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# High-resolution snake venom inhibition profiling combined with HPLC-HRMS-SPE-NMR for identification of antivenomous constituents in *Clausena excavata*

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## Background

On average 100,000 persons are bitten by venomous snakes in China each year, with a mortality rate of 5-10%<sup>1</sup>. According to the clinical report, *Gloydius blomhoffi brevicaudus*, *Deinagkistrodon acutus*, *Naja naja atra* and *Trimeresurus stejnegeri* bites are most common.<sup>2,3,4</sup>



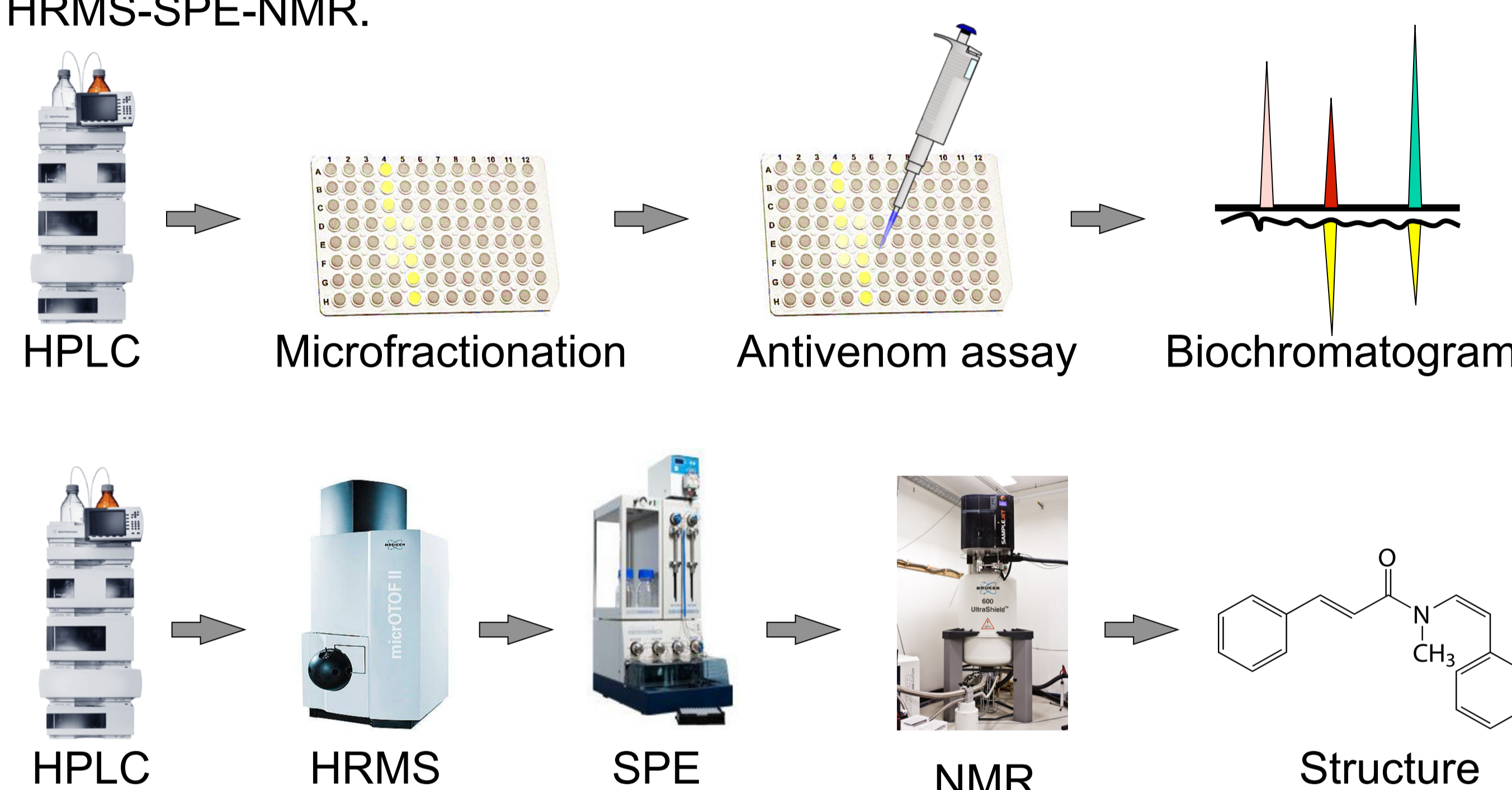
**Figure 1.** *Gloydius blomhoffi*, *Deinagkistrodon acutus*, *Naja naja* & *Trimeresurus stejnegeri*

