NMR-spectroscopic screening of crude venom of *Mesobuthus Cyprius*

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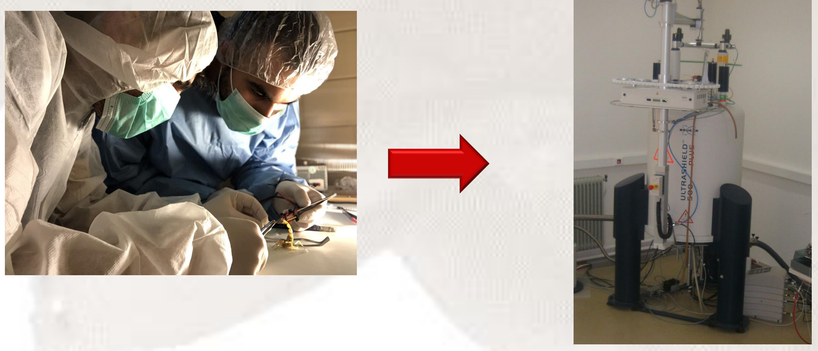
Introduction

Mesobuthus cyprius, one of the two endemic scorpions in Cyprus, belongs in the family of Buthidae which is geographically distributed worldwide and is the largest of the scorpion families [1]. Moreover, from a clinical perspective, Buthidae is the most important scorpion family as several members of this family are toxic to mammals and can be dangerous to humans. Even though *Mesobuthus cyprius* was discovered in 2000 using molecular phylogenetics there are no other published data regarding the peptide and protein composition, the toxicity, or any other activity of the venom. It is impressive, that a broad variety of bioactive substances in scorpion venoms may be considered as a source for drug discovery and development.

Direct NMR spectroscopic analysis of unpurified biological extracts is a powerful tool for the discovery of natural products especially in complex mixtures like venoms. It permits partial or complete structural characterization of its major components as well as of many minor components. Herein, we report the application of 1D and 2D NMR spectroscopy for the first time in the analysis of the *Mesobuthus cyprius* venom and the identification of a wide range of biomolecules and peptides.



Experimental

Scorpion Venom. Venom samples were provided from MedVenom Ltd, Cyprus. The venom was milked by using electric stimulation of the venom gland and subsequently lyophilized.

Sample Preparation. Venom was obtained in lyophilized form, generally as a white powder. To each sample was added 0.6 ml (5-mm NMR tubes) of D_2O (100 atom % D). The resulting suspension was subjected to sonication for 1 min and then centrifuged for 10 min. The supernatant was removed and analyzed via NMR spectroscopy.

NMR spectroscopy. NMR experiments were performed on a Bruker AV500 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 298 K using the Topsin 3.2 suite. Water suppression in the 1D ^{1}H NMR and the 2D ^{1}H-^{1}H NOESY spectra were achieved by the use of an excitation sculpting pulse sequence. The mixing time in the 2D ^{1}H-^{1}H TOCSY was set at 70 ms and for ^{1}H-^{1}H NOESY at 600 ms in order to obtain the maximum intensity. 2D ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC NMR experiments were recorded using standard Bruker software.

Results & Discussion

Citric acid was identified as one of the major components of *Mesobuthus cyprius* species. Citric acid or citrate is a common constituent of spider, scorpion, snake etc venoms. The exact role of citric acid has not been fully understood. One of the functions that have been proposed is its protection ability through inhibition of toxins within the venom gland. It can act as an inhibitor of proteins such as calcium-ion-dependant phospholipase A2 (PLA_2) neurotoxins, as well as Zinc-ion-dependant venom metalloprotease hemorrhagic toxins which are present in scorpion venom [2]. Citrate was identified and characterized as one of the major components with 1D and 2D NMR spectroscopy (Fig. 1) within the crude extract of the venom. The two characteristic protons $-CH_2$ resonances were observed at 2.47 and 2.61 ppm and a characteristic cross-peak of the TOCSY spectrum is presented in Fig. 1B.

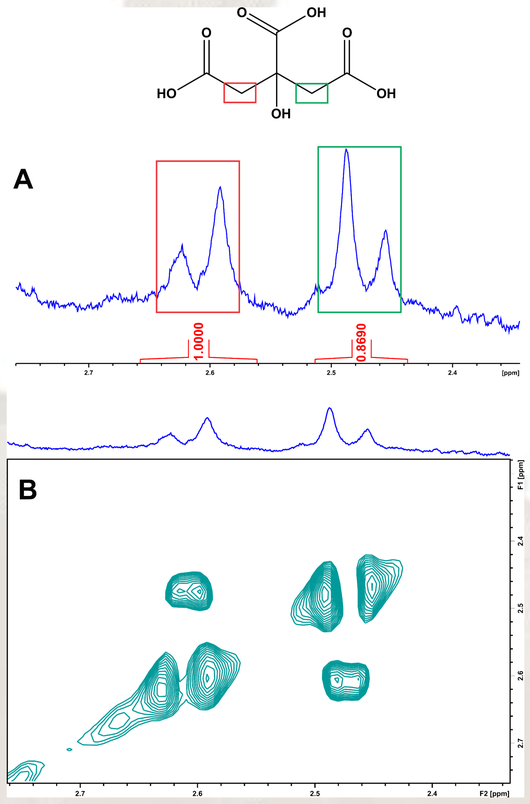


Figure 1: **A.** ^{1}H NMR and **B.** 2D TOCSY NMR of citric acid in the *Mesobuthus cyprius* venom.

