

Response letter

Reviewer 3

Venom are sources of novel chemical entities with promising therapeutic applications. They are also valuable compounds to study the ecology and evolution of venomous animals. In this sense, this research article is an interesting study that brings novel insights into biochemistry and biological effects of *Psytalla horrida* venom.

1. Some sentences should be supported by references. Please double check the lines (31-32: recent studies, which?; 42-43, 55-57, 74-75, and so on) and the entire manuscript.

The lines mentioned by the reviewer are mostly opening sentences that introduce a particular topic. They are followed by a more detailed description, and we refer here to the relevant publications. However, we carefully checked the manuscript and added references in cases where this may not have been clear.

2. Lines 118-119, 152, 154, 193, 208 and so on. Scientific names are always italicised.

When copying the text of our original file to the Word template provided by Toxins, it seems that we have missed to correctly convert the originally italicized species names. We carefully checked and revised all species names in the updated version of the manuscript.

3. Authors should standardise the graphs and colours. Controls and fractions are represented using different colours in different figures. For example, in figure 2 fraction A is green and in figure 3 fraction A is blue.

The colors in the boxplots are standardized for the respective type of assay (yellow for insect cell viability, blue/green for bacterial growth, and red for erythrocyte integrity). Therefore, the blue/green color in Figures 2 and 5 refers to the bacterial growth inhibition assays and not to a specific treatment. We did not adhere to this color scheme when analyzing the calcium imaging experiments (Figure 3) because the visualization of the individual fluorescence profiles is more complex. Given the large number of different treatments in this study (five controls, two fractions, 18 redulysin peptides, one Vpf2), we believe that an assay-based color scheme is most appropriate.

4. Why the effect of crude venom was not analysed? It would be interesting include the effect of whole venom in figure 2.

The effects of *P. horrida* PMG venom were analyzed in detail in a previous study (Fischer et al. 2020). However, we now added a supplementary Figure S2 showing the effects of 0.2 mg/ml crude PMG venom extracts on insect cell viability, bacterial growth and erythrocyte integrity.

5. Figure 3B. Please include bar length.

We added a scale bar in Figure 3B.

6. Figure 5 B. This representation is uncommon for antibacterial effect. Why the authors have not assessed the MIC?

To reduce complexity and identify redulyisin peptides that are generally antimicrobially active, we performed a first simple antimicrobial screening at 10 μ M using all peptides (Figure 5B). The dose-response curves shown in Figure 5C were only performed with the four most potent redulyisin peptides (based on additional screenings at concentrations below 10 μ M). For those peptides, ED50 values are given in Figure 5C as a suitable alternative to the MIC.

7. I recommend the authors to use *in silico* approaches to corroborate the haemolytic effect of peptides. This additional evaluation contributes to expand the use of *in silico* methods and assess their potential.

Although the *in silico* prediction of hemolytic activities of venom peptides is certainly interesting, here we have intentionally focused on experimental approaches, testing individual candidate peptides in a number of assays. In addition, while numerous *in silico* prediction tools have been described, almost all of the web-based tools for prediction are either non-accessible or seem to no longer exist. This holds true for e.g.: HLPpred-Fuse, ToxinPred2, ToxiPred, HemoPI, NTXpred, ClanTox, TOXIFY, ATSE etc. Other prediction tools such as ToxDL or ToxIBLT seem to be unable to predict hemolytic activity. We have used the prediction tool HemoPred (<http://codes.bio/hemopred/>) to assess the hemolytic potential of the 18 peptides tested in our assays. However, HemoPred does not provide any threshold criteria and out of 18 predictions, only 9 matched the results of our hemolytic activity assay. We would therefore restrain from using this information, also because we cannot evaluate the robustness of the *in silico* prediction.

Please also double check the statistical analysis shown in Figure 5B.

We are not sure which test this comment refers to specifically. We checked the statistical analysis again but could not find any mistakes. Details on the statistical tests used in this study are provided in Supplemental Table S1.