As for the treatment of snakebite patients, usually the first choice is the corresponding antiserum. Despite antiserum can alleviate the lethal effect of venom to some extent, it has minimal preventive effect on local tissue damage caused by snakebite which could even result in physical disability. The venom toxins responsible for these local effects are phospholipase A2, proteases and hyaluronidase enzymes.<sup>5,6</sup> Traditional medicines are normally used in Chinese hospitals to prevent tissue necrosis after snakebites. Furthermore, snakebites often happen in remote areas where there is lack of basic medical facilities and no access to antivenom. For these snakebite victims traditional medicines are important to heal the wound and resist tissue necrosis. *Clausena excavata* is a common plant used against snakebite in China.<sup>7</sup>

## Procedure

The procedure used in this work was divided into three steps:

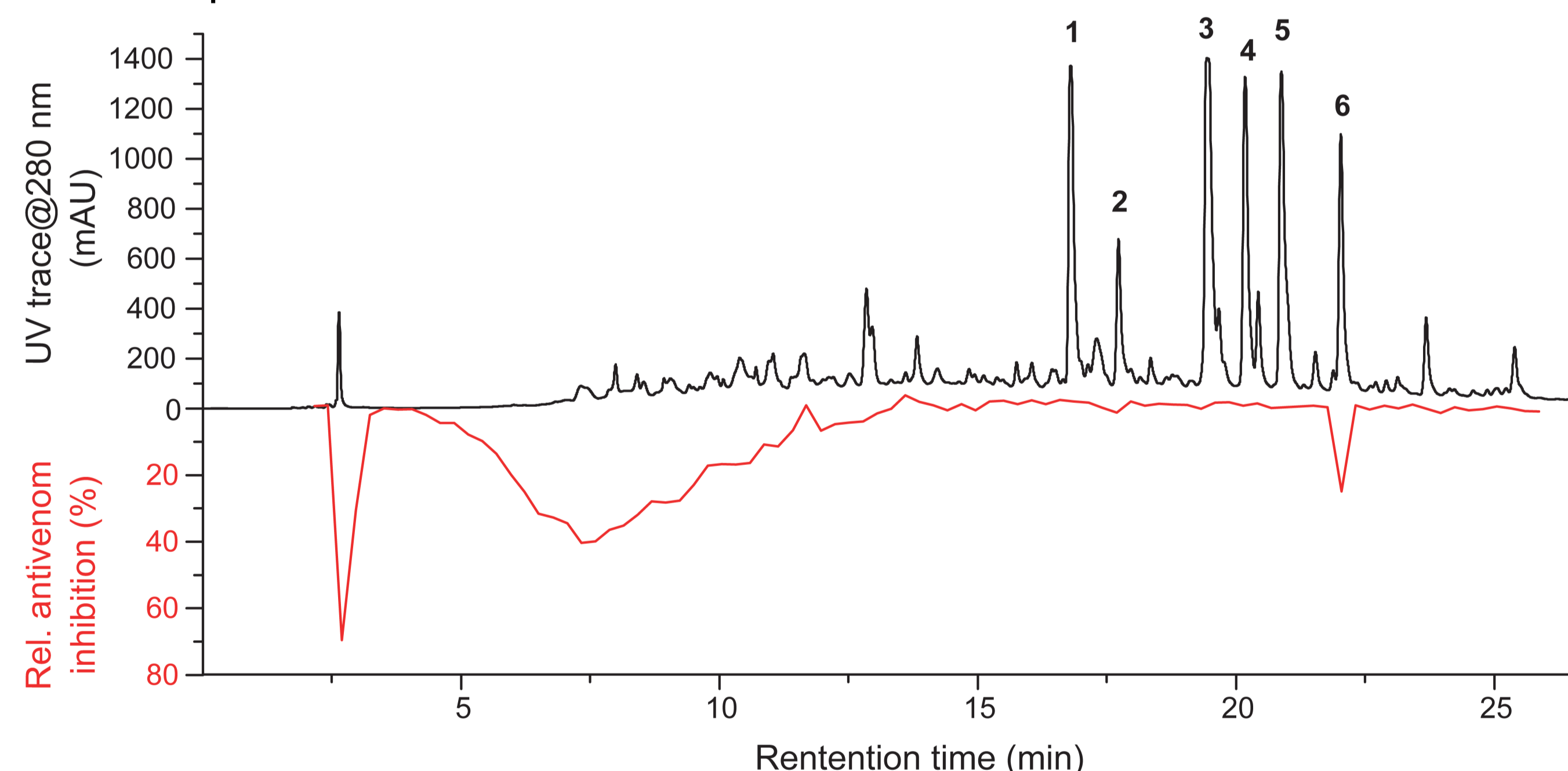
1. Extracts of *C. excavata* were tested at 1 mg/mL in hyaluronidase, phospholipase A2, and protease inhibition assays against *Gloydius blomhoffi*, *Deinagkistrodon acutus*, *Naja naja* and *Trimeresurus stejnegeri* venom.
2. Extracts with more than 90% inhibition were fractionated into microplates and biochromatograms were constructed.
3. Bioactive constituents from the biochromatograms were analyzed by HPLC-HRMS-SPE-NMR.



