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Title: Physico-Chemical and Mineral Variability of Apis mellifera Bee Venom Across Seasons and Feeding Regimes

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Section: Natural Products Chemistry

Special Issue: Bee Product Chemistry: Identification and Characterization of Natural Compounds, 2nd Edition

Dear Reviewer,

Thank you for taking the time to review our manuscript. We greatly appreciate the suggestions made to improve our paper.

We have addressed your comments as follows:

This paper examines how nutritional supplements and seasonal floral sources affect the physico-chemical and mineral profile of Apis mellifera bee venom. The topic is interesting and the experimental design is reasonably comprehensive. However, several issues need to be addressed before the manuscript can be considered for publication.

Major Issues:

1. There is a clear disconnect between the Introduction and the Results. The Introduction emphasizes the potential of dietary supplements to modulate venom composition (Line 57), but the data consistently show that supplementation has minimal effects. This contradiction should be acknowledged and discussed, why do your results differ from the expectations set by the cited literature?

A: We thank the reviewer for this important observation. We agree that the Introduction highlights findings from previous studies suggesting that essential oils and probiotic supplements may influence certain biochemical characteristics of bee products, including venom. Compared with other studies, our results show that, under the conditions of this experiment, supplementation produced only minor and inconsistent effects, whereas seasonal dynamics and floral origin were the dominant drivers of physico-chemical and mineral variability. We have now clarified this point in the Results and Discussion section and aligned it with the conclusions of the manuscript (marked with blue).

2. Pb levels up to 36.18 µg/g are reported without any discussion of safety implications. Given that bee venom is used therapeutically, the authors need to address whether such levels pose a health risk, ideally with reference to relevant regulatory limits.

A: Thank you for your comment. Given that the permitted daily exposure to lead for oral, parenteral, and inhalation routes is 5 µg/day (according to the ICH Q3D(R2) Guideline for Elemental Impurities), and that the usual maintenance dose in venom immunotherapy is 100 µg of bee venom, the maximum measured concentration of 36.18 µg/g remains well below this limit ($36.18 \mu\text{g/g} \times 0.0001 \text{ g} = 0.0036 \mu\text{g}$).

3. The ninhydrin colorimetric method described in Section 3.6 measures free amino acids, not total amino acids. Please correct the terminology or describe the hydrolysis step if one was performed.

A: We thank the reviewer for this valuable observation. We agree that the ninhydrin colorimetric assay quantifies free amino acids unless preceded by a hydrolysis step. In our study, the determination of amino acids was performed after acid hydrolysis, which allows quantification of total amino acids. To

clarify this point, we have revised Section 3.6 to include a detailed description of the hydrolysis procedure. The updated method now specifies that 3 mg of dried bee venom were subjected to acid hydrolysis with 6 N HCl at 110 °C for 24 h, under nitrogen to prevent oxidative degradation. After hydrolysis, samples were dried to remove excess HCl, reconstituted, and subsequently reacted with ninhydrin for colorimetric quantification at 570 nm using alanine as the calibration standard. This correction ensures that the terminology “total amino acids” is accurate and consistent with the analytical procedure employed. We have updated the manuscript accordingly to avoid ambiguity and to accurately reflect the methodology used.

4. The Introduction states that antioxidant capacity was evaluated (Line 101), but no such data appear in the paper. Either add the data or remove the claim.

A: We thank the reviewer for pointing out this inconsistency. The evaluation of antioxidant capacity was initially considered but was not included in the final experimental design. To avoid misleading the reader, we have removed the reference to antioxidant capacity from the Introduction and ensured that no such claims remain

5. Climate data come from a single hive and are discussed only descriptively. Incorporating temperature/humidity into the statistical analyses (e.g., correlation matrix) would substantially strengthen this aspect of the work.

A: We thank the reviewer for this constructive suggestion. In the revised manuscript, we have clarified the role of temperature and humidity in interpreting seasonal variations in venom composition. Our objective was to assess how these meteorological variables influence colony foraging activity, metabolic intensity, and biochemical processes involved in venom biosynthesis. This ecological interpretation is supported by the observed seasonal dynamics of hive weight variations, as well as by the cited literature. We were unable to integrate temperature and humidity into multivariate statistical analyses for two methodological reasons:

- Climatic data were recorded at the hive level, not at the level of individual venom samples. This resulted in a single pair of temperature–humidity values for each sampling period, insufficient to ensure the replication required for robust statistical models or correlation analyses.

- The number of sampling points is limited. With only a few seasonal intervals, including meteorological variables in correlation matrices or regression models would not produce statistically reliable or interpretable results.

For these reasons, temperature and humidity were used as contextual ecological variables, intended to support the interpretation of seasonal patterns, rather than as independent predictors in statistical models. This has been clarified in the manuscript, and the descriptive link between meteorological conditions, hive weight dynamics, and venom composition has been strengthened.

Minor Issues:

1. The last two paragraphs of the Introduction (lines 91-106) are essentially duplicated. Please remove one.

A: Thank you for your comment. The paragraph has been removed.

2. Figure captions for Figs. 3 and 4 are too brief, they should be informative enough to stand alone.

A: We have revised the figure captions for Figs. 3 and 4.

Figure 3. Hierarchical clustering dendrogram illustrating the similarity relationships among bee venom samples based on their physico-chemical and mineral parameters. The clustering pattern highlights how samples group according to seasonal conditions and floral origin, revealing distinct profiles associated with rapeseed, acacia, and sunflower flows. This visualization provides an integrated overview of multivariate variability and supports the interpretation that environmental and botanical factors are the primary drivers shaping venom composition.

Figure 4. Pearson correlation matrix illustrating the relationships among environmental variables (temperature, humidity), hive-weight dynamics, and the physico-chemical and mineral parameters of bee venom. Positive and negative correlations are represented by color intensity, highlighting how seasonal conditions and forage availability co-vary with venom composition. The matrix reveals clusters of strongly associated variables, supporting the interpretation that natural floral resources and ecological factors are the primary drivers shaping venom quality and colony metabolic activity.

3. Reference formatting needs attention: missing page numbers (e.g., 34, 57), apparent errors (e.g., 47, 50), and inconsistent use of DOIs.

A: We have carefully rechecked the references and made corrections where inconsistencies were identified. Thank you!

We are grateful for your time and effort to review our paper, and we hope we have successfully addressed all your queries!

Sincerely,
The authors.