species that appears only in five isolated localities, some of which are related to Bosnia and Herzegovina: southern Velebit, Poštak, Dinara, Troglav and Kamešnica mountains. Scientific literature on *V. ursinii* deals only with its morphology, ecology and distribution range due to the species' conservation problems, while its venom composition and properties have not been investigated.

Karst viper (*V. ursinii macrops*) from southern Velebit, Croatia (photo by Dušan Jelić)

The Karst Viper in Croatia favours high-mountain dry grasslands, on southern and south-eastern exposures, at altitudes from 1100 to 1900 m above the see level. Because of that, meadow vipers are medicinally less significant than other *Viper* species. Majority of envenoming generally display mild and negligible local symptoms only, which resolve spontaneously within a couple of days without any medical treatment or antivenom therapy. This may be associated with short length of their flanges (2-3 mm) and a very low amount of injected venom (1-4 mg in dry weight), which cannot cause serious systematic symptoms.

AIM

Here we investigate for the first time the composition and biological activity of the *Vum* venom, in comparison to the venom of *Vipera ammodytes ammodytes (Vaa)*, the most venomous European snake.

RESULTS AND DISCUSSION

Table 1. *The lethal toxicity (as LD_{50} in µg) and haemorrhagic potency (as MHD in µg)* of *Vum* venom in comparison to *Vam* venom. Results are given as mean ± SE.

	V. ammodytes ammodytes	*V. ursinii macrops*
LD_{50} (µg)	4.4 – 13.7*	37.0 ± 0.1 (*n*=3)
MHD (µg)	21.6 – 42.8*	34.1 ± 4.8 (*n*=4)

*depending on the geographical location (as determined by Halassy *et al.* (2011))

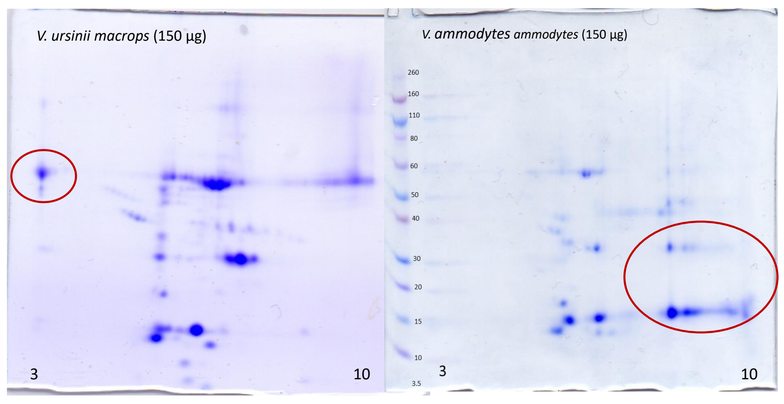


Figure 1. 2D electropherograms of *Vum* and *Vam* venoms. Regions indicating the most pronounced qualitative differences in the proteomes of *Vum* and *Vam* venoms are indicated by circles.

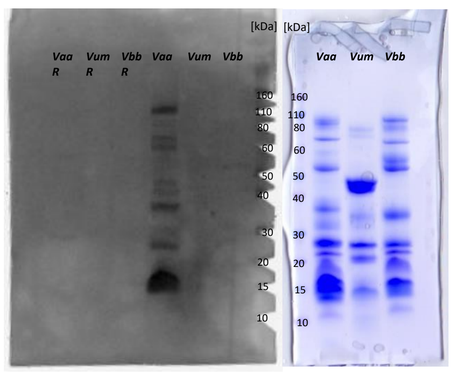


Figure 2. Western blot detection of ammodytoxins in *Vaa*, *Vum* and *Vbb* venoms. SDS-PAGE of venoms was performed under reducing (lanes 1-3) and non-reducing (lane 4-6) conditions. Detection was performed with anti-Atx antibodies. Each lane contains 40 µg of venom.

The *Vum* venom is less lethally toxic in mice than the *Vaa* venom (Table 1.), however the pattern of mice dying indicates the presence of a strong neurotoxic component. Interestingly, 2D electrophoresis (Figure 1.), as well as Western blot of non-reduced Vum venom with anti-Atx (Figure 2.) and ELISA (Figure 3.) revealed a lack of ammodytoxin-like proteins in *Vum* venom. These are well known neurotoxic components of *Vaa* venom with Mw of 14 kDa and highly basic pI. MS analysis (Figure 4.) and ELISA (Figure 3.) confirmed the presence of only non-toxic ammodytin-like phospholipases.
Metalloproteinases are the most abundant components of *Vum* venom. Accordingly, *Vum* venom exhibited strong haemorrhagic activity, comparable to that of *Vaa* venom (Table 1.). Antiserum specific for *Vaa* haemorhagins recognised *Vum* haemorrhagins with comparable affinity. Moreover, *Vum* venom was shown highly instable when dissolved probably due to its very strong proteinases. (Figure 5.).