**Figure 2.** Flowchart of the procedure used in this work

## High-resolution snake venom profiling

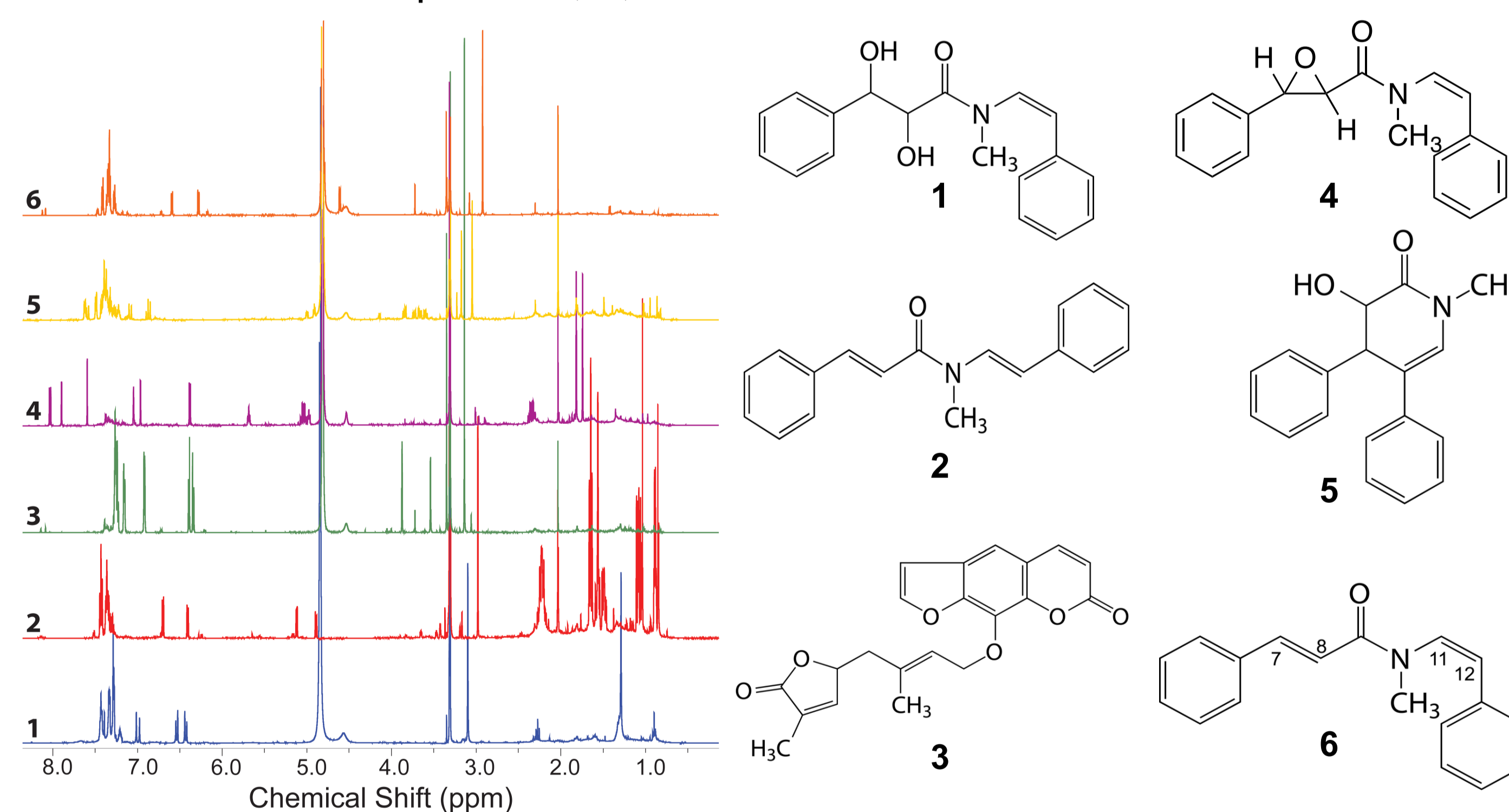
Crude ethanol extract of *Clausena excavata* showed 99.5% inhibition against *Deinagkistrodon acutus* venom in the hyaluronidase assay, and was therefore subjected to high-resolution hyaluronidase profiling. The biochromatogram is shown below the HPLC trace in **Figure 3**, and allowed pinpointing of peak 6 as an active constituent. The active hump between 5 and 13 min is presumably due to the presence of tannins.<sup>8</sup>



**Figure 3.** Biochromatogram of ethanol extract of *Clausena excavata* from hyaluronidase assay against *Deinagkistrodon acutus* venom.

## HPLC-HRMS-SPE-NMR analysis

Analysis of HRMS and NMR data obtained via HPLC-HRMS-SPE-NMR led to identification of the metabolites as clausenalansamide A (**1**), lansiumamide I (**2**), indicolactone (**3**), clausamide-I (**4**), 2(1*H*)-Pyridinone (**5**) and lansiumamide B (**6**). The 7,8-*trans* double bond and the *cis* double bond at position 11 and 12 might be important for the antivenomous activity of compound **6** by comparing the structure with compound **1**, **2**, **4** and **5**.



**Figure 4.** <sup>1</sup>H NMR spectra and structure of compounds **1-6**

## Perspectives and concluding remarks

Compounds **1-6** were purified by preparative scale HPLC and subjected to the activity test in the hyaluronidase assay against *Deinagkistrodon acutus* venom. Compounds **1-5** showed no activity in the test. The IC<sub>50</sub> value of lansiumamide B (**6**) was very close to the value of the standard hyaluronidase inhibitor aristolochic acid, which indicate lansiumamide B might be a promising inhibitor against snakebite of *Deinagkistrodon acutus*.

## In conclusion, this study showed:

- ✓ High-resolution snake venom inhibition profiling allowed fast pinpointing of a bioactivity constituent, *i.e.*, lansiumamide B (**6**).
- ✓ High-resolution hyaluronidase assay allows subsequent HPLC-HRMS-SPE-NMR analysis to be targeted bioactive constituents only.

## References:

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