Results & Discussion

Furthermore, the purine nucleoside, **adenosine**, which is a known constituent of arthropods venoms including the venom of the scorpion [3] was also identified with 1D and 2D NMR spectroscopy in the crude extract of *Mesobuthus cyprius* venom. A wide range of physiological effects of adenosine in mammalian targets has been characterized, including inducing vasodilation, causing increased vascular permeability, increasing blood coagulation time, and inhibiting neurotransmitter release [2]. Additionally, **adenosine monophosphate** was identified within the venom of *Mesobuthus cyprius*. Adenosine monophosphate has hypotensive properties and acts in a similar way to facilitate envenomation in mammalian aggressors. Figure 2 illustrates the TOCSY NMR cross-peaks of the two characteristic $-CH_2$ protons of adenosine and adenosine monophosphate of the crude *Mesobuthus cyprius* venom extract.

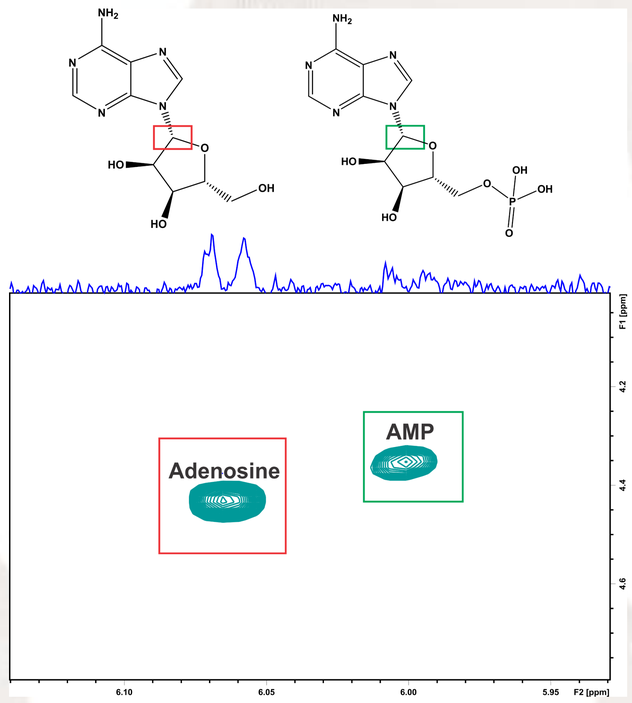


Figure 2: 2D TOCSY NMR of the prurine nucleosides, adenosine and adenosine monophosphate, within the *Mesobuthus cyprius* crude extract venom.

Results & Discussion

Finally, hyaluronidase, which is an enzyme that cleaves hyaluronic acid (HA) in the extracellular matrix is highly present in venoms of several arthropods such as spiders, snakes and scorpions [4]. Hyaluronidase facilitates the diffusion of toxins from the bite or sting site to circulation and can cause local damage. Additionally, the enzyme can indirectly contribute to venom toxicity through the inflammatory properties of the HA fragments. Herein, a characteristic pattern of hyaluronidase 2D NMR was identified within the *Mesobuthus cyprius* crude extract venom suggesting that the enzyme is also present. Figure 3 demonstrates a selected region of the 2D TOCSY NMR spectrum with three characteristic cross-peaks of the enzyme. Further, investigation will be conducted including HPLC and LC-MS characterization.

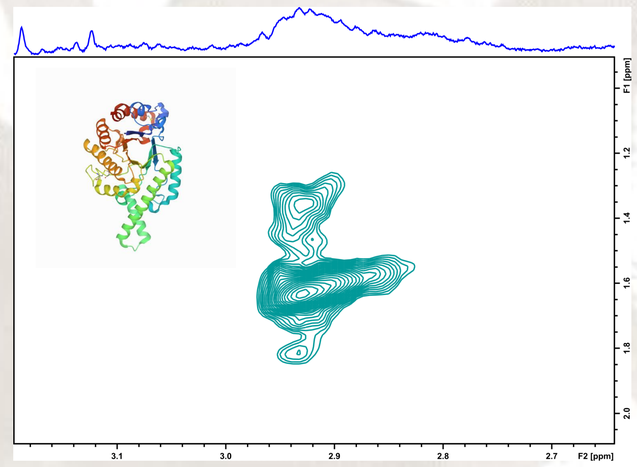


Figure 3: 2D TOCSY NMR of a selected region with three characteristic cross-peaks of hyaluronidase within the *Mesobuthus cyprius* crude extract venom (PDB entry 1FCQ [5]).

Conclusions

Citric acid has been characterized as a major component of *Mesobuthus cyprius* crude extract venom. Additionally, the purine nucleosides, adenosine, adenosine monophosphate were also characterized with 1D and 2D NMR spectroscopy and characteristic pattern cross-peaks of the enzyme hyaluronidase were observed within the scorpion venom. Further analysis will be conducted with NMR spectroscopy and the results will be compared with the extensive analytical studies (LC-MS, MALDI-TOF, etc). To our knowledge, this is the first NMR study of the *Mesobuthus cyprius* venom.

Conflicts of Interest: The authors declare no conflict of interest

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