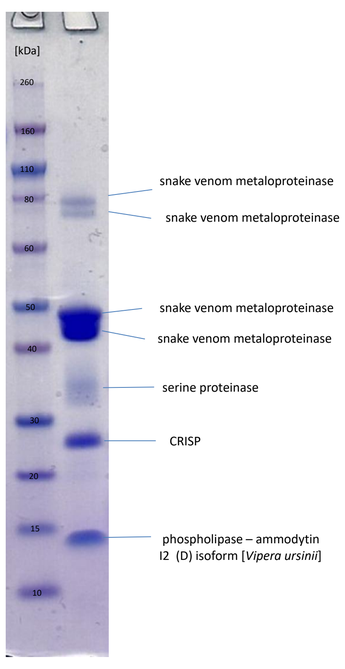


Figure 4. SDS-PAGE analysis of *Vum* venom under non-reducing conditions and proteins identified in individual bands by ion trap LC-ECI-MS/MS analysis of their tryptic fragments
line 1 - Molecular weight standards;
line 2 - *Vum* venom (40 µg).

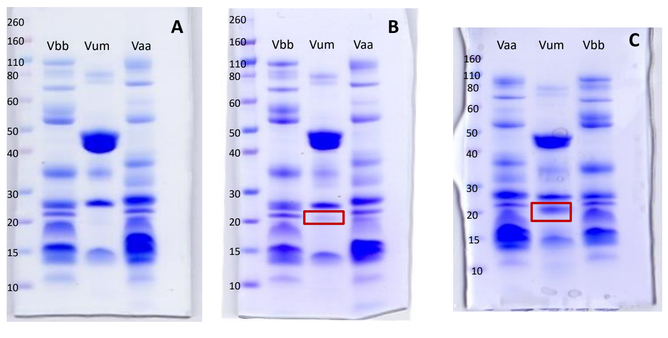


Figure 5. Instability of *Vum* venom after freezing and thawing. SDS-PAGE analysis of *Vum*, *Vaa* and *Vbb* venoms performed with freshly prepared solutions (A), with stored at -20 °C and thawed once (B) or twice (C). Protein band that appears after repeated thawing in *Vum* venom is indicated.

MATERIALS AND METHODS

ANIMALS, SNAKE VENOMS AND ANTIVENOMS

Vum venom was a pooled sample obtained by milking of >10 adult snakes caught at the southern Velebit isolated locality and released afterwards. It was air dried and stored in the dark at 4 °C until use. Air-dried crude *Vaa* venom was from Institute of Immunology Inc., Croatia. Commercial freeze-dried *Vipera berus berus* (*Vbb)* venom (Serpentarium of the Central Trade Base „Zoo-obyedinenie" Khimky, Moscow District, Russia) was the generous gift of Dr. Juri Siigur from NICPB, Tallinn, Estonia.

Rabbit sera against *Vaa* venom and its components (anti-H, anti-Atx, anti-$AtnI_2$) were produced according to our *in house* immunisation scheme on a small scale.

Mice and rats used for *in vivo* assays were bred at the Institute of Immunology. Procedures, handling and animal work were in accordance to the Croatian Law on Animal Welfare (2013) which complies with EC Directive 2010/63/EU.

MEASUREMENT OF LETHAL TOXICITY AND HAEMORRHAGIC ACTIVITIES

The lethal toxicity expressed as the median lethal dose (LD_{50}) was determined according to the European Pharmacopeia (Ph.Eur. 01/2008:0145). The minimum haemorrhagic dose (MHD) of venom is defined as the least amount of venom (mg per dry mass) which, when injected intradermally into rats, results in haemorrhagic lesion of 10 mm diameter 24 h later and was determined as described in Lang Balija *et al.* (2005). All results are given as mean from at least three determinations ± standard error (SE).

ELISA FOR DETECTION OF HAEMORRHAGIN-LIKE, AMMODYTOXIN-LIKE OR AMMODYTIN-LIKE PROTEINS IN Vum VENOM

ELISA assay for detection of haemorrhagin-, ammodytoxin- or ammodytin-like proteins in *Vum* venom was performed as previously described (Kurtović *et al.* 2014).

ELECTROPHORESIS, PROTEIN DETECTION AND IDENTIFICATION

Electrophoresis (1D and 2D) was performed using Bis-Tris precast gels (4-12%) according to manufacturer's instructions (Invitrogen, ThermoFisher Scientific). Detection of proteins was performed using Coomassie Brilliant Blue R250 or Western blotting, as described in Brgles et *al.* (2014). Identification of proteins was performed using mass spectrometry (MS), as described in Leonardi et *al.* (2012). Protein bands were excised from the gel and subjected to tryptic digestion. Subsequently, peptides were extracted and subjected to PMF and MS/MS identification.

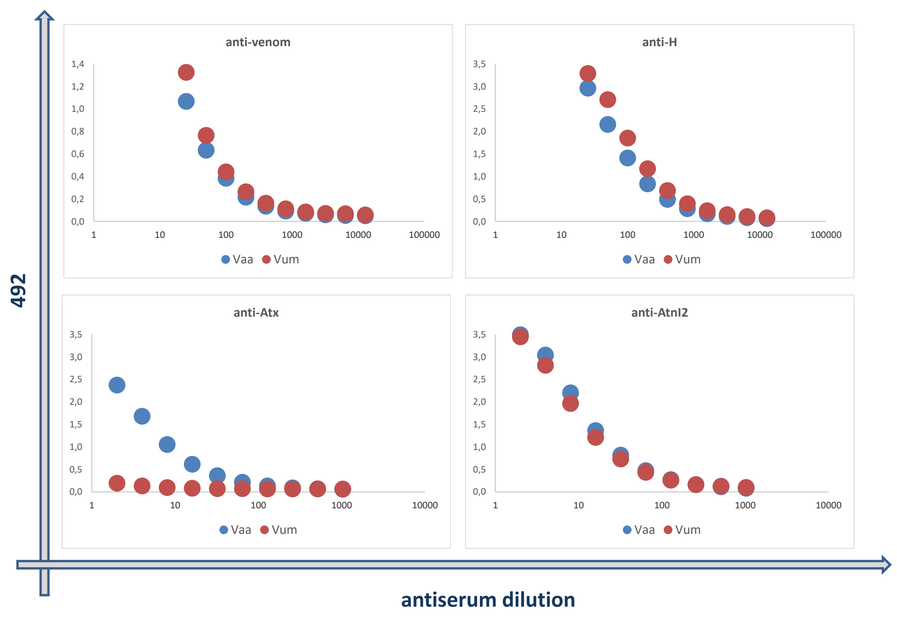
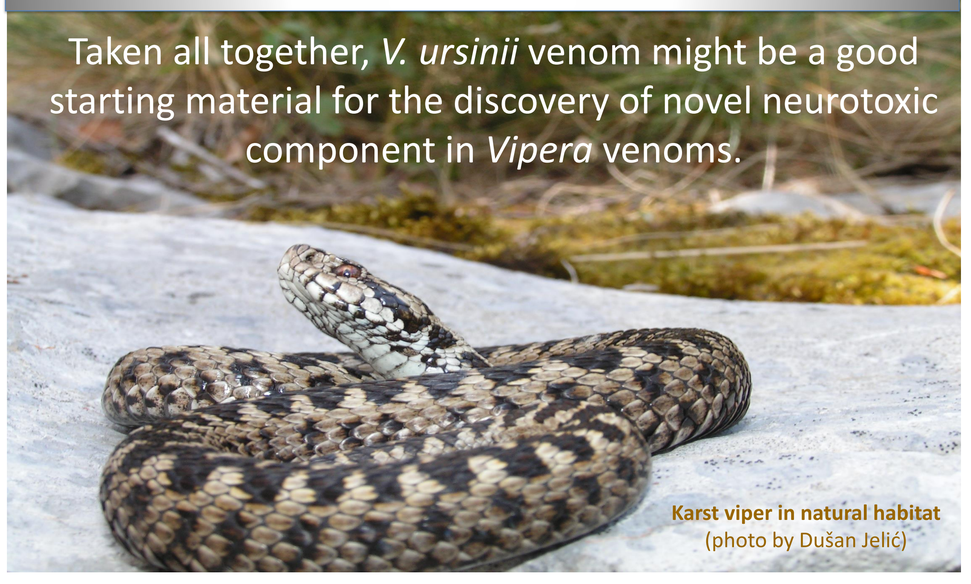


Figure 3. The lack of Atx-like proteins in *Vum* venom demonstrated by ELISA. *Vaa* and *Vum* coated wells were reacted with anti-*Vaa* venom, anti-H, anti-Atx and anti-$AtnI_2$ sera.

CONCLUSION

Taken all together, *V. ursinii* venom might be a good starting material for the discovery of novel neurotoxic component in *Vipera* venoms.



Karst viper in natural habitat (photo by Dušan Jelić)

LITERATURE